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Survival assessment and Optimization of BCR/ABL-KD amplification protocol for detection of Imatinib resistant mutations in Ph+ Chronic Myeloid Leukemia patients from Pakistan

Research Article

Afia Muhammad Akram[1*], Tanveer Akhtar[1] and Zafar Iqbal [2]

1Department of Zoology, Faculty of Biological Sciences, University of the Punjab, Lahore-54590, Pakistan.

2College of Applied Medical Sciences, King Saud bin Abdulaziz University for Health Sciences, Riyadh, Saudi Arabia.

***Correspondence:** Afia Muhammad Akram, Department of Zoology, Faculty of Biological Sciences, University of the Punjab, Lahore-54590, Pakistan. Phone: +92 3324794782 Email address: afiamakram@gmail.com;
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Summary

Translocation between parts of BCR and ABL genes is the baseline abnormality for chronic myeloid leukemia (CML) genesis. To overcome this malignancy, Imatinib mesylate, a tyrosine kinase inhibitor (TKI) is being used as the first line treatment option. Certain point mutations arising in the kinase domain of ABL part constitute resistance against drug therapy. By knowing the underlying mutation, resistance can be addressed either by dose adjustment or choosing second generation TKI's. The present studies aimed to investigate survival probability of patients in relation to their clinical features and to optimize an efficient as well as reliable protocol for RT-PCR based amplification of BCR-ABL kinase domain, and its direct sequencing analysis for mutation detection. Since this procedure has been established for the first time in Pakistan, reproducible and amplifiable products of 1306bp (b2a2) and 1380bp (b3a2), carrying BCR-ABL KD were successfully achieved after trial and error. Their sequencing analysis revealed a total of fourteen point mutations, six in Imatinib responders and eight in resistant CML patients. Thus the mutation detection supported the usefulness of this protocol in both, Imatinib sensitive and resistant patients of chronic myeloid leukemia.

I. Introduction:

Chronic myeloid leukemia (CML) is a stem cell disorder of hematopoietic linage, characterized by unnecessary proliferation of white blood cells, mainly the myeloid cells (Florean et al. 2011). The chromosomal translocation between chromosome 9 and 22 i.e. t(9;22)(q34;q11) forms juxtaposition of ABL1 (Abelson murine leukemia) gene on chromosome 9 and BCR (Break Point Cluster Region) gene on chromosome 22 (Hehlmann et al. 2007). The resultant fusion oncogene BCR-ABL anchored in chromosome 22 also known as Philadelphia chromosome (Ph) is the distinctive feature of CML (Nowell, 1960). This rearrangement of genes induces a conformational variation in ABL protein, which makes it a hyperactive tyrosine kinase enzyme. The tyrosine kinase activity of BCR-ABL fusion oncogene blocks apoptosis and activates the pathophysiological cascade of CML (Cang and Liu, 2008; Jabbour and Kantarjian, 2012). To overcome this malignancy a molecularly targeted drug, Imatinib mesylate (Gleevec®/Glivec®, Novartis, Hanover, NJ, USA) formerly called STI571 is recommended as first line treatment option. Imatinib specifically combines to the ATP binding site of BCR-ABL fusion oncoprotein and inactivates the surge by restraining phosphorylation of tyrosine residues of the substrate (Zoubir et al. 2010; Horne et al. 2013). Patient's response towards therapy varies with respect to disease phase, so the chronic phase patients show most effectiveness (Gugliotta et al. 2011). Some individuals do not respond to Imatinib (IM) therapy at all which is termed as primary resistance, whereas some may respond at first but relapse occurs later on, known as secondary or acquired resistance (Quintás-Cardama et al. 2009; Baccarani et al. 2013). Point mutations present in BCR-ABL kinase domain (KD) are the major cause of IM resistance. Genetic study of this particular domain in IM resistant CML patients have revealed more than 100 point mutations translating altered amino acids. Various techniques, like DNA sequencing, denaturing HPLC and other methods have been established for the purpose of identifying possible point mutations (Volpe et al. 2009). Such substituted nucleotides affect imatinib sensitivity, by altering amino acids involved in drug binding or in regulatory regions of ABL-KD (Moore et al. 2013). In Pakistan, Imatinib is being used as first line tyrosine kinase inhibitor for treatment of CML patients as per FDA recommendations (Aziz et al. 2007). Cytogenetic analysis of bone marrow and fluorescence *in situ* hyberdization (FISH) are routinely practiced to diagnose and monitor CML patients receiving imatinib treatment. However, use of molecular methods like real time quantitative PCR for drug response monitoring and BCR-ABL mutation testing for management of drug resistance in CML are not available anywhere in the country, possibly due to shortage of trained manpower and laboratories with facilities for molecular analysis (Usmani et al. 2009). According to recommendations of European LeukemiaNet (ELN) and National Comprehensice Cancer Network (NCCN) USA, chronic and/or advanced phase CML patients undergoing imatinib or other TKI therapy should be screened for BCR-ABL KD mutations for a better management of TKI resistance. They can show a better disease prognosis either by dose escalation or switching over to second line tyrosine kinase inhibitors like Nilotinib, Dasatinib, Bosutinib and Ponatinib (Khorashad et al. 2008; Soverini et al. 2011). As medical facilities in Pakistan are tolerated by the patients themselves, use of commercial kits for PCR or sequencing based therapy monitoring could not have been applied. Therefore, there was a need to develop a low-cost PCR and BCR-ABL KD sequencing protocol for monitoring TKI response and

determination of drug resistance in Pakistani CML patients undergoing imatinib therapy.

Primary concern of the present investigation was to estimate survival outcome of chronic myeloid leukemia patients in relevance to their different clinical features. In addition, we focused to optimize a resourceful method for BCR-ABL kinase domain amplification and direct sequencing analysis, keeping in view the socio-economic circumstances of the country. This will help physicians to develop a better understanding of disease prognosis and management of drug resistance.

II. Materials and Methods:

A. Sample collection

A total of 125 CML patients receiving imatinib treatment from Jinnah hospital and Mayo hospital, Lahore were included in this study. Permission from hospital authorities and patient consent was obtained to collect blood samples. Clinical characteristics of concerned patients are given in Table 1.

B. Survival estimation by statistical analysis of clinical data

Various clinical features of CML patients at the time of diagnosis were noted. Cytogenetic and hematologic response definitions were followed from previously published response criteria (Baccarani et al. 2006; O'Brien et al. 2009; Baccarani et al. 2013; Iqbal et al. 2013). Kaplan-Meier survival analysis (IBM SPSS version 19, Chicago, IL, USA) was performed to estimate patient's survival duration with reference to their clinical features and disease conditions.

C. Selection of Samples for protocol optimization

To standardize the amplification and sequencing assay for BCR-ABL kinase domain, the collected samples were categorized into two groups i.e. Imatinib responder and resistant CML patients with treatment duration of 6 months and onwards. Ten samples from each group were selected for the procedure. Sample numbers CML 57, 66, 67, 68, 69, 77, 79, 81, 85 and 88 represent those showing primary response to tyrosine kinase inhibitor Imatinib. Whereas, sample numbers CML 93, 97, 107, 109, 115, 116, 118, 121, 124 and 129 were Imatinib resistant patients.

