Sensitive Detection of Pre-Existing BCR-ABL Kinase Domain Mutations in CD34+ Cells of Newly Diagnosed Chronic-Phase Chronic Myeloid Leukemia Patients Is Associated with Imatinib Resistance: Implications in the Post-Imatinib Era

Zafar Iqbal1,9,12,15, Aamer Aleem2,9, Mudassar Iqbal3,15, Mubashar Iqbal Naqvi4,15, Ammara Gill5,15, Abid Sohail Taj6, Abdul Qayyum7, Najeeb ur-Rehman8, Ahmad Mukhtar Khalid9, Ijaz Hussain Shah1, Muhammad Khalid10, Riazul Haq11,12, Mahwish Khan12,15, Shahid Mahmood Baig13, Abid Jamil14, Muhammad Naeem Abbas15, Muhammad Absar15, Amer Mahmood16, Mahmood Rasool17, Tanveer Akhtar15

1 College of Applied Medical Sciences, King Saud Bin Abdulaziz University for Health Sciences (KSAU-HS), National Guards Health Affairs, Riyadh, Kingdom of Saudi Arabia, 2 Department of Medicine, Division of Hematology/Oncology, College of Medicine and King Khalid University Hospital, King Saud University, Riyadh, Kingdom of Saudi Arabia, 3 Foreign Faculty, Asian Medical Institute, Kant City, National Surgical Centre, Bishkek, Kyrgyzstan, 4 Computer Sciences and Bio-informatics Laboratory, Government. Elementary School Chak 19 S.B., Sargodha, Pakistan, 5 Springfield, Missouri, United States of America, 6 Institute of Radiotherapy and Nuclear Medicine, Peshawar, Pakistan, 7 Department of Oncology, Pakistan Institute of Medical Sciences, Islamabad, Pakistan, 8 Medilaser, Lahore, Pakistan, 9 School of Biological Sciences, University of Sargodha, Sargodha, Pakistan, 10 Department of Oncology, Allied Hospital and Punjab Medical College, Faisalabad, Pakistan, 11 Health Centre, University of Texas San Antonio, San Antonio, Texas, United States of America, 12 Institute of Molecular Biology and Biotechnology and Centre for Research in Molecular Medicine, the University of Lahore, Lahore, Pakistan, 13 Human Molecular Genetics Group, Health Biotechnology Division, National Institute for Biotechnology and Genetic Engineering, Faisalabad, Pakistan, 14 Post-graduate Medical Institute, Hayatabad Medical Complex, Peshawar, Pakistan, 15 Higher Education Commission Program in “Hematology Oncology and Pharmacogenetic Engineering Sciences (HOPES)”, HOPES Group, Health Sciences Research Laboratories, Department of Zoology, University of the Punjab, Lahore, Pakistan, 16 Stem Cell Unit, Department of Anatomy, College of Medicine, King Saud University, Riyadh, Kingdom of Saudi Arabia, 17 Centre of Excellence in Genomic Medicine Research, King Abdulaziz University Jeddah, Kingdom of Saudi Arabia

Abstract

Background: BCR-ABL kinase domain mutations are infrequently detected in newly diagnosed chronic-phase chronic myeloid leukemia (CML) patients. Recent studies indicate the presence of pre-existing BCR-ABL mutations in a higher percentage of CML patients when CD34+ stem/progenitor cells are investigated using sensitive techniques, and these mutations are associated with imatinib resistance and disease progression. However, such studies were limited to smaller number of patients.

Methods: We investigated BCR-ABL kinase domain mutations in CD34+ cells from 100 chronic-phase CML patients by multiplex allele-specific PCR and sequencing at diagnosis. Mutations were re-investigated upon manifestation of imatinib resistance using allele-specific PCR and direct sequencing of BCR-ABL kinase domain.

Results: Pre-existing BCR-ABL mutations were detected in 32/100 patients and included F311L, M351T, and T315I. After a median follow-up of 30 months (range 8–48), all patients with pre-existing BCR-ABL mutations exhibited imatinib resistance. Of the 68 patients without pre-existing BCR-ABL mutations, 24 developed imatinib resistance; allele-specific PCR and BCR-ABL kinase domain sequencing detected mutations in 22 of these patients. All 32 patients with pre-existing BCR-ABL mutations had the same mutations after manifestation of imatinib-resistance. In imatinib-resistant patients without pre-existing BCR-ABL mutations, we detected F311L, M351T, Y253F, and T315I mutations. All imatinib-resistant patients except T315I and Y253F mutations responded to imatinib dose escalation.

Conclusion: Pre-existing BCR-ABL mutations can be detected in a substantial number of chronic-phase CML patients by sensitive allele-specific PCR technique using CD34+ cells. These mutations are associated with imatinib resistance if affecting drug binding directly or indirectly. After the recent approval of nilotinib, dasatinib, bosutinib and ponatinib for treatment of chronic myeloid leukemia along with imatinib, all of which vary in their effectiveness against mutated BCR-ABL forms, detection of pre-existing BCR-ABL mutations can help in selection of appropriate first-line drug therapy. Thus, mutation testing using CD34+ cells may facilitate improved, patient-tailored treatment.

Citation: Iqbal Z, Aleem A, Iqbal M, Naqvi MI, Gill A, et al. (2013) Sensitive Detection of Pre-Existing BCR-ABL Kinase Domain Mutations in CD34+ Cells of Newly Diagnosed Chronic-Phase Chronic Myeloid Leukemia Patients Is Associated with Imatinib Resistance: Implications in the Post-Imatinib Era. PLoS ONE 8(2): e55717. doi:10.1371/journal.pone.0055717

Editor: Steven R. Ellis, University of Louisville, United States of America

Received September 10, 2012; Accepted December 29, 2012; Published February 8, 2013

Copyright © 2013 Iqbal et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
Introduction

Chronic myeloid leukemia (CML) is a hematopoietic stem cell disorder characterized by the t (9; 22) chromosomal translocation. This translocation results in the formation of BCR-ABL fusion gene, which is central to the pathogenesis of CML. The BCR-ABL gene exhibits constitutive tyrosine kinase activity, resulting in myeloid proliferation [1]. Imatinib mesylate, a tyrosine kinase inhibitor (TKI), induces durable responses in the majority of CML patients and is currently the standard of care for CML [2,3]. However, imatinib resistance, usually due to BCR-ABL kinase domain (KD) point mutations, remains a significant problem in the management of CML patients [4,6]. BCR-ABL mutations have varying effects on the patient’s sensitivity to imatinib and other TKIs, and may cause partial or complete resistance depending upon the nature and location of the mutations [3,7–10]. The presence of KD mutations has been studied mostly in the advanced phase of CML (accelerated phase and blast crisis), in chronic phase (CP) patients who develop resistance to imatinib, and in Philadelphia-positive (Ph+) acute lymphoblastic leukemia [5,10–13].

