Detection of BCR-ABL kinase domain mutations in CD34$^+$ cells from newly diagnosed chronic phase CML patients and their association with imatinib resistance

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ABSTRACT

BCR-ABL kinase domain (KD) mutations, the most common cause of imatinib resistance, are infrequently detected in newly diagnosed chronic-phase chronic myeloid leukemia (CP-CML) patients. Recent studies indicate pre-existing mutations (PEMs) can be detected in a higher percentage of CML patients using $CD34^+$ stem/progenitor cells, and these mutations may correlate with imatinib resistance. We investigated KD mutations in $CD34^+$ stem cells from 100 CP-CML patients by multiplex ASO-PCR and sequencing ASO-PCR products at the time of diagnosis. PEMs 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 PEMs exhibited imatinib resistance. Of 68 patients without PEMs, 24 developed imatinib resistance. Mutations were detected in 21 of these patients by ASO-PCR and KD sequencing. All 32 patients with PEMs had the same mutations. In imatinib-resistant patients without PEMs, we detected F311L, M351T, Y253F, and T315I mutations. All imatinib-resistant patients without T315I and Y253F mutations responded to imatinib dose escalation. In conclusion, BCR-ABL PEMs can be detected in a substantial number of CP-CML patients when investigated using $CD34^+$ stem/progenitor cells. These mutations are associated with imatinib resistance, and mutation testing using $CD34^+$ cells may facilitate improved, patient-tailored treatment.

Keywords: BCR-ABL, Chronic myeloid leukemia, chronic phase, kinase domain mutations, imatinib, $CD34^+$ cells, pre-existing mutations.

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 mutation [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 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]. Recent 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 [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.

MATERIALS AND METHODS

Patients and definitions

One hundred newly diagnosed CP-CML patients were included in the study. All patients gave informed consent, and the study was approved by the institutional ethics committees of the four participating centers. All patients had newly diagnosed CP-CML at the time samples were taken, 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 [24-26]. Complete hematologic response (CHR), complete cytogenetic response (CCR), and a partial cytogenetic response were defined according to previously published response criteria [24-26]. Briefly, CHR required the normalization of blood counts: leucocytes counts <10,000/mm^3; normal differential counts without blasts, promyelocytes, or myelocytes; platelet counts from 150,000/mm^3 to 450,000/mm^3; and no evidence of extramedullary disease. CCyR was defined as 0% Ph^+ cells in metaphase BM samples, and a partial cytogenetic response (PCyR) was defined as presence of 0–35% 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). Complete molecular response (CMR) was defined as BCR-ABL fusion transcript negativity according to nested reverse transcriptase-PCR. We could not record the major molecular response due to the non-availability of real-time quantitative PCR.

Resistance patterns were adopted as defined by the LeukemiaNet guidelines [25]. 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 [27].

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 in fetal calf serum (FCS) and cryopreserved in liquid nitrogen until required [28]. 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) [29].

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 [30]. RNA and DNA quality was checked by spectrophotometry, gel electrophoresis, and by the amplification of the ABL gene [14, 30]. As BCR-ABL PEMs are 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, 31-32]. PCR amplifications were performed exactly as reported,

without changing any of the reagents, PCR mix formulations and thermal profile[31]. A 30-µL mixture PCR mix was prepared as: 2 µL of DNA (corresponding to 100 ng of DNA); 1× TaqGold reaction buffer (Applied Biosystems, Foster City, CA); 1.5 mM $MgCl_2$; 250 mM each of dATP, dCTP, dGTP, and dTTP (Pharmacia, Uppsala, Sweden); 0.5 U of AmpliTaq Gold polymerase (Applied Biosystems); and 50 pmol of forward and reverse ASO primer for each mutation. Thermocycling conditions consisted of 5 min at 94°C, 30 cycles of denaturation at 94°C for 25 s, annealing at the indicated temperature for 25 s, and extension at 72°C for 30 s, and a final extension for 5 min at 72°C. The sequences of ASO primers specific for each mutation with the corresponding annealing temperatures are given in Table 2. HL60 cell line was used as negative control in ASO-PCR reactions. Although we use pre-validated ASO-PCR assays as 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 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, 31, 33].

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, 31-32] 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 [33] using an automated ABI377 sequencer (Applied Biosystems). HL60 cell line was used as a negative control in PCR and sequencing while KCL22 cell line was used as a positive control. Sequences were analyzed with Sequence Analysis software V3.3 and Sequence Navigator software V1.0.1. 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, 31, 33].