D. RNA Isolation, complementary DNA synthesis and its integrity check

Isolation of RNA from blood samples was performed using TRIzol® LS reagent (Chomczynski and Sacchi, 1987; Liedtke et al. 1994) with some modifications. Quantitative analysis of RNA was performed spectrophotometrically at 260/280 wavelength, while its quality was assessed by native agarose gel electrophoresis (Smithies, 1955).

Complementary DNA was prepared by reverse transcription of RNA, to be used as template in nested PCR reaction. The RT-reaction protocol was adopted with slight variations (Van Dongen et al. 1999). The integrity of cDNA was evaluated by amplification of housekeeping genes GAPDH. The primer sequences GAPDH forward: 5′-ACCACAGTCCATGCCATCA-3′ and GAPDH reverse: 5′-TCCACCACCCTGTTGCTGTA-3′ were used in the reaction (Asad et al. 2012).

E. Optimization of nested RT-PCR amplifications

The primers used in first round of nested PCR were B2A forward: 5′-TTCAGAAGCTTCTCCCTGACAT-3′ and Abl4065 reverse: 5′-CCTTCTCTAGCAGCTCATACACCTG-3′. While, for the second round BcrF4 forward: 5′-ACAGCATTCCGCTGACCATCAATA-3′ and U396 reverse: 5′GCCATAGGTAGCAATTTCCC-3′ was used (Willis et al. 2005).

To amplify whole BCR-ABL fusion gene it was necessary to select a long range enzyme . For this we selected Long PCR enzyme mix by Thermo Scientific™, USA. The reagent concentrations for the nested PCR reaction were standardized as; 5µl of 10X Long enzyme buffer with $MgCl_2$, 5µl dNTPs (2mM), 1µl of each forward and reverse primers (20pMol), 0.5µl Long PCR enzyme Mix (2.5u), 1µl cDNA as a templetate and Nuclease free water to make the total reaction volume upto 50µl. Thermal cycling profile for nested PCR was standardized after executing several annealing temperatures to minimize the arrival of non-specific DNA bands. The optimized thermal profile for the First round of RT-PCR was; Preliminary denaturation at 94°C for 3 min followed by 10 cycles of denaturation of double stranded DNA at 94°C for 20 sec, annealing of primers to DNA template at 62°C for 30 sec and extension to form multiple copies of DNA strands at 68°C for 1:30 min, followed by 20 cycles of denaturation of double stranded DNA at 94°C for 20 sec, annealing at 62°C for 30 sec and extension at 68°C for 3 min, followed by a post amplification extension at 68ºC for 10 min and hold at 4°C for infinite (Applied Biosystems 2720 thermal cycler). The second round of reaction was carried out with the same conditions except annealing temperature to be as 52°C. PCR products were analyzed by gel electrophoresis with 1.2% agarose gel. To avoid any contamination of non-specific DNA fragments in sequencing reaction, PCR products were purified from agarose gel. For this purpose, Quick gel extrcation Kit by Invitrogen (Cat # K2100-12) was used. 40µl of nested PCR product was run on 1.2% agarose gel at 90V for 35min. While viewing the gel on transilluminator (UVP PhotoDoc-It™ Imaging System), PCR product of required size was cut carefully, using a sharp blade for each sample separately. Later on they were subjected to purification procedure as per protocol instructions. 10µl of the purified PCR products were again run on 2% agarose gel to ensure their purification before sequencing.

F. Optimization of Abl-Kinase domain amplification from healthy individuals

It was indispensable to amplify and sequence the kinase domain of Abl gene from healthy population so that those from CML patients could be compared to them. For this purpose blood samples from 5 healthy individuals were collected and RNA isolation and cDNA synthesis were performed. To amplify Abl kinase domain, same set of primers used for sequencing reaction was operated i.e. forward primer (3306F) 5′-TGGTTCATCATCATTCAACGG-3′ and reverse primer (4000R) 5′-GGACATGCCATAGGTAGCA-3′. The master mix was prepared as follows; 5µl of 10X Taq buffer with KCl, 5µl $MgCl_2$ (25mM), 5µl dNTPs (2mM), 1µl of each forward and reverse primers (20pMol), 0.5µl Taq DNA polymerase (recombinant) (2.5u) by Thermo Scientific™, USA (#EP0401), 2µl cDNA as templetate and Nuclease free water to make the total reaction volume upto 50µl. The thermal profile for the reaction was; Preliminary denaturation at 95°C for 5 min followed by 35 cycles of denaturation of double stranded DNA at 95°C for 30 sec, annealing of primers to DNA template at 52°C for 35 sec and extension to form multiple copies of DNA strands at 72°C for 40 sec. Final extension was proceeded at 72°C for 10 min. The amplified segments were subjected to direct sequencing.

G. Direct Sequencing

The amplified PCR products were subjected to direct sequencing analysis to for detection of any possible point mutations present in ABL-kinase domain of CML patients. Nested RT-PCR products of processed samples were sequenced by Sanger method. The forward primer (3306F) 5′-TGGTTCATCATCATTCAACGG-3′ and reverse primer (4000R) 5′-GGACATGCCATAGGTAGCA-3′ were used for sequencing reaction (Willis et al. 2005). Standard procedure for BigDye® Terminator v3.1Cycle Sequencing Kit from Applied Biosystems, USA was followed as per manufacturer's instructions.

Following reagents were used for preparation of reaction mixture; Ready reaction premix (25x) 4µl, Big dye sequencing buffer (5x) 2µl, primer 3.2 pmol, template 30 ng and DEPC treated water making total volume upto 20µl. ABI-3130XL and ABI-3730 genetic analyzers (Applied Biosystems)

was used with 36 cm capillary arrays and POP-7 polymer to sequence the nested PCR products.
The sequenced templates were analyzed using Sequencing Analysis (Applied Biosystems) version 5.2, "DNASTAR®-SeqMan ™II" and Molecular Evolutionary Genetics Analysis (MEGA) V 5.05. The DNA sequences from both groups i.e. CML patients and healthy persons were aligned against the reference sequence from NCBI (GenBank accession number: M14752.1). Whole kinase domain including P-loop, C-Helix, SH2 contact and Activation loop were closely studied to find any possible nucleotide substitutions.

III. Results

A. Clinical characteristics and survival curves

Clinical history and disease background of the patients concluded following interpretation.

I. Signs and symptoms at the time of diagnosis.

Splenomegaly was reported in 86.4% (108/125), fever in 50.4% (63/125), hepatomegaly in 19.2% (24/125), fatigue in 17.6% (22/125), weight loss in 12% (15/125) and musculoskeletal pains in 7.2% CML patients **(Table 1; Figure 1A)**.