BCR-ABL KD mutations can exist in the newly diagnosed CP-CML patients and may affect the outcome of imatinib treatment [14–18]. There are limited data available from imatinib-naive patients in CP-CML regarding the incidence of KD mutations, and the correlation of these mutations with the therapeutic response in unselected patients has not been established [14,17–18]. Although KD mutations are infrequently detected in newly diagnosed CP-CML patients [18], KD mutations were found in a substantial number of patients when CD34+ stem cell were analyzed [19,20]. Previous studies indicated that a small population of CD34+ CML (stem/progenitor) cells are less responsive to imatinib and other TKIs, and act as a reservoir for the emergence of imatinib-resistant subclones [19,21–23]. Thus, the detection of pre-existing mutations (PEMs) in primitive stem/progenitor (CD34+) cells may have therapeutic and prognostic implications and is likely to be helpful in optimizing the management of CML patients, specifically after availability of three tyrosine kinase inhibitors as first-line treatment of CML which vary in their effectiveness against different BCR-ABL mutants as well as after FDA approval of ponatinib for TKI-resistant CML, particularly the most aggressive T315I-mutant CML [19–23]. Large-scale studies to assess the role of BCR-ABL PEMs in CD34+ cells and their correlation with imatinib therapy in CP-CML are lacking. To address this issue, we analyzed 100 newly diagnosed CP-CML patients for BCR-ABL PEMs in CD34+ CML cells using allele-specific oligonucleotide polymerase chain reaction (ASO-PCR) and sequencing, and studied the outcome of these patients after imatinib treatment.

Design and Methods

Ethics Statement

Patients’ samples were collected from the following centers. 1) Department of Oncology, Allied Hospital and Punjab Medical College Faisalabad, Pakistan. 2) Pakistan Institute of Medical Sciences Hospital, Islamabad, Pakistan. 3) Khyber Teaching Hospital & Hayatabad Medical Complex, Peshawar, Pakistan. 4) Institute of Radiotherapy and Nuclear Medicine, Peshawar, Pakistan, & 5) HOPES, Department of Zoology, University of the Punjab, Lahore, Pakistan. All patients gave written informed consent, and the institutional ethics committees of the participating centers approved the study as well as contents of the written consent.

Patients and Definitions

One hundred newly diagnosed CP-CML patients were included in the study. The study was conducted from March 2006 until February 2010 and 4 centers participated in the study while experiments were carried out at HOPES, Department of Zoology, University of the Punjab, Lahore, Pakistan. All patients had newly diagnosed CP-CML at the time of sample collection, and patients with accelerated-phase or blast-crisis CML were excluded. Patients’ clinical characteristics are given in Table 1.

CP was defined by the presence of less than 15% blasts, less than 20% basophils, and less than 30% blasts and promyelocytes in the peripheral blood and bone marrow (BM) and no extramedullary blastic disease [24]. Complete hematologic response (CHR), complete cytogenetic response (CCyR), and a partial cytogenetic response were defined according to previously published response criteria [24]. Briefly, CHR required the normalization of blood counts; leucocytes counts <10,000/mm3; normal differential counts without blasts, promyelocytes, or myelocytes; platelet counts from 150,000/mm3 to 450,000/mm3; and no evidence of extramedullary disease. CCyR was defined as 0% Ph+ cells in metaphase BM samples, and a Major (partial) cytogenetic response (PCyR) was defined as the presence of 1–35% Ph+ cells in BM. Other categories included minor cytogenetic response (36–65% Ph+ cells in BM) and minimal cytogenetic response (66–95% Ph+ cells in BM). Molecular response was defined as BCR-ABL fusion transcript negativity according to nested reverse-transcriptase PCR. We could not record the major molecular response (MMR) due to the non-availability of real-time quantitative PCR during the study, but a real-time quantitative PCR was performed on archived samples preserved in 10% DMSO and 90% FBS stored at −80°C using IPSOGEN BCR-ABL Mbr FusionQuant® Kit (Catalogue FQPP-10-CE) at the end of the study after the availability of real-time PCR (ABI 7500 real-time PCR, Applied Biosystems, USA). A 3-log reduction in BCR-ABL transcripts was considered an MMR.

Resistance patterns were adopted as defined by the Leukemia-Net guidelines [24]. Primary or intrinsic resistance was defined by the failure to achieve CHR by 3 months, any cytogenetic response
by 6 months, partial cytogenetic response by 12 months, and complete cytogenetic response by 18 months. Acquired or secondary resistance was defined as the loss of previous hematological, cytogenetic, or molecular responses, sustained CHR that was followed by transformation to the accelerated or blastic phase, Ph\(^+\) clonal evolution, or the emergence of clinically relevant BCR-ABL KD mutations predicted to confer resistance [25].

Isolation of CD34\(^+\) CML Stem/Progenitor Cells

BM mononuclear cells were isolated by Ficoll-Hypaque (Sigma Diagnostics, St Louis, MO) density gradient separation (specific gravity, 1.077) for 30 min at 400\(\times\)g. The cells were then suspended in a solution of 10% dimethylsulfoxide (DMSO) in fetal calf serum (FCS) and cryopreserved in liquid nitrogen until required [26]. Before use, cells were thawed and stained with antibodies to CD34 directly conjugated to fluorescein isothiocyanate (Becton Dickinson Immunocytometry System, San Jose, CA). After staining for 30 min at 4\(\degree\)C, the cells were washed twice in phosphate-buffered saline containing 2% FCS (Stem Cell Technologies Inc.) and resuspended in 2 \(\mu\)g/mL propidium iodide (Sigma). CD34\(^+\) cells were collected by fluorescence-activated cell sorting (FACS) using a FACSVantage cell sorter (Becton Dickinson, San Jose, CA) [27].

