

Abl Kinase Domain Mutations in Imatinib-treated Egyptian Patients with Chronic Myeloid Leukemia

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Abstract

Background: Point mutations within *ABL* kinase domain (AKD) of the *BCR-ABL* gene are the most common cause of resistance to Imatinib Mesylate (IM) treated Chronic Myeloid Leukemia (CML) patients.

Objectives: To assess the frequency, type and impact of AKD mutations on prognosis in a cohort of Egyptian CML patients.

Patients and methods: Serial measurements of *BCR-ABL* transcripts level in 175 IM treated CML patients were performed using real time quantitative polymerase chain reaction (RQ-PCR). Mutation screening was performed by allele specific oligonucleotide polymerase chain reaction (ASO-PCR) in 72 patients including all 42 non-optimal responders; 28 resistant patients, 18 suboptimal responders in addition to 30 patients randomly selected with stable/decreasing transcript level representing an optimal responder category.

Results: AKD mutations were detected in 16/28 resistant patients (57%) at time of >2-fold rise in *BCR-ABL* transcript and in none of the 44 optimal or suboptimal responders (0%) with decreasing or stable transcript levels. From 16 positive patients, P-loop mutations were detected in 9 patients; Q252H in 3 patients (19%), Y253H in 2 patients (12%), Y253F in 2 patients (12%) and E255K in 2 patients (12%). T315I was detected in 1/16 (6%) patient. Regarding non P-loop mutation; V299L was detected in one patient (6%), M351T in 4 patients (25%), F359V in 2 patients (12%). One patient had both Y253H and E255K mutations. Ten/16 (62%) patients carrying mutations experienced disease progression versus 1/56 (2%) in non mutation group ($p=0.001$). Median progression free survival (PFS) and overall survival (OS) of the mutation group was 13.5 months and 37.5 months, respectively versus 42.6 months in non mutation group ($p=0.001$). The estimated PFS and OS at 49 months in patients with mutations were 37.5% and 56.3% respectively versus 98.2% in non mutation carriers ($p<0.001$). Mutations detected in chronic phase (CP) were mostly non P-loop (5/6, 83%) while mutations detected in accelerated phase (AP) and acute blastic crisis (ABC) were mostly located in P-loop and gate keeper regions (8/10, 80%). Patients harboring P-loop mutations/T315I showed poorer PFS and OS; 14 months (7.5-38) and 10 months (3-40) versus 42 months (39-45) and 42 months (9-45) in non-P-loop mutations carriers, respectively ($p=0.003$ and $p=0.017$).

Conclusion: A rise in *BCR-ABL* transcript of >2-folds in IM resistant patients may signal progression that implies testing for AKD mutations and early planning for second generation tyrosine kinase inhibitors (TKIs). P-loop mutations are significantly associated with advanced CML phases and poorer OS than non-P loop mutations. ASO-PCR is a valuable tool for detection of mutations in countries where sequencing facilities are not available.

Keywords: Abl kinase domain mutation screening; *BCR-ABL* transcripts; IM resistance

Introduction

Chronic myeloid leukemia (CML) is characterized by a reciprocal chromosomal translocation t(9;22)(q34;q11) resulting in the *BCR-ABL* oncogenic fusion gene, which encodes the cytoplasmic *BCR-ABL* protein. The *BCR-ABL* protein has constitutive tyrosine kinase activity that mediates cellular transformation and leukemogenic effects [1].

Imatinib Mesylate (IM) the selective inhibitor of the *BCR-ABL* tyrosine kinase, competitively targets the adenosine 5'-triphosphate (ATP) binding site of the AKD and blocks downstream signal transduction pathways leading to growth arrest and apoptosis [2]. Because of its excellent safety and important therapeutic benefit for patients with CML, IM has become the standard of care for the treatment of CML. However, despite high rates of hematologic, cytogenetic and molecular responses, refractoriness or acquired resistance after initial response to IM is observed in a significant proportion of patients [3].

Point mutations within the AKD are emerging as the most frequent mechanism for reactivation of *BCR-ABL* kinase activity [4]. These mutations affect amino acids involved in IM binding or in regulatory regions of the AKD and result in decreased sensitivity to the drug [5]. It is postulated that mutations within the AKD can prevent IM from binding by changing the conformation of the *BCR-ABL* protein [6].

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Molecular monitoring of *BCR-ABL* transcript levels for CML patients treated with IM has proved effective in defining patient response and provides important guide for clinical management and has prognostic relevance, in addition that it can indicate timing of mutation testing. Knowing the AKD mutational status is clinically useful, since it may prompt a change in therapy [7].

Biochemical and cellular assays have demonstrated that different AKD mutations result in varying levels of resistance [8,9]. Clinical studies have shown that the site of mutation within the kinase domain may affect disease outcome. The different mutations may therefore require different strategies to overcome resistance such as dose escalation for those that confer moderate resistance, or shifting to second generation TKIs or even transplantation for more resistant mutations [10].

The aim of the current study was to perform mutation analysis in a series of IM treated CML Egyptian patients of different response category in light of serial *BCR-ABL* measurements by RQ-PCR. We report on their type, frequency, relation to IM response and impact on clinical