II. Drug History, blood cell count and Hb level in blood. Newly diagnosed CML patients are treated with Hydroxyurea before administration of Imatinib. In our studies 40.8% (42/125) patients had been on Hydroxyurea for a period of 6 months or more. Normal white cell count (05-1.0x10^9/L) was reported in 36.8% (46/125), whereas 63.2% (79/125) had white cell count exceeding the normal range. Platelet count within the normal range (100-450 x10^9/L) was presented by 83.2% (104/125) patients, however 16.8% (21/125) had them more than 450 x10^9/L. Blast cells less than 5% were reported in 68.8% (86/125) individuals, while 57.6% (72/125) presented lower levels of Hemoglobin (Hb) (<10g/dl) **(Table 1; Figure 1B)**.

III. Gender and age groups of patients. Among the collected samples, male patients 52% (65/125) were greater in number than females 48% (60/125), respectively **(Table 1; Figure 1C)**.
Kaplan-Meier survival analysis determined 7 years survival probability of 100% in female patients, while in males 5 years estimated survival is 86%, which lowers to 32% by 7 years from disease diagnosis (p=0.004) **(Figure 2A)**.
Both pediatrics n=8 (6.4%) and adults n=117 (93.6%) (19-65 years) were part of the study **(Table 1; Figure 1D)**. A non-significant (p=0.600) difference between survival probabilities of both groups was observed because of smaller number of pediatric patients. However, adult patients had a survival rate of 93% (5 years) and 70% (7 years) from the time of diagnosis **(Figure 2B)**.

IV. Disease phase and Sokal Risk. Chronic phase was presented by 64% (80/125), accelerated phase by 23.2% (29/125) and blast phase by 12.8% (16/125) CML patients at the time of diagnosis **(Table 1; Figure 1E)**. Kaplan-Meier analysis estimated 7 year survival rate for CP, AP and BP as 98%, 87% and 46% respectively (p=0.035) **(Figure 2C)**.
A low sokal risk was anticipated in 19.2% (24/125), intermediate-risk in 46.4% (58/125) and high-risk in 34.4% (43/125) patients **(Table 1; Figure 1F)**. The estimated survival probability (p=0.012) in low risk patients was 100% for 7 years, while it was 97.9% for 5 years in patients with intermediate risk.
In high risk patients, survival possibility was 82% for 5 years and 31% for 7 years **(Figure 2D)**.

V. Cytogenetic and Hematologic response of CML patient. Complete

cytogenetic response (CCyR) was presented in 53.6% (67/125), partial cytogenetic response (PCyR) in 13.6% (17/125), minor cytogenetic response in 5.6% (7/125) and minimal cytogenetic response was achieved in 20% (25/125) CML patients. However, 7.2% (09/125) presented no cytogenetic response **(Table 1; Figure 1G)**. A survival probability (p=0.008) of 100% was estimated in patients achieving CCyR and partial CyR, while 78.8% for those attaining minor CyR and 60% for minimal CyR at 7 years of treatment **(Figure 2E)**.

In our study 85.6% (107/125) CML patients presented complete hematologic response (CHR) while 6.4% (08/125) showed partial hematologic response (PHR) and 8% (10/125) were observed with no HR **(Table 1; Figure 1h)**. The Kaplan-Meier survival analysis revealed that patients with complete HR show survival probability of 100% at 7 years of disease onset, however this prospect is 75% for individuals with partial HR and 38.3% for those with no HR (p=0.00) **(Figure 2F)**.

VI. Amplification of Abl kinase domain from CML patients and healthy individuals

It is observed in nested RT-PCR that products of first round rarely appear in agarose gel electrophoresis, so was the case in our experiment (Figure 3A). In present studies well amplified BCR-ABL fusion oncogene of length 1306bp (b2a2) or 1380bp (b3a2) was obtained in Imatinib responder and resistant samples of CML patients, after second round of nested RT-PCR reaction **(Figure 3; B and C)**. In addition, amplified PCR product of ABL kinase domain (780bp) from healthy individuals of the same population was also achieved **(Figure 3D)**.

Sr. No.	Patient Characteristics	No. of Patients (n=125)
1	**Gender**	Females 6(
		Males 6!
2	**Age groups**	Pediatrics (<18 yrs.) 0!
		Adults (19-65 yrs.) 1!
3	**Presenting features at the time of diagnosis**	Splenic enlargement 1(
		Fever 6!
		Hepatomegaly 2!
		Fatigue 2!
		Weight loss 1!
		Musculoskeletal pains 0!
4	**Phase at the time of blood collection**	Chronic 8(
		Accelerated 2!
		Blast 1(
5	**Sokal risk**	Low 2!
		Intermediate 5!
		High 4!
6	**Cytogenetic response**	Complete 6!
		Partial 1!
		Minor 0!
		Minimal 2!
		No response 0!
7	**Hematological Response**	Complete 1(
		Partial 0!
		No 1(
8	**Previous Treatment (>6 months)**	Hydroxyurea 4!
9	**White cell count (x10^9/L)**	5-10 4(
		>10 7!
10	**Platelet count (x10^9/L)**	100-450 1(
		>450 2!
11	**Blast cells in PB**	Less than 5% 8(
12	**Hemoglobin level in PB**	<10g /dl 7!

PB= Peripheral blood

Table 1: Clinical data of CML patients (n=125) collected, showing various features of patients included in this study.