Detection of Pre-existing BCR-ABL Mutations

RNA and DNA were extracted from FACS-sorted CD34\(^+\) cells using TriZol and DNAzol (Invitrogen Life Technologies, Carlsbad, CA) methods, respectively [28]. RNA and DNA quality was checked by spectrophotometry, gel electrophoresis, and by the amplification of the ABL gene [14,18]. As BCR-ABL PEMs are known to be rare among wild-type CBR-ABL and thus cannot be detected by sequencing the whole BCR-ABL KD, we employed a very sensitive ASO-PCR assay for this purpose which has already been optimized and clinically validated using appropriate positive and negative controls elsewhere [31]. This assay can detect 18 of the most clinically relevant and common BCR-ABL mutations [14,29–30]. PCR amplifications were performed exactly as reported, without changing any of the reagents, PCR mix formulations and thermal profile [29]. The sequences of ASO primers specific for each mutation with the corresponding annealing temperatures are given in Table 2. HL60 cell line (ATCC \#CCL-240\(^{TM}\)) was used as a negative control in ASO-PCR reactions. Although we used pre-validated ASO-PCR assays and reproduced those assays using exactly same reaction conditions, reagents and PCR mix formulation, to eliminate the possibility of false-positive results ASO-PCR products were sequenced on both strands using an automated ABI377 sequencer (Applied Biosystems). Sequences were analyzed with Sequence Analysis software V3.3 and Sequence Navigator software V1.0.1 (Applied Biosystems). A mutation was considered present only if it was detected in both strands in two or more independent ASO-PCR amplified products [14,29–31].

Imatinib Treatment and Response Monitoring

All patients were treated with 400 mg of imatinib/day. Clinical studies were performed in collaboration with CML treatment centers. Patients were monitored every 2 weeks for hematological response and every 3 months for cytogenetic and molecular response during imatinib treatment and follow-up. Secondary resistance, as described previously, was also monitored. For imatinib-resistant patients, second-generation TKIs were not available due to financial constraints. However, imatinib-resistant patients were treated with 600–800 mg of imatinib/day, irrespective of presence or absence of PEMs [7]. Patients were monitored regularly every 2 weeks for hematological response and every 12 weeks for cytogenetic and molecular responses after imatinib dose escalation.
Detection of Mutations After the Manifestation of Imatinib Resistance

All imatinib-resistant patients, irrespective of their PEM status, were investigated for BCR-ABL mutations using ASO-PCR [14,29–30], as well as by DNA sequencing of the RT-PCR–amplified whole BCR-ABL KD. For RT-PCR and DNA sequencing of the BCR-ABL KD, we adopted the protocol described by Branford and Hughes [31] using an automated ABI377 sequencer (Applied Biosystems). HL60 cell line (ATCC # CCL-240TM) was used as a negative control in PCR and sequencing while KCL22 cell line (DSMZ # ACC 519) was used as a positive control. Sequences were analyzed with Sequence Analysis software V3.3 and Sequence Navigator software V1.0.1 (Applied Biosystems). To confirm mutation detection by sequencing, the opposite strand of the PCR product was sequenced. Moreover, the whole procedure of RNA extraction, RT-PCR, and sequencing was repeated once. Detection of the mutation was confirmed only if the same mutation was detected in both DNA strands as well as in the repeat analysis [15,29,31].

Table 2. Sequences of ASO primers and corresponding annealing temperatures (bold nucleotides in the primers denote nucleotide changes corresponding to mutations).

| Primer name | Primer polarity | Nucleotide change | 5’–3’ sequence | ℓL (bp) | ℓA Tm (°C) |
|-------------|----------------|------------------|----------------|---------|-----------|
| 1. M244V-F  | F              | A1094G           | GAACGCGACGACATCACC| 19      | 65.7      |
| 2. L248V    | F              | C1106G           | ACCATGAAACACACAGG| 16      | 55        |
| 3. G250E    | F              | G1113A           | GAACGACAGCCTGGGGCCAGA | 18      | 56        |
| 4. Q252H(a) | F              | G1120C           | AGCTGGGCGGGGCCCAC | 17      | 62        |
| 5. Q252H(b) | F              | G1120T           | AGCTGGGCGGGGCCAT | 17      | 62        |
| 6. Y253H    | F              | T1121C           | GCTGGGCGGGGCCCAGC | 17      | 62        |
| 7. Y253F    | F              | A1122T           | CTGGGCGCGGGGCAGT | 17      | 55        |
| 8. E255K    | F              | G1127A           | GCGGGGCGCACAGGGA | 18      | 68        |
| 9. E255V    | F              | A1128T           | GCGGGGCGAGCTGGGGT | 19      | 58        |
| 244 R       | R(1–9)         | –                | GCAATGAAGCCCTGGAC | 19      |           |
| 10. F311L   | F              | T932C            | CACCGGGAGGCCCAC | 17      | 62        |
| –           | R(10)          | –                | CCCCCACTGCTGGTAGGT | 20      |           |
| 11. T315I   | F              | C1308T           | GGGACCTGTATATACATCA | 21      | 63.4      |
| 12. F317L   | F              | C1315G           | CGGTCTATATACATCAGTTG | 24      | 54        |
| 315 R       | R(11–12)       | –                | GGTAGAAAGTTCCTTCCAG | 22      |           |
| 13. M343T   | F              | T1392C           | GTGGTGCTGTGATCAC | 17      | 62        |
| 14. M351T   | F              | T1416C           | CACCTGATCTGTCAGCACC | 22      | 70        |
| 351 R1      | R(13–14)       | –                | GCCCTGAGAATCTTTCAGT | 20      |           |
| 15. E355G   | –              | A1428G           | GTCAACGGTCATGACAGT | 21      | 56        |
| 16. F359V   | F              | T1439G           | GAGTATCTGAGAGAAGAAGA | 22      | 50        |
| 351 R2      | R(15–16)       | –                | ATGCCAAAGGCTGTTTGG | 19      |           |
| 17. H396R   | F              | A1551G           | GGACACTACACAGCCCG | 18      | 62.5      |
| 369 R       | R(17)          | –                | GGACACTACACAGCCCG | 18      |           |
| 18. F486S   | F              | T1821C           | TCTGACCGGCCCTTCCTC | 17      | 62        |
| 486 R       | R(18)          | –                | AGCTTTCTGGATCCAGA | 18      |           |