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 (Figure 2 and 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 12–18 months of imatinib treatment (400 mg/day), all 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 1-3). Regarding the 68 patients without PEMs, imatinib resistance developed in 24 (24/68, 35.3%) patients. BCR-ABL mutations (alone or in combination) were found in 21 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 (12/21, 57.1%) and F311L (15/21, 71.5%) were the most common mutations in this group of patients, whereas M351T was detected in 11/21 (52.4%) 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. Imatinib-resistant CML patients with and without PEMs significantly differed with respect to time-to-detection-of-mutations (0 vs. 22.4±4.1 months, p=0.001), 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%) in the two groups,

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-crises (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 [34]. These naturally occurring genetic variants of BCR-ABL are known as pre-existing BCR-ABL mutations (PEMs) [14]. 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 in 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], 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, 3) we did not have facilities like denaturing HPLC to detect each and every mutation. Furthermore, detection of PEMs using ASO-PCR in a group of CML patients and detection of the same mutations after a period of time 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. 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]. Many patients in our area present late due to a lack of education, poor knowledge, 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. It is tempting to speculate that this patient population may have a different disease biology, but we have no convincing evidence to support this notion [26].

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]. Recently, Jiang et al showed for the first time that these mutations were present even in very primitive ($CD34^-CD38^-$) stem cells [20]. These findings 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, 34-35]. 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, 34].

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 is 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 this group of the patients with F311L and/or M351T mutations, 16 of 20 patients 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.

Currently, screening for BCR-ABL mutations is not recommended in newly diagnosed CP-CML patients [25] 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, 25, 36, 39]. 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 [25]. Our study revealed that using sensitive techniques, BCR-ABL KD mutations may be found in a substantial number of patients and correlate with the response to imatinib therapy. After the recent approval of two second-line TKIs—dasatinib and nilotinib—by the FDA for the frontline treatment of CML, knowledge about the presence and type of mutations may facilitate timely decision making regarding the choice of 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 TKI. For patients with mutations such as T315I, which is known to confer resistance to all currently approved TKIs, one of the newer agents such as ponatinib (AP24534) which is effective against this mutation [40-41], or allogeneic transplantation must be considered.

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 [42,43]. Population based studies have shown lower efficacy of imatinib in CML patients when compared to the clinical trial results. Lucas et el reported 49% imatinib failure by 24 months and suggested caution in extrapolating clinical trial data to the general CML population [44]. 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 [45]. 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 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.

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CONFLICT OF INTEREST:

No conflict of interest by any of the authors related to this article.

AUTHORS' CONTRIBUTIONS:

ZI planned the study, designed and performed the experiments, analyzed the data, wrote the paper, and supervised the overall work; MI performed the experiments and analyzed the data; AA analyzed the data and wrote the paper; MIN analyzed the data; AHT analyzed the clinical data; TJG analyzed the clinical data; AST analyzed the clinical data; AQ analyzed the clinical data; NUR analyzed the data; MF performed the experiments; HIS analyzed the clinical data; MK analyzed the clinical data; WXQ analyzed the data; AMK analyzed the data; MK performed experiments; SMB analyzed the data; AJ analyzed the clinical data; MNA performed the experiments and analyzed the experimental data; TA analyzed the data and supervised the overall work.

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Table 1. Patient characteristics

Patient characteristics		**Number (n=100)**
Age (years)	Median	35
	Range	12-70
Gender	Male	69
	Female	31
Splenic enlargement		87
Hemoglobin < 10.0 g/dl		55
WBC count (mm^3)	50-100	15
	>100	72
Platelet count	100-450	72
	>450	19
Mode of diagnosis		
Philadelphia chromosome positive (Ph+)		99
BCR-ABL fusion oncogene positive (BCR/ABL+)		100
BCR-ABL splice variants		
	b2a2	37
	b3a2	63
Follow-up (months)	Median	30
	Range	8-48