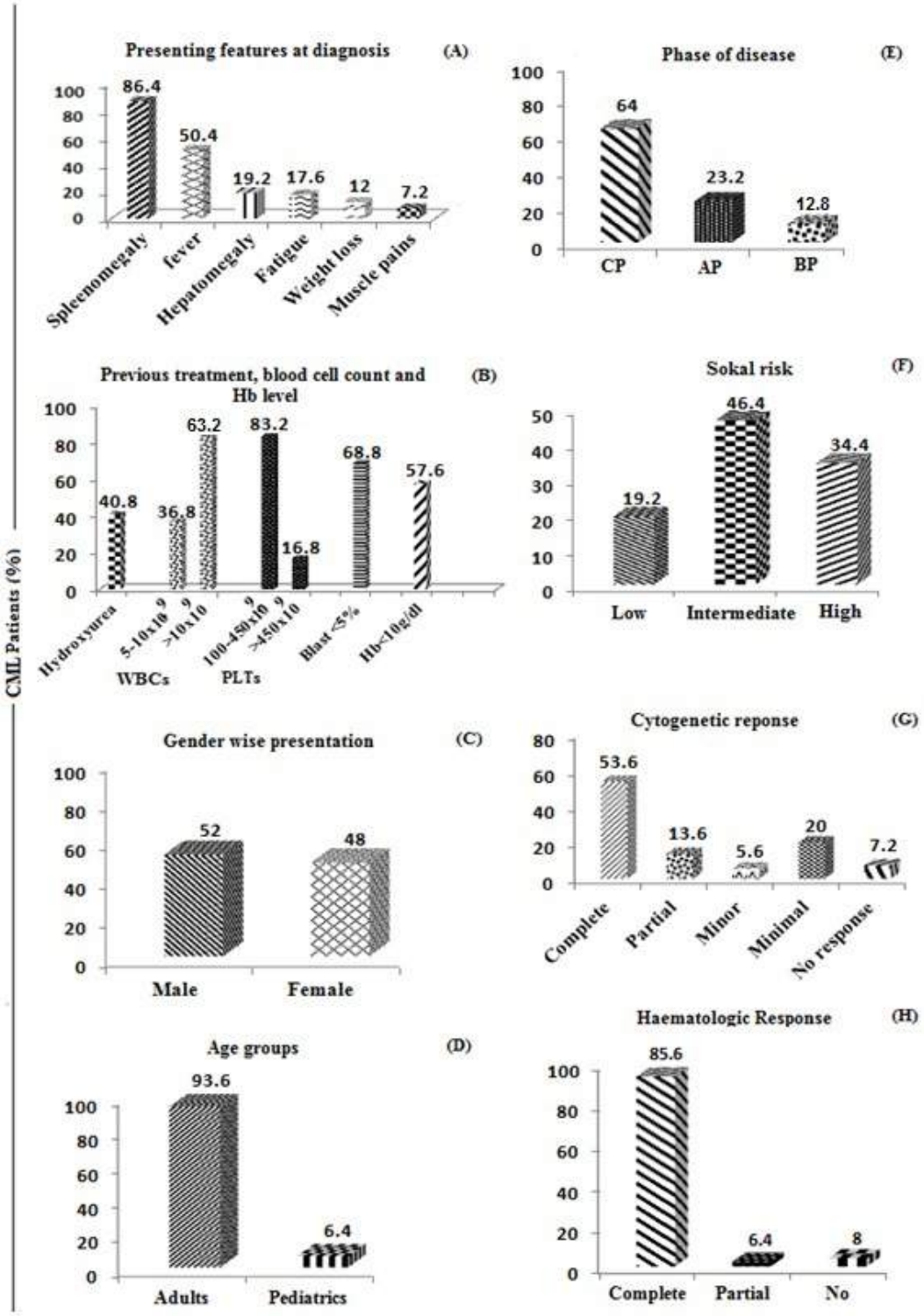

Figure 1: Showing CML patients (n=125) exhibiting particular diagnostic, hematologic and cytogenetic characteristics at the time of blood sample collection.
*CP: Chronic phase, AP: Accelerated phase, BP: Blast phase.

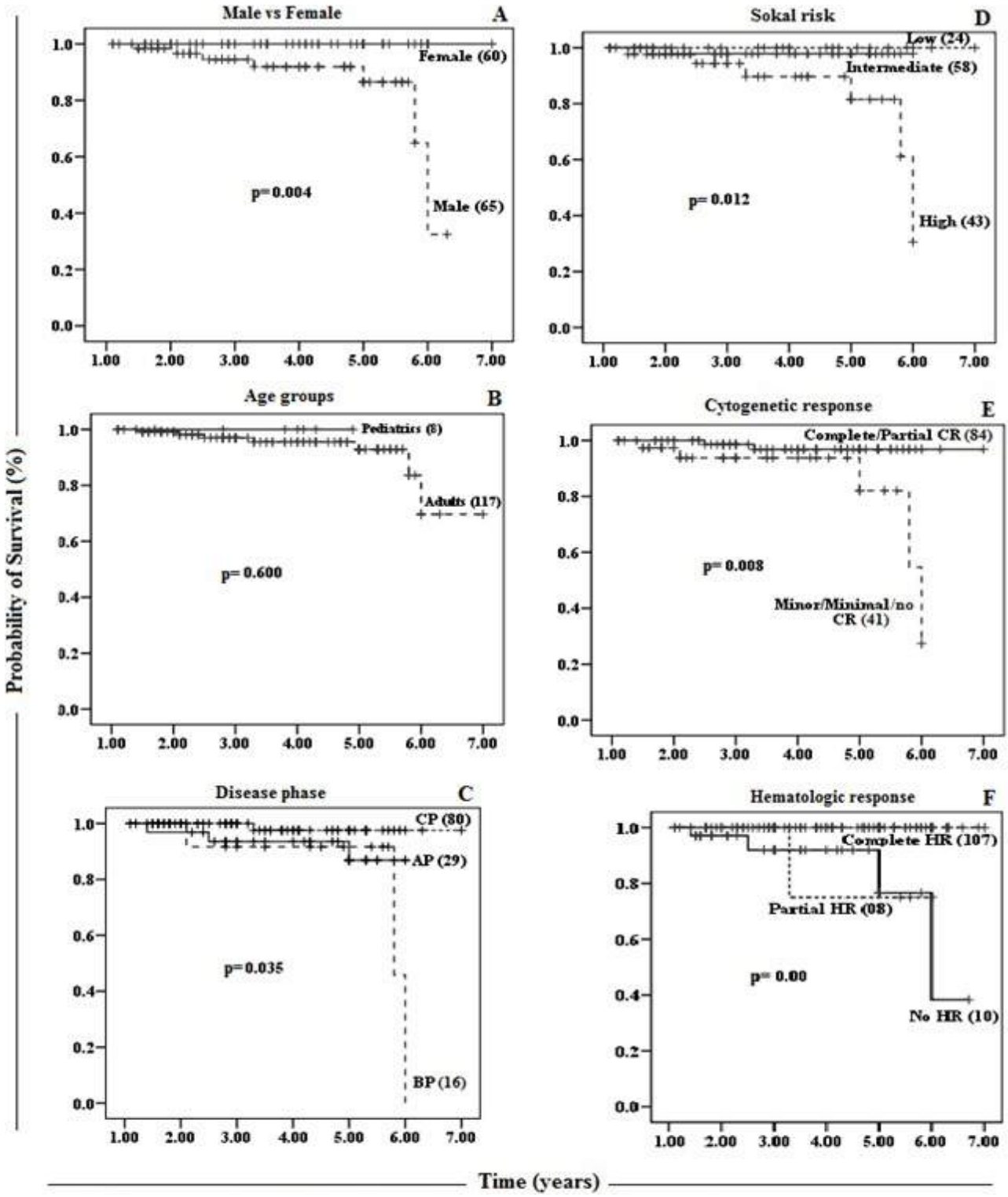

Figure 2: Kaplan-Meier plots showing cumulative survival probability of 125 CML patients according to different variables. (A) Gender wise (B) Age groups (C) Disease phase (D) Sokal risk (E) Cytogenetic response (F) Hematologic response. Significance was determined by Log-rank test. *CP: Chronic phase, AP: Accelerated phase, BP: Blast phase, CR: Cytogenetic response, HR: Hematologic response.