*Substitutions of amino acids; positions according to GenBank no. AAB60394for ABL type 1a.*

**Changes of nucleotide; positions according to GenBank no. M14752.

ℓL (bp) = Primer length in base pairs.

ℓA Tm = Annealing temperature in degree Celsius.

Statistical Analysis

Various clinical parameters, frequencies of imatinib resistance, and clinical response rates were compared in the two subgroups of patients with and without PEMs by Chi-square test using “Statistical Package for Social Sciences (SPSS)” software, version 17. A p-value of <0.05 was considered significant.

Results

Pre-existing and Post-resistance BCR-ABL Mutations

BCR-ABL PEMs were detected in 32 out of 100 (32%) patients (Table 3). We found three mutations, namely T315I, F311L, and M351T, either alone or in combination, as PEMs in this group of CML patients. The frequencies of the M351T, F311L, and T315I mutations were 87.5%, 50%, and 37.5%, respectively, either alone or in combination. Thus, M351T was the most common PEM, whereas T315I was the least common PEM detected (Figure 2).

After a median follow-up of 30 months (range 8–48), patients with BCR-ABL PEMs exhibited imatinib resistance (32/32, 100%). Upon re-investigation of BCR-ABL mutations in these patients using ASO-PCR and DNA sequencing, all patients had the same PEMs (Figure 1 and 2). Regarding the 68 patients without PEMs, none of them exhibited imatinib resistance (68/68, 0%).
Figure 1. Detection of BCR-ABL mutations by ASO-PCR and DNA sequencing. (-ve control = negative control, bp = base pair, PEM = pre-existing BCR-ABL mutations, C = Cytosine, T = Thymine, A = Adenine, G = Guanine). HL60 cell line was used as a negative control in ASO-PCR and sequencing while KCL 22 was used as positive control in RT-PCR and DNA sequencing.

Figure 2. Comparison of the frequencies of pre-existing BCR-ABL KD mutations and mutations detected after manifestation of imatinib resistance in CML patients.

doi:10.1371/journal.pone.0055717.g001

doi:10.1371/journal.pone.0055717.g002
imatinib resistance developed in 24 (24/68, 35.3%) patients. BCR-ABL mutations (alone or in combination) were found in 22 of these patients (Table 3; Figure 2). By DNA sequencing, we were able to detect Y253F mutation in one of the patients as an acquired mutation (not as a PEM). T315I (12/22, 54.5%) and F311L (15/22, 68.2%) were the most common mutations in this group of patients. Two patients harboring a T315I mutation (alone or in combination with F311L/M351T) did not respond to imatinib dose escalation, whereas four patients had partial cytogenetic responses (4/20, 20%). Fifteen CML patients without PEMs responded to dose escalation with complete hematological, cytogenetic, and molecular responses to imatinib (20/32, 62.5%), 16 patients (16/20, 80%) exhibited complete cytogenetic response; minor CyR: minor cytogenetic response; MMR: major molecular response.

Association of Mutations with Clinical Parameters

No significant association was found between BCR-ABL KD mutations and clinical parameters such as age, gender, type of BCR-ABL splice variant, white blood cell count, hemoglobin level, and platelet count (data not shown). Imatinib-resistant CML patients with and without PEMs significantly differed with respect to frequency of imatinib resistance (100% vs. 33.3%, p = 0.01), CHR (71.9% vs. 91.2%, p = 0.05), partial hematological response (28.1% vs. 4.4%, p = 0.01), minor cytogenetic response (15.6% vs. 8.8%, p = 0.05), and MMR (0% vs. 41.2%, p = 0.001), while no significant difference was found in terms of CCyR (53.1% vs. 55.9%), time to development of resistance (16.5 vs. 18.2 months), and time to progression of disease (27.6 vs. 32.3 months) in the two groups. All CCRs correlated with MMRs on archived samples.

Management of Resistant Patients

Resistant patients were treated with 600–800 mg of imatinib/day irrespective of PEM status. Patients harboring the T315I mutation (alone or in combination with F311L/M351T) did not exhibit any response, and progressed to accelerated-phase or blastic crises (12/37, 32.5%). In this group of patients with F311L/M351T PEMs (20/32, 62.5%), 16 patients (16/20, 80%) exhibited complete hematological, cytogenetic, and molecular responses to dose escalation, whereas four patients had partial cytogenetic responses (4/20, 20%). Fifteen CML patients without PEMs harboring a T315I mutation (alone or in combination with F311L/M351T/Y253F) did not respond to imatinib dose escalation and progressed to an advanced phase, whereas 7 out of 9 (77.8%) patients harboring F311L/M351T mutations responded to dose escalation with complete hematological, cytogenetic, and molecular responses.

Discussion

CP-CML comprises of two types of cells. The majority of cells of the leukemic clone comprise a more mature type that is sensitive to TKIs. A small population of stem/progenitor (CD34+) cells is less sensitive to TKIs and is usually responsible for the development of resistance to therapy [21,22]. The BCR-ABL fusion gene is highly unstable in these primitive CML cells, and it is associated with frequent genetic alterations and mutations in BCR-ABL itself as well as in other genes such as p53 even in the absence of imatinib exposure [30]. These naturally occurring genetic variants of BCR-ABL are known as pre-existing BCR-ABL mutations (PEMs) [7]. Although the mechanism of clinical resistance to imatinib in CML varies widely, BCR-ABL KD point mutations are the leading cause of imatinib resistance [1,11]. If these mutations are present in critical regions of BCR-ABL, they can affect the binding of BCR-ABL protein with TKIs. The impaired binding of imatinib to these BCR-ABL mutants results in an inadequate response or loss of response. The mutant strains proliferate under selective pressure of TKIs after treatment initiation, leading to drug resistance [4,11]. These mutations are likely to be present at an early stage of disease evolution and become clinically manifested due to selective overgrowth after imatinib treatment [20,23].