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	Length (bases)	Annealing temperature
1. M244V-F	Forward	A1094G	GAACGCACGGACATCACC**G**	19	65.7
2. L248V	Forward	C1106G	ACCATGAAGCACAAG**G**	16	55
3. G250E	Forward	G1113A	GAAGCACAAGCTGGGCG**A**	18	56
4. Q252H(a)	Forward	G1120C	AGCTGGGCGGGGGCCA**C**	17	62
5. Q252H(b)	Forward	G1120T	AGCTGGGCGGGGGCCA**T**	17	62
6. Y253H	Forward	T1121C	GCTGGGCGGGGGCCAG**C**	17	62
7. Y253F	Forward	A1122T	CTGGGCGGGGGCCAGT**T**	17	55
8. E255K	Forward	G1127A	GCGGGGGCCAGTACGGG**A**	18	68
9. E255V	Forward	A1128T	GCGGGGGCCAGTACGGGG**T**	19	58
244 R	Reverse (for 1-9)		GCCAATGAAGCCCTCGGAC	19	
10. F311L	Forward	T932C	CACCCGGGAGCCCCCG**C**	17	62
	Reverse (for 10)		CCCCTACCTGTGGATGAAGT	20	
11. T315I	Forward	C1308T	GCCCCCGTTCTATATCATCA**T**	21	63.4
12. F317L	Forward	C1315G	CCGTTCTATATCATCACTGAGTT**G**	24	54
315 R	Reverse (for 11-12)		GGATGAAGTTTTTCTTCTCCAG	22	
13. M343T	Forward	T1392C	GTGGTGCTGCTGTACA**C**	17	62
14. M351T	Forward	T1416C	CCACTCAGATCTCGTCAGCCA**C**	22	70
351 R1	Reverse (for 13-14)		GCCCTGAGACCTCCTAGGCT	20	
15. E355G	Forward	A1428G	GTCAGCCATGGAGTACCTAG**G**	21	56
16. F359V	Forward	T1439G	GAGTACCTAGAGAAGAAAAAC**G**	22	50
351 R2	Reverse (for 5-16)		ATGCCCAAAGCTGGCTTTG	19	
17. H396R	Forward	A1551G	GGACACCTACACAGCCC**G**	18	62.5
369 R	Reverse (for 17)		GGACACCTACACAGCCCG	18	
18. F486S	Forward	T1821C	TCTGACCGGCCCTCCT**C**	17	62
486 R	Reverse (for 18)		AGCTTTCTGGTCTCAGGA	18	

*Substitutions of amino acids; positions according to GenBank no. AAB60394

for ABL type 1a.

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

M14752.

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

Group	Subcategory	Number of patients (%)	Hematological response Number (%)			Cytogenetic response Number (%)				CMR Number (%)
			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 (41.2)
	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)

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; CMR: complete molecular response

FIGURE LEGENDS

Figure 1. Detection of BCR-ABL mutations by ASO-PCR and DNA sequencing (-ve control= (negative control). HL60 cell line used as a negative control in ASO-PCR and 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.

Figure 3. Comparison of patients with and without BCR-ABL PEMs in relation to imatinib resistance.

Figure 1A: Mutation T3151 (1-3: PEM; 4: -ve control; 5: 100bp DNA Ladder; 6-8: Mutations after manifestation of IM resistance (IMR))

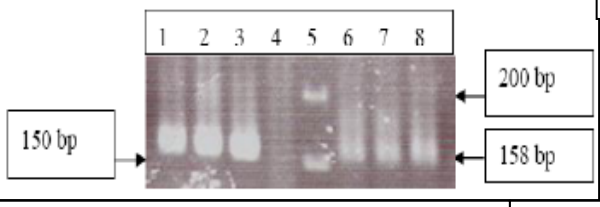

Figure 1B: Mutation F311L (1: -ve control; 2: 100bp DNA Ladder; 3-4: PEM; 5-6: Mutations after manifestation of IMR)

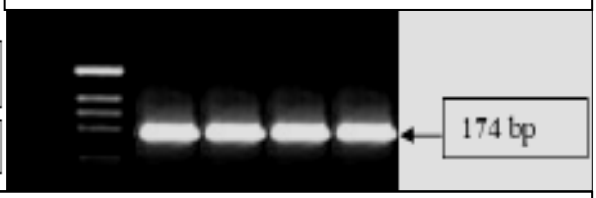

Figure 1C: Mutation M351T (1: PEM; 2: Mutation after manifestation of IMR; 3: -ve control; 5:100bp DNA adder

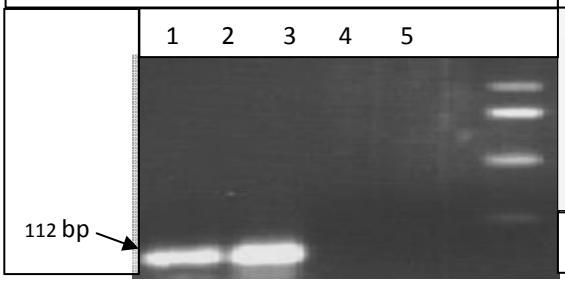

Figure 1D: Confirmation of PEM by DNA sequencing