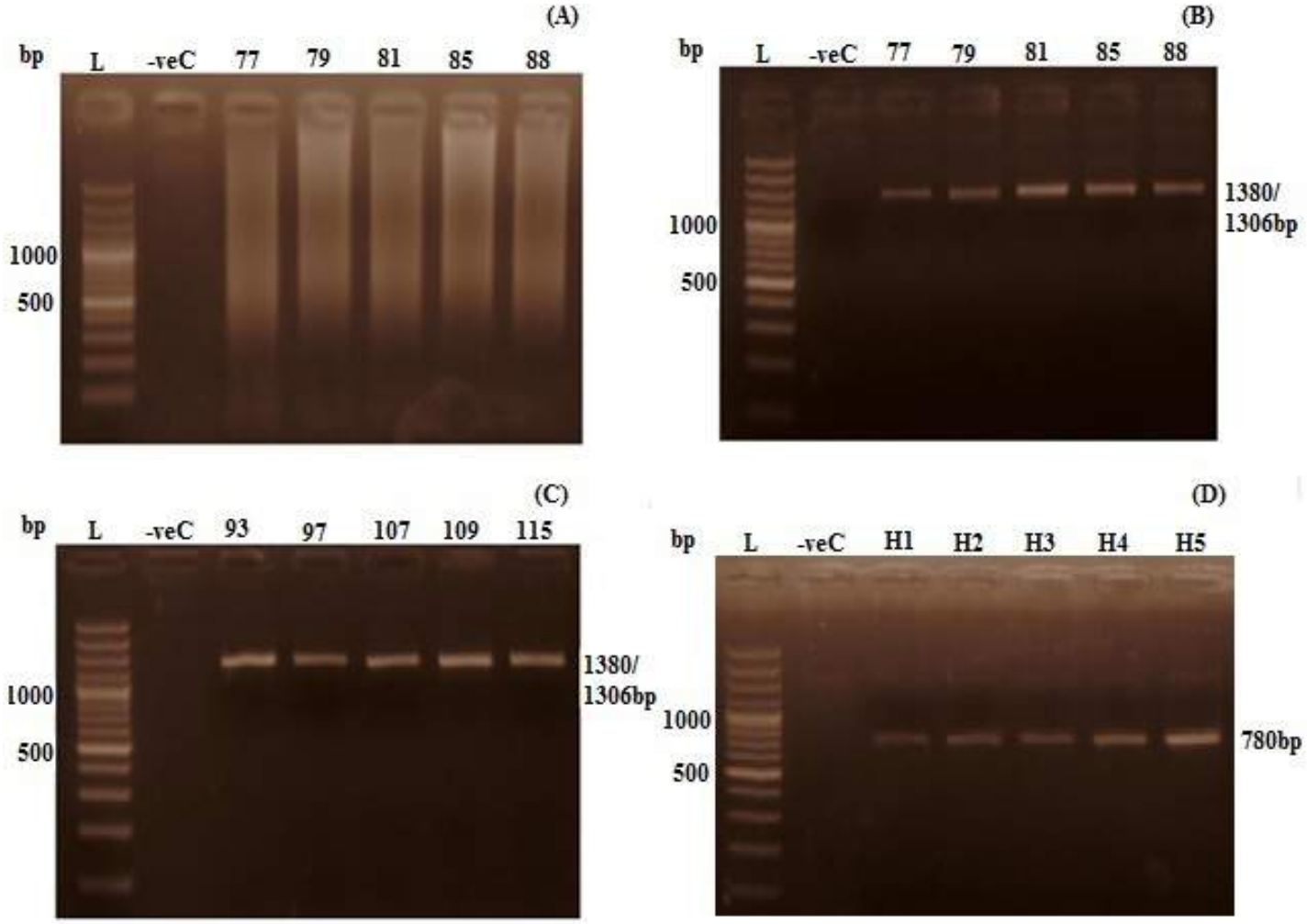

Figure 3: Nested PCR products of both groups of CML patients; round 1 (A), round 2 (B & C) and ABL-KD amplification from healthy individuals (D) on 1.0% agarose gel (100V, 30min).
bp= Base pairs, L= DNA Ladder (Thermo™ SM# 0323), -ve C= Negative control.

VI.Direct Sequencing analysis

The electropherograms of detected point mutations in Imatinib sensitive (**Figure 4**) and resistant **(Figure 5)** CML patients are shown. Among Imatinib responders, ABL-KD mutations were found in three out of ten (3/10) samples i.e. CML 67 (E355G, R367X), CML 69 (P309P/L) and CML 81 (V228A, W261R, M278L). However, four out of ten (4/10) samples were detected positive for mutations in Imatinib resistant patients i.e. CML 93(G250W), CML 109 (A269A/A, T394A), CML 118 (Y253H), CML 124 (E292E/E, E355E/G, V371V/V, Y393H). Rest of the samples of both groups had no nucleotide alterations in the analyzed domain **(Table 2)**.

Sample no.	n-PCR (BCR-ABL) +ve/-ve	Treatment duration	Response towards therapy		Imatinib sensitive		Imatinib resistant	
			Cytogenetic	Hematologic	Wild type	Mutant	Wild type	Mutant
CML 57	+	2 yrs	CCyR	CHR	+			
CML 66	+	3 yrs 9 months	CCyR	CHR	+			
CML 67	+	3 yrs	CCyR	CHR		+		
CML 68	+	2 yrs 8 months	CCyR	CHR	+			
CML 69	+	4 yrs 9 months	CCyR	CHR		+		
CML 75	+	3 yrs 9 months	CCyR	CHR	+			
CML 79	+	5 yrs	CCyR	CHR	+			
CML 81	+	2 yrs 7 months	CCyR	CHR		+		
CML 85	+	3 yrs 1months	CCyR	CHR		+		
CML 88	+	1 yr 5 months	CCyR	CHR	+			
CML 93	+	1 yr 2 months	PCyR	PHR				+
CML 97	+	6 months	Minor CyR	No HR			+	
CML 107	+	1 year 4months	Minimal CyR	CHR			+	
CML 109	+	8 months	Minimal CyR	CHR				+
CML 115	+	6 months	Minimal CyR	CHR			+	
CML 116	+	6 months	Minimal CyR	CHR			+	
CML 118	+	6 months	Minor CyR	No HR				+
CML 121	+	1 yr 3 months	Minimal CyR	No HR			+	
CML 124	+	1 yr 5 months	PCyR	CHR				+

*CCyR= Complete cytogenetic response, PCyR=Partial cytogenetic response, CHR= Complete Hematologic response, PHR= Partial hematologic response.

Table 2: Clinical characteristics and presence or absence of mutations in Imatinib sensitive and resistant CML patients screened for BCR-ABL kinase domain mutations through direct sequencing analysis.