Our study demonstrated that BCR-ABL PEMs might be found in a substantial number of newly diagnosed CP-CML patients if sensitive techniques such as ASO-PCR are used to assess CD34+ stem/progenitor cells, and these PEMs can significantly affect the outcome of imatinib therapy. BCR-ABL PEMs have been reported previously in newly diagnosed CP-CML patients in some studies [14–15,17,20,31], whereas others failed to detect any mutations in CP-CML patients before treatment initiation despite using sensitive techniques [18]. Most of these studies were limited by a small sample size and CD34+ cell population was not specifically targeted for mutation detection. Ours is the largest study to date on the incidence of naturally occurring BCR-ABL KD mutations using CD34+ cells and their association with imatinib resistance. Although more than 50 BCR-ABL mutations have been reported, we analyzed for the 18 most common mutations as: 1) they cover more than 90% of the mutations responsible for imatinib resistance and not all the mutations are clinically relevant [18,20], 2) these 18 mutations can be detected by ASO-PCR which is the most sensitive technique to detect low copy number mutations like pre-existing BCR-ABL mutations. Furthermore, detection of PEMs using ASO-PCR in a group of CML patients and detection of the same mutations after a period of time after manifestation of imatinib resistance in that group using ASO-PCR as well as sequencing, is an indirect proof of validity of ASO-PCR for PEM detection with a minimal possibility of false positive results, which was done in our study.

Table 3. Clinical, cytogenetic, and molecular follow-up studies of CML patients with and without BCR-ABL PEMs who received imatinib treatment.

| Group(s) | Characteristics | N (%) | Hematological response N (%) | Cytogenetic response N (%) | MMR N (%) |
|----------|-----------------|-------|-----------------------------|---------------------------|----------|
|          |                 |       | CHR | PHR | No HR | CCyR | PCyR | Minor CyR | Minimal CyR |          |
| Group 1  | Patients with PEM (A) | 32 (100) | 23 (71.9) | 9 (28.1) | 17 (53.1) | 7 (21.9) | 3 (9.4) | 5 (15.6) | - |          |
|          | Patients without PEM (B = C+D) | 68 (100) | 62 (91.2) | 3 (4.4) | 3 (4.4) | 38 (55.9) | 19 (27.9) | 5 (7.4) | 6 (8.8) | 28 (63.6) |
|          | Patients with PEM, resistant to imatinib (C) | 24 (68) | 19 (79.2) | 2 (8.3) | 3 (12.5) | 7 (29.2) | 11 (45.8) | 2 (8.3) | 4 (16.7) | - |          |
|          | Patients without PEM, susceptible to imatinib (D) | 44 (68) | 43 (97.7) | 1 (2.3) | - | 31 (70.5) | 8 (18.2) | 3 (6.8) | 2 (4.5) | 28 (63.6) |

N: number of patients, PEMs: pre-existing mutations; IM: imatinib; CHR: complete hematological response; PHR: partial hematological response; CCyR: complete cytogenetic response; MCyR: major cytogenetic response; minor CyR: minor cytogenetic response; MMR: major molecular response.

doi:10.1371/journal.pone.0055717.t003
The reasons for the presence of PEMs in almost one-third of our CP-CML patients are not entirely clear. We selected CD34+ cells to detect KD mutations because this compartment of primitive cells is likely to be the source of many of these mutations [14] and, in combination with the use of a sensitive technique such as ASO-PCR in a larger number of patients, may explain our findings [19]. Furthermore, it is also known that patients with advanced CP-CML are more likely to exhibit various KD mutations and primary resistance [5,33]. Many patients in our area present late due to poor knowledge, lack of education, and the use of traditional remedies before seeking medical advice. Therefore, we cannot rule out the possibility that our patient population may be skewed toward a higher-risk group of CP-CML patients [19]. This could explain the higher mutation detection rate in some of these patients.

To the best of our knowledge, there are no studies of KD mutation detection in CML patients comparing whole mononuclear cells (MNC) with CD34+ cells, so we can only hypothesize that by selecting CD34+ cells, the likelihood of detection of low level mutations is increased. Our findings are also supported by the work performed by Chu et al, who reported that KD mutations, when studied in CD34+ cells, were present even during complete cytogenetic remission in 5 of 13 CML patients treated with imatinib [19]. Recent studies indicate that residual BCR/ABL+ progenitors persist despite undetectable molecular disease in CML patients responsive to imatinib [32]. Furthermore, Jiang et al recently showed that KD mutations were present even in very primitive (CD34+CD38−) stem cells [20]. Overall, these data suggest that CD34+ progenitor/stem cell compartment usually harbors KD mutations in CP-CML, and these low level mutations are more likely to be detected in CD34+ cell population, as compared to MNC. These findings also support the idea that primitive CML cells have an intrinsic tendency to continuously acquire new mutations independent of therapy. Some of the mutations would be expected to confer imatinib resistance; others could lead to disease progression. It is the nature and timing of these mutations at diagnosis and during imatinib treatment that may explain the variable clinical responses in different patients [6,15–16,20,33–34]. Thus, the CML patients labeled clinically as imatinib responders and non-responders display significant differences in the frequencies of mutant BCR-ABL transcripts present in their pretreatment CD34+ cells [20,33].