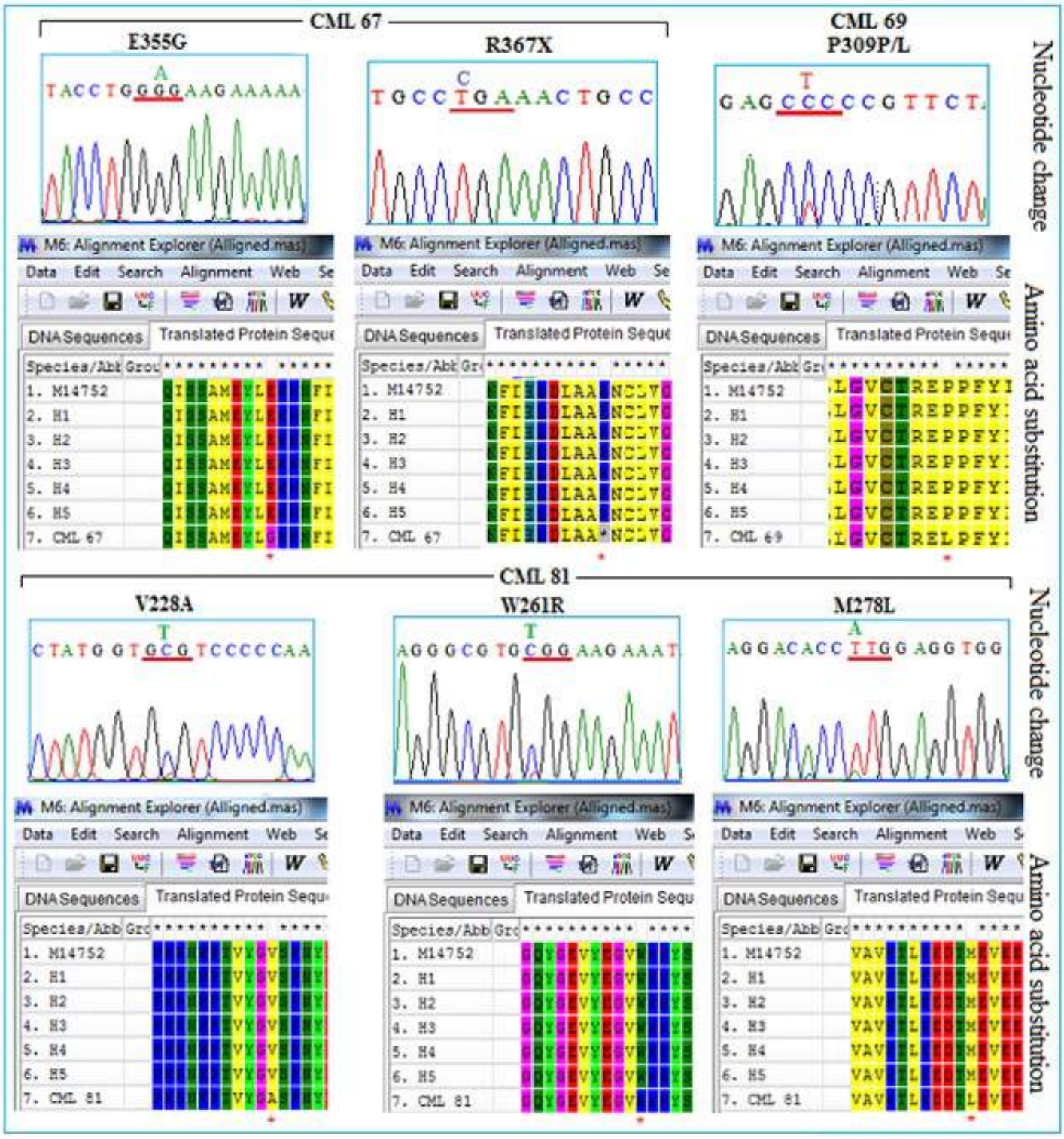

Figure 4: Electropherograms showing nucleotide alterations and subsequent amino acid substitutions in ABL1 kinase domain of Imatinib responder CML patients (no. 67, 81, 85). M14752.1 is the reference sequence, whereas H1-5 refers to sequences of healthy individuals from Pakistani population.

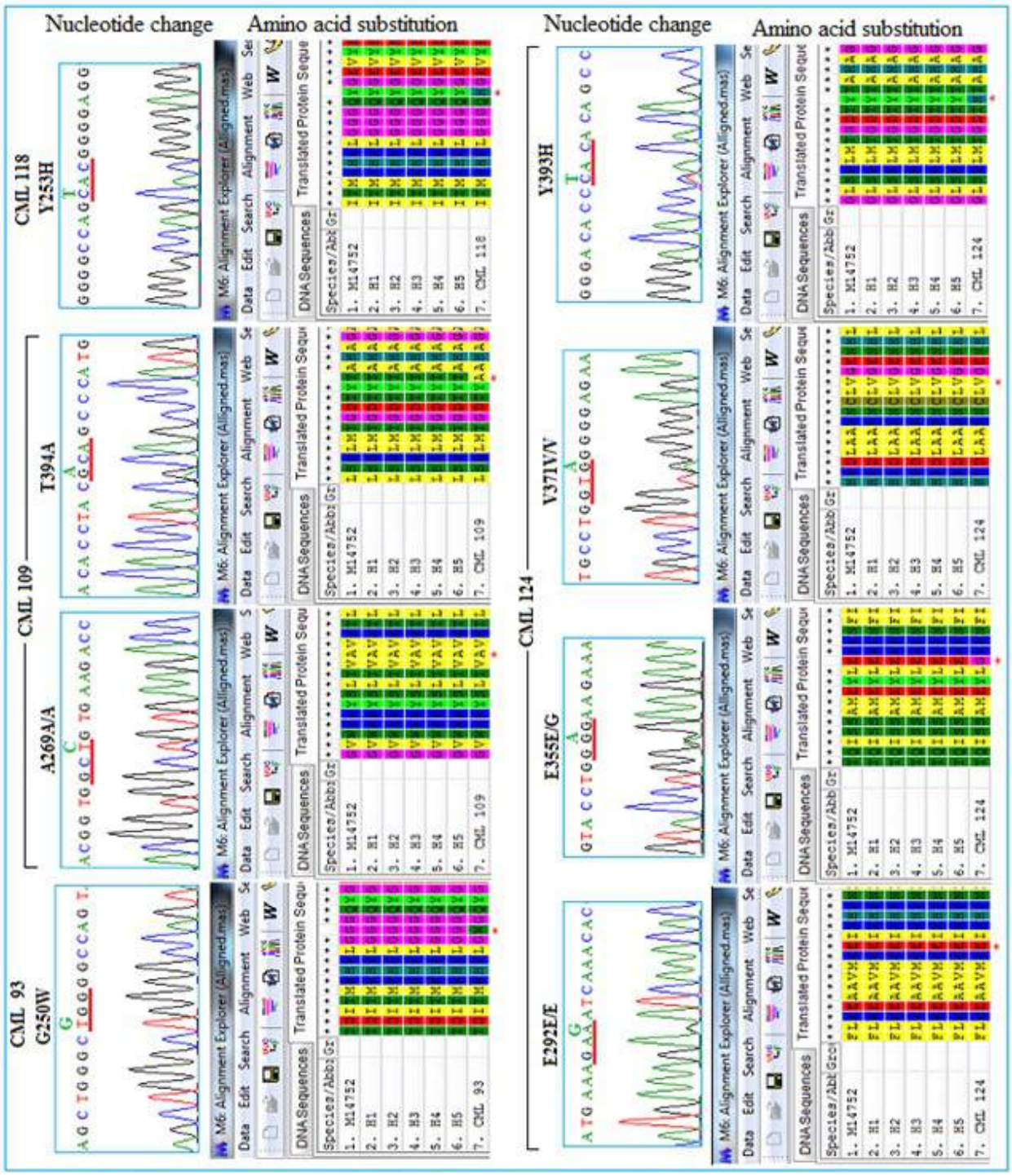

Figure 5: Electropherograms showing nucleotide alterations and subsequent amino acid substitutions in ABL1 kinase domain of Imatinib resistant CML patients (no. 93, 109, 118, 124). M14752 is the reference sequence, whereas H1-5 refers to sequences of healthy individuals from Pakistani population.

IV. Discussion

Imatinib mesylate, administered as the first-line treatment in newly diagnosed CML patients bring over a better prognosis and even progression free survival. Nevertheless, Hughes et al. (2009) and Hochhaus (2011) reported intolerance or resistance against the therapy in approximately 30% cases. At initial stages, a suspected patient of CML (WBC > 80-100 X 10^9/L) is treated with Hydroxyurea until the confirmation of disease, this helps to keep white cell count close to normal (Cortes and Kantarjian, 2012). Considering the data obtained and analyzed in our studies, we found spleen enlargement to be the most frequently (86.4%) observed sign in CML patients at initial diagnosis **(Figure 1A)**. This is in agreement to the finding of (Luo and Levitt 2008), that massive splenomegaly is often linked to CML. Compatibly, earlier studies from Pakistan as well as other countries mentioned splenomegaly as the most recurrently reported symptom (82.2%-100%) (Jameel and (Jamil 2006; Aziz and Qureshi, 2008; Karimi et al. 2008; Ahmed et al. 2009; Aziz et al. 2010; Koffi et al. 2010). Contrary to this, spleen discomfort was reported in 20% and 58.1% CML patients in French and Mexican CML patients, respectively (Millot et al. 2005; Aguayo, et al. 2008).

Our study samples presented fatigue (17.6%), hepatomegaly (19.2%) and fever (50.4%) as common signs and symptoms at diagnosis **(Figure 1A)**. This incidence varies considerably in previously reported findings in which these occurrences are stated as 22-100%, 21.7-100% and 40-64%, respectively (Jameel and Jamil 2006; Aguayo et al. 2008; Aziz and Qureshi 2008; Karimi et al. 2008; Efficace et al. 2011)
_ENREF_39_ENREF_42_ENREF_44.

We observed weight loss in 12% and musculoskeletal pains in 7.2% patients at initial diagnosis (Figure 1A). Conversely, in earlier studies weight loss had been described in 17.5-73% of CML patients_ENREF_45. Whereas, musculoskeletal pains in 7.5-35.6% patients at the time of diagnosis (Millot et al. 2005; Jameel and Jamil 2006; Aziz and Qureshi 2008).

Substantial over proliferation of white blood cells ranging between 12.8-860 x 10^9/L, was observed in 63.2% patients in our findings **(Figure 1B)**. If CML patients express a WBC count more than 10 x 10^9/L after at least 3 months, they represent absence of complete hematological response (Jabbour et al. 2011; Baccarani et al. 2013). In previous studies, elevated WBC count had been reported in 40.7-77.3% of patients with CML (Millot et al. 2005; Jameel and Jamil 2006; Usman et al. 2007; Aziz et al. 2010; Jabbour et al. 2011). Normal range for platelets in peripheral blood is 50-450 x 10^9/L. Among CML patients included in our investigation, 16.8% had an accelerated platelet count (486-812 x 10^9/L) **(Figure 1B)**. In concord, higher platelets count had been stated in 10.2-21.4% CML patients in other literature (Jameel and Jamil 2006; Usman et al. 2007; Aziz et al. 2010). In our study samples, 56% had lower levels of hemoglobin (<10g/dl) **(Figure 1B)** which is in agreement to the previous findings stating decreased levels of Hb in 15-59.3% CML patients (Jameel and Jamil 2006; Usman et al. 2007; Aziz et al. 2010; Jabbour et al. 2011)

In the present invstigation, number of male patients was greater than female with a ratio of 1.08:1 **(Figure 1C)**. However, in earlier studies this ratio is stated as 1.1:1 to 1.74:1 (Jameel and Jamil 2006; Syed et al. 2006; Usman et al. 2007; Jemal et al. 2009; Aziz et al. 2010; Cortes and Kantarjian 2012) . The Kaplan-Meier analysis revealed 100% (7 year) and 86% (5 year) survival in female

and male patients respectively **(Figure 2A)**. In agreement to our finding, deaths in Pakistani male CML patients are reported 33.1% more than females (Jameel and Jamil, 2006), which supports the impression that deaths reported in male CML patients is significantly higher (Hochhaus et al. 2009).

As the incidence of CML in children is uncommon (Suttorp and Millot, 2010), very few pediatric patients were part of our study **(Figure 1D)**. So, the survival analysis non-significantly showed 100% (5 year) survival in pediatric and 70% (7 year) in adult patients **(Figure 2B)**. Though, childhood CML has not been widely worked upon, a finding explains elevated mortalities in young adults of age range 15-29 years (Thomas et al. 2010). Correspondingly, suggestions have been made that disease outcome in pediatrics and young adults is comparable (Suttorp, 2008; Gupta et al. 2010).

Disease phase is also important to determine overall survival of patients. In our findings CP-CML patients were higher in number than AP and BP **(Figure 1E)**, with survival probabilities of 98% (7 year), 87% and 46% (5 year), respectively **(Figure 2C)**. In agreement, the respective survival probabilities for CP, AP and BP were stated as 92%, 74% and 38% in previous studies (Aziz et al. 2007). In a case study, prolonged survival time for 19 years has been reported in AP-CML patient (Wiernik et al. 2011). This is indicative of better treatment outcome and improved survival time in IM treated advanced phase CML patients.

It has been suggested that correlation of sokal risk with survival of CML patients depends upon treatment duration (Corm et al. 2011), lower the sokal risk, higher are the chances for good prognosis (Heaney and Holyoake, 2007). It was documented that till 2001, there was a significant difference in mortalities among intermediate and high risk patients, but this difference between the two groups reduced since 2001 (Kantarjian et al. 2012). Contrary to this, Kaplan-meier curve in our studies showed 97.9%, 82% and 31% survival probability in low, intermediate and high risk patients of CML respectively **(Figure 2D)**. Similarly, in an earlier report from Pakistan this incidence is stated as 70.3% in low, 56.1% in intermediate and 43.8% in high risk patients (Aziz et al. 2007). This difference from other populations can be for the reason of regional variations and ethnic diversity.