Different BCR-ABL mutations have prognostic significance and vary in their effects on the sensitivity to standard doses and dose escalations of imatinib and as well as to other TKIs [3,6–7,13,36–39]. All of our resistant patients were treated with imatinib dose escalation to 600–800 mg daily irrespective of their PEM status. We did not have an opportunity to treat imatinib-resistant patients with second-line TKIs because these agents were not obtainable due to the high cost and lack of funding (only imatinib was supplied free of cost to these patients by a non-governmental organization). Twelve patients with T315I PEM (alone or in combination with F311L and/or M351T) did not respond to imatinib dose escalation, and they progressed to accelerated-phase or blast-crisis. In the group of patients with PEM, 16 of 20 patients with F311L and/or M351T mutations exhibited complete hematological, cytogenetic, and molecular responses to dose escalation, whereas the other four patients exhibited partial cytogenetic responses. Fifteen CML patients without PEMs harboring T315I mutation (alone or in combination with F311L, M351T, and/or T253L mutations) did not respond to imatinib dose escalation, as expected, and progressed; whereas 7 out of 9 patients harboring F311L and/or M351T mutations responded to dose escalation, achieving complete hematological and cytogenetic responses. Overall, 31 CML patients remained resistant to imatinib even after dose escalation.

We detected more than one mutation in some of the patients. These mutations could be either in the same clone or two different clones, and cannot be confirmed by direct ASO-PCR or sequencing techniques. In order to know if such multiple mutations in the same patient are present in the same or different clones, one need to sub-clone the PCR fragments, select at least 20 or more different clones and perform sequencing of each clone. Although it may be interesting to study the clone specificity and the response to treatment in patients with “multiple mutations in two or more different clones” and “multiple mutations in the same clone”, the clinical value of determining this clone specificity of multiple mutations remains to be established.

Currently, screening for BCR-ABL mutations is not recommended in newly diagnosed CP-CML patients [24] because the frequency of mutations in these patients was found to be low in previous studies, these mutations may not necessarily correlate with response, and the screening costs are prohibitive [18,24,36,39–40]. According to the European LeukemiaNet guidelines for CML management, mutation analysis of CP-CML patients treated with imatinib should be performed when there is evidence of inadequate response or loss of response [24]. Our study revealed that using sensitive techniques and CD34+ cell population, BCR-ABL KD mutations may be found in a substantial number of patients and correlate with response to imatinib therapy and pre-treatment mutation detection may have important clinical implications in the post-imatinib era. FDA has approved two second-line TKIs–dasatinib and nilotinib–for the frontline treatment of CML. BCR-ABL mutations respond differently to three tyrosine kinase inhibitors approved for first-line treatment of CML, e.g., V253F and F250E mutations resistant to imatinib can respond to nilotinib or/and dasatinib, T315A shows better response to imatinib though resistant to Dasatinib and Nilotinib, while some mutations are less sensitive to nilotinib (E255K/V and F359V/C) or dasatinib (F317L and V299L) [41]. In this scenario, knowledge about the presence and type of mutations may facilitate timely decision making regarding the choice of first-line therapy at the time of diagnosis. Patients with mutations known to confer resistance to standard or high doses of imatinib can benefit from an upfront treatment with a second-line TKIs and vice versa. For patients with mutations such as T315I which is known to confer resistance to all TKIs currently approved for first-line treatment of CML, one of the newer agents such as ponatinib (AP24534) which is effective against this mutation [41] and very recently been approved by FDA for TKI-resistant CML [42], or allogeneic transplantation must be considered.

Second-generation TKIs induce cytogenetic responses in around 50% of patients with CP-CML in whom imatinib treatment has failed. Although two of the second lines TKIs have been approved for first line therapy of CML, we still find the applicability of this study for the future because of the cost issues. Imatinib patent is about to finish in the near future and with the availability of generic forms of imatinib, the cost difference between imatinib and 2nd line TKIs is going to be substantial. Stratification of patients based on mutations before the start of therapy may have significant cost savings.

We acknowledge the fact that there is high incidence of imatinib resistance in our study patients. Patients with CML vary in their response to treatment and although the basis for this variation is not known, it has been attributed to the biologic heterogeneity of the disease. Some of the factors implicated in poor response to CML therapy include low level of expression of molecular
transporter hOCT1 and multidrug resistance gene (MDR1) polymorphisms [43–44]. Population based studies have shown lower efficacy of imatinib in CML patients when compared to the clinical trial results. Lucas et al reported 49% imatinib failure by 24 months and suggested caution in extrapolating clinical trial data to the general CML population [45]. Possible causes of inferior results in the community setting include less strict conditions than in the clinical trials, lesser motivation and poorer compliance with the treatment. Marin et al recently showed that lack of adherence to treatment was an important factor leading to poor results in CML patients [46]. Poor compliance, inclusion of patients in the late chronic phase and genetic variability are the possible explanations for high resistance in our study.

In summary, we found that by using sensitive techniques like ASO-PCR in CD34+ cells, BCR-ABL KD mutations could be detected in almost one-third of CP-CML patients at the time of diagnosis and were found to be associated with the outcome of imatinib therapy. Therefore, testing for BCR-ABL mutations in CD34+ CML stem/progenitor cells using validated sensitive assays like allele-specific PCR may be cost-effective and should be considered before the start of TKI therapy, particularly in patients who present in the late CP. Larger population-based studies with longer follow-up times are needed to estimate the true incidence of KD mutations in this group of patients and determine whether screening is useful in management planning in present scenario of availability of second generation and third generation TKIs for different resistant forms of CML.

Acknowledgments

We acknowledge the help and collaboration of Professor Moustapha Kassem working at Department of Endocrinology Molecular Endocrinology Laboratory (KMEB), Department of Endocrinology, Odense University Hospital and University of Southern Denmark, and visiting professor at Stem Cell Unit, Department of Anatomy, College of Medicine, King Saud University and King Khalid University Hospital, Riyadh, Kingdom of Saudi Arabia, for CML stem/progenitor cells isolation and characterization. Help and collaboration of Professor Sai-Juan Chen & Prof Zhu Chen, Directors Shanghai Institute of Hematology, Rui-Jin Hospital, Shanghai Jiao-Tong University School of Medicine, China for giving us an opportunity of training in leukemia stem cell characterization, BCR-ABL mutation detection and functional characterization of leukemia genes. This work was partially supported by the College of Medicine Research Center, Deanship of Scientific Research, King Saud University, Riyadh, Saudi Arabia. Research funding provided by Higher Education Commission Pakistan is also acknowledged.