Cytogenetic response is another essential prognostic factor of chronic myeloid leukemia. Factually, the improved survival outcome is linked to attainment of CCyR, either by older therapies or Imatinib (Druker et al. 2006). In the present investigation, patients showing CCyR and PCyR have shown 7 year survival probability of 100%. This probability is 78.8% for those achieving minor CyR, 60 % for minimal CyR and 0% for those who achieve no response at 6 years since the beginning of treatment **(Figure 2E)**. This is indicative of the fact that Imatinib sensitive/responder patients with improved cytogenetic response, present less or no Ph+ metaphases and vice versa (Jabbour et al. 2009). Our results are comparable to the early findings, stating survival probability of 89% in patients achieving CCyR (Aziz et al. 2010). Similarly, hematologic response is a clinically important factor in CML response monitoring. In our investigation 85.6% patients exhibited CHR with survival probability of 100% **(Figure 1h; 2F)**. In another investigation complete hematologic response was reported in 93.75% patients (Gupta and Prasad, 2007). Similarly another studies reported that 90% CML patients under observation show some sort of hematologic response, either complete or partial (Razmkhah et al. 2010). Whereas, CHR was observed in 71% patients in accelerated phase and 52% in those with blast

phase of the disease (Cortes et al. 2011).

Above mentioned hematological parameters and response outcomes are significantly important to bridge their association with contributory point mutations. Knowing and understanding the ABL-KD mutations can be helpful to establish an understanding for disease condition and progression (Ramirez and DiPersio, 2008). In almost 50-90% CML patients with acquired resistance, such alterations have been detected earlier (Skorski, 2008; Soverini et al. 2011). Direct sequencing is the recommended method for detection of mutations in ABL-KD_ENREF_26. Other methods such as Denaturing-High Performance Liquid Chromatography (DHPLC), DS combined with DHPLC, Allele specific oligonucleotide-PCR techniques i.e. amplification refractory mutation system (ARMS) PCR and ligation-PCR (LPCR) are also in use for this purpose (Ernst et al. 2009; Soverini et al. 2011)_ENREF_69. In the current investigation Imatinib responder and resistant CML patients were brought under investigation, to detect resistance mediating mutations in drug binding site. Such mutations can cause or contribute in either primary or secondary IM resistance along the course of treatment (Soverini et al. 2011)_ENREF_67).

Among Imatinib responders all ten patients either Imatinib sensitive-mutatnt (IMS-m) or Imatinib sensitive-wild type (IMS-w) were in CP of disease (400mg/day) and showed CCyR and CHR at the time of sample collection. We found six (6) point mutations in imatinib sensitive CML patients. The subsequent amino acid substitutions are V228A, W261R, M278L, P309P/L E355G and R367X **(Figure 4)**. One patient manifested single alteration in amino acid residues while one had two and one had three mutated sites in ABL-KD. The mutation V228A has been reported in $CD34^{+}$ $CD38^{-}$ stem cells isolated from chronic phase CML patients (Jiang et al. 2007). In the same context researchers previously reported W261L as imatinib resistant mutation in CML patients (Ernst et al. 2008; Soverini et al. 2011). As mentioned above, we have found amino acid substitution at residue 261 from Try to Arg instead of Leu. Similarly in past investigations M278L mutation from the C-helix is reported to render drug resisance by activating the kinase (Hochhaus and La Rosee 2004; Martinelli et al. 2005; Azam and Daley 2006). The mutation P309P/L is observed in one patient in our study, which is not previously reported in literature. However, E355G from SH2 contact is studied to have intermediate sensitivity to imatinib causing low level resistance but may respond to dose escalation (Jones et al. 2009). The amino acid R367 (SH2 contact) is reported to have important role in drug binding and its interactions (Tokarski et al. 2006). In our sample the mutation in coding nucleotides for this amino acid has altered it to an unknown mutant. Thus, in context of previous reports, it can be anticipated that there is a possibility for patients in our studies to establish secondary resistance against imatinib along the course of disease.

Among IM resistant patients, we found a total of eight (8) mutations. Patients categorized as resistant in our studies, showed varying response towards Imatinib treatment. Among Imatinib-resistant wild type (IMR-w), one patient showed minor CyR though five had minimal CyR. Whereas three had CHR and three showed no HR at the time of sample collection. Imatinib-resistant mutant (IMR-m) patients exhibited mutations namely G250W, Y253H, A269A/A, E292E/E, E355E/G, V371V/V, Y393H and T394A **(Figure 5)**. Sample CML 93 had PCyR and PHR over a period of more than 12 months, and it manifested single mutation. CML 118 also showed single

mutation but had minor CyR and no HR over 6 months of treatment duration. Whereas CML 109 with minimal CyR and CHR had two mutations, and CML 124 with PCyR and CHR had four point mutations in ABL-KD.

In a therapy optimization study, the point mutation G250W was listed among resistant mutations against TKI therapy (Katouli and Komarova, 2010). Likewise, Y253H is reported to confer high-level resistance to Imatinib and even Nilotinib (Branford et al. 2009; Jones et al. 2009). In our studies we found a silent mutation at amino acid residue 269 of ABL-KD that produces Ala as does the wild type. However A269T mutation producing Thr instead of Ala has been reported in past studies (Jiang et al. 2007). Similarly our analysis revealed another silent point mutation E292E/E from C-helix, whereas amino acid substitution E292K has been reported previously (Azam et al. 2006). A heterozygous mutation E355E/G was observed in our studies, whereas in past investigations E355G had been stated as a mutation that cause low to moderate degree of resistance against imatinib and second line kinase inhibitors (von Bubnoff et al. 2005; Soverini et al. 2011). Another silent mutation at residue V371 was found in present findings, however V371A is mentioned previously to cause resistance in Imatinib treated patients of CML (Soverini et al. 2011). Mutations Y393H and T394A in activation loop had been found in our analysis, whereas Y393C is reported in an earlier report (Jiang et al. 2007).

Thus it can be summarized that there is no significant clinical marker for underlying point mutations among imatinib sensitive or resistant CML patients. As mentioned above, patients with minimal CyR and no HR are mutation free, while those with PCyR and CHR showed one, two and even four mutations each. In addition, here we have discussed four new silent mutations A269A/A, E292E/E, P309P/L and V371V/V and two new mutations causing amino acid substitutions Y393H and T394A, found in our analysis.

We concluded here, that patient characteristics i.e. cytogenetic and hematological parameters are an important tool to establish their survival outcomes and to map disease prognosis. In addition, the protocol for BCR-ABL kinase domain amplification and its sequencing analysis have been established successfully. This procedure is cost effective and highly valuable as RNA extraction was performed by TRIzol method. Similarly cDNA synthesis and PCR amplification was performed using reagents by Thermo Scientific™, easily available in the country. Reproducible and amplifiable products were achieved with very low chances of error. This standardized procedure is a gateway to detect possible ABL-KD mutation in IM sensitive and resistant CML patients, which was an unseen prospect since now in the country.

It has been revealed from various studies that any point mutations present in Imatinib naïve or sensitive CML patients, can bring about resistance in a long run and can affect treatment outcomes. Early detection of such underlying anomalies in patients sensitive to Imatinib therapy will be helpful to better understand disease progression and timely alterations in treatment strategy. Also, mutation analysis in Imatinib resistant patients can prove helpful in opinion to opt for Imatinib dose acceleration, second generation TKIs or bone marrow transplant (BMT). The direct sequencing analysis is therefore a recommended tool for disease monitoring and response evaluation. The present contribution by optimization of RT-PCR protocol for ABL KD amplification will prove helpful in this regard.

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