Author Contributions

Conceived and designed the experiments: ZI MI RH MNA. Analyzed the data: ZI MI AA MIN AHT AST AQ NUR IHS M. Khalid SMB AMK AJ MNA MA TA AM M. Khan MR. Contributed reagents/materials/analysis tools: ZI MI RH M. Khan MNA. Wrote the paper: ZI AA MA AM.

References

1. Goldman JM, Melo JV (2003) Mechanisms of Disease: Chronic myeloid leukemia-advances in biology and new approaches to treatment. N Engl J Med 349: 1451–1464.
2. Goldman JM (2007) How I treat chronic myeloid leukemia in the imatinib era. Blood 110: 2929–2937.
3. Kantarjian H, Cortes J (2008) BCR-ABL tyrosine kinase inhibitors in chronic myeloid leukemia: using guidelines to make rational treatment choices. J Natl Compr Canc Netw 6: Suppl 2: S37–S42; quiz S15–S14.
4. Gorre ME, Mohammed M, Elwood K, Hsu N, Paquette R, et al. (2001) Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science 293: 876–880.
5. Soverini S, Colarossi S, Gnaill R, Rosti G, Castagnetti F, et al. (2006) GIMEMA Working Party on Chronic Myeloid Leukemia. Contribution of ABL kinase domain mutations to imatinib resistance in different subtypes of Philadelphia-positive patients: by the GIMEMA Working Party on Chronic Myeloid Leukemia. Clin Cancer Res 12: 7347–7359.
6. Shah NP, Nicoll JM, Nagar B, Gorre ME, Paquette RL, et al. (2002) Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. Cancer Cell 2: 117–125.
7. Kantarjian HM, Talpaz M, O’Brien S, Giles F, Garcia-Manero G, et al. (2005) Dose escalation of Imatinib mesylate can overcome resistance to standard-dose therapy in patients with chronic myelogenous leukemia. Blood 101: 473–475.
8. Baranńska M, Lewandowski K, Gnaill R, Isola M, Lewandowska M, et al. (2005) Komarnicki M. Dasatinib treatment can overcome imatinib and nilotinib resistance in a CML patient carrying F359I mutation of BCR-ABL oncogene. J Appl Genet 49: 201–203.
9. Cortes J, Kantarjian H (2008); Beyond dose escalation: clinical options for relapse or resistance in chronic myelogenous leukemia. J Natl Compr Canc Netw 6: Suppl 2: S27–S30.
10. Nicollini FE, Corm S, Leˆ QH, Sorel N, Hayette S, et al. (2006) Mutation status and clinical outcome of 89 imatinib myelosup-pressant resistant chronic myelogenous leukemia patients: a retrospective analysis from the French intergroup of CML (EFS/LMC MRGTP). Leukemia 20: 1061–1066.
11. Apperley JF (2007) Part I: Mechanisms of resistance to imatinib in chronic myeloid leukemia. Lancet Oncol 8: 1018–1029.
12. Pfeffer H, Wassmann B, Pavlova A, Wunderle L, Oldenburg J, et al. (2007) Kinase domain mutations of BCR-ABL frequently precede imatinib-based therapy and give rise to relapse in patients with de novo Philadelphia-positive acute lymphoblastic leukemia (Ph+ ALL). Blood 110: 727–734.
13. Soverini S, Vitale A, Porro A, Gnaill R, Colarossi S, et al. (2011) Philadelphia-positive acute lymphoblastic leukemia patients already harbor BCR-ABL kinase domain mutations at low levels at the time of diagnosis. Haematologica 96: 532–537.
14. Roche-Lestienne C, Soenen-Cornu V, Gradel-Duflous N, Lal JL, Philippe N, et al. (2002) Several 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 100: 1014–1018.
15. Roche-Lestienne C, Lal JL, Darré S, Facon T, Preudhomme C (2003) A mutation conferring resistance to Imatinib at the time of diagnosis of chronic myelogenous leukemia. N Engl J Med 348: 2926–2926.
16. Kreuzer KA, Le Coutre P, Landt O, Na IK, Schwarz M, et al. (2003) Preexistence and evolution of imatinib myelosuppressant-resistant clones in chronic myelogenous leukemia detected by a PNA-based PCR clamping technique. Ann Hematol 82: 284–289.
17. Carella AM, Garuti A, Cirmena G, Catania G, Rocco I, et al. (2010) Kinase domain mutations of BCR-ABL identified at diagnosis before imatinib-based therapy are associated with progression in patients with high Sokal risk chronic phase chronic myeloid leukemia. Leuk Lymphoma 5: 275–278.
18. Willis SG, Lange T, Demelini S, Otto S, Crossman L, et al. (2005) High-sensitivity detection of BCR-ABL kinase domain mutations in imatinib-naive patient’s correlation with clonal cytogenetic evolution but not response to therapy. Blood 106: 2128–2133.
19. Chu S, Xu H, Shah NP, Snyder DS, Forman SJ, et al. (2005) Detection of BCR-ABL kinase mutations in CD34+ cells from chronic myelogenous leukemia patients in complete cytogenetic remission on imatinib mesylate treatment. Blood 105: 2993–2998.
20. Jiang X, Forrest D, Nicolini F, Turhan A, Guilhot J, et al. (2010) Properties of CD34+ CML stem/progenitor cells that correlate with different clinical responses to imatinib mesylate. Blood 116: 2112–2121.
21. Graham SM, Jorgensen HG, Allan E, Pearson C, Akram MJ, et al. (2002) Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. Blood 99: 319–325.
22. Copeland M, Hamilton A, Elrick JI, Baird JW, Allan EK, et al. (2006) Dasatinib (BMS-554825) targets an earlier progenitor population than imatinib in primary CML, but does not eliminate the quiescent fraction. Blood 107: 4532–4539.
23. Jiang X, Zhao Y, Smith C, Gasparetto M, Turhan A, et al. (2007) Chronic myeloid leukemia stem cells possess multiple unique features of resistance to BCR-ABL targeted therapies. Leukemia 21: 926–933.
24. Baccarani M, Saglio G, Goldman J, Hochhaus A, Simonsson B, et al. (2006) European LeukemiaNet. Evolving concepts in the management of chronic myeloid leukemia: Recommendations from an expert panel on behalf of the European LeukemiaNet. Blood 108: 1809–1820.
25. Branford S, Rudzik Z, Parkinson I, Grigg A, Taylor K, et al. (2004) Real-time quantitative PCR analysis can be used as a primary screen to identify patients with CML treated with imatinib who have BCR-ABL kinase domain mutations. Blood 104: 2926–2932.
26. Holyoake TL, Jiang X, Jorgensen HG, Graham S, Akram MJ, et al. (2001) Primitive quiescent leukemia cells from patients with chronic myeloid leukemia spontaneously initiate factor-independent growth in vitro in association with up-regulation of expression of interleukin-3. Blood 97: 720–728.
Natural BCR-ABL Mutants in Newly Diagnosed CP-CML

PLOS ONE | www.plosone.org 9 February 2013 | Volume 8 | Issue 2 | e55717

27. Hao QL, Smogorzewska EM, Barsky LW, Crooks GM (1998) In vitro identification of single CD34\(^+\)CD38\(-\) cells with both lymphoid and myeloid potential. Blood 91: 4145–4151.
28. Radich JP, Gehly G, Gooley T, Bryant E, Cillit RA, et al. (1995) Polymerase chain reaction detection of the BCR-ABL fusion transcript after allogeneic marrow transplantation for chronic myeloid leukemia: results and implications in 346 patients. Blood 85: 2632–2638.
29. Kang HY, Hwang JY, Kim SH, Geh HG, Kim M, et al. (2006) 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: 659–662.
30. Iqbal Z, Siddique RT, Qureshi AM, Khalid AM (2004) Case study of primary imatinib resistance and correlation of BCR-ABL multiple mutations in chronic myeloid leukemia. Therapy 1: 249–254.
31. Wongboonma W, Thongnoppakhun W, Auewarakul CU (2012) BCR-ABL kinase domain mutations in tyrosine kinase inhibitor-naive and -exposed Southeast Asian chronic myeloid leukemia patients. Exp Mol Pathol 92: 259–265.
32. Chu S, McDonald T, Lin A, Chakraborty S, Huang Q, et al. (2011) Persistence of leukemia stem cells in chronic myelogenous leukemia patients in prolonged remission with imatinib treatment. Blood 118: 5565–5572.
33. Leder K, Fojo T, Skaggs B, Gorre M, Sawyer CL, et al. (2011) Fitness conferred by BCR-ABL kinase domain mutations determines the risk of pre-existing resistance in chronic myeloid leukemia. PLoS One 6: e27662.
34. Branford S, Hughes T (2006) Detection of BCR-ABL mutations and resistance to imatinib mesylate. Methods Mol Med125: 93–106.
35. Jiang X, Saw KM, Eaves A, Eaves C (2007) Instability of BCR-ABL gene in primary and cultured chronic myeloid leukemia stem cells. J Natl Cancer Inst 99: 689–693.
36. Sorel N, Bonnet ML, Guiller M, Guilhot F, Brizard A, et al. (2004) AG. Evidence of ABL-kinase domain mutations in highly purified primitive stem cell populations of patients with chronic myelogenous leukemia. Biochem Biophys Res Commun 323: 728–730.
37. Khorashad JS, de Lavalade H, Apperley JF, Milojkovic D, Reid AG, et al. (2008) Finding of kinase domain mutations in patients with chronic phase chronic myeloid leukemia responding to imatinib may identify those at high risk of disease progression. J Clin Oncol 26: 4806–4813.
38. O’Brien S, Berman E, Moore JO, Pinilla-Ibarz J, Radich JP, et al. (2011) NCCN Task Force Report: Tyrosine kinase inhibitor therapy selection in the management of patients with chronic myelogenous leukemia. J Natl Compr Canc Netw 9: S1–S25.
39. Hughes T, Saglio G, Branford S, Soverini S, Kim DW, et al. (2009) Impact of baseline BCR-ABL mutations on response to nilotinib in patients with chronic myeloid leukemia in chronic phase. J Clin Oncol 27: 4204–4210.
40. Sherbenou DW, Wong MJ, Hanawya A, McGreevy LS, Harrell P, et al. (2007) Mutations of the BCR-ABL-kinase domain occur in a minority of patients with stable complete cytogenetic response to imatinib. Leukemia 21: 489–493.
41. O’Hare T, Shakespeare WC, Zhu X, Ede CA, Rivera VM, et al. (2009) AP24534, a pan-BCR-ABL inhibitor for chronic myeloid leukemia, potently inhibits the T315I mutant and overcomes mutation-based resistance. Cancer Cell 16: 401–412.
42. Dolgin E (2013) As leukemia options grow, drugs jockey to be first-line therapies. Nature Medicine 19, 7–7; doi: 10.1038/nm0113–7.
43. Wang L, Giannoudis A, Lane S, Williamson P, Firoz Mohamed M, et al. (2008) Expression of the uptake drug transporter hOCT1 is an important clinical determinant of the response to imatinib in chronic myeloid leukemia. Clin Pharmacol Ther 83: 250–264.
44. Duhooq S, Bouchet S, Turcq B, Lippert E, Etienne G, et al. (2008) Multidrug resistance gene (MDR1) polymorphisms are associated with major molecular responses to standard-dose imatinib in chronic myeloid leukemia. Blood 112: 2024–2027.
45. Lucas GM, Wang L, Austin GM, Knight K, Wattmough SJ, et al. (2008) A population study of imatinib in chronic myeloid leukemia demonstrates lower efficacy than in clinical trials. Leukemia 22: 1963–1966.
46. Marin D, Bazeos A, Mahon FX, Ellason L, Milojkovic D, et al. (2010) Adherence is the critical factor for achieving molecular responses in patients with chronic myeloid leukemia who achieve complete cytogenetic responses on imatinib. J Clin Oncol 28: 2381–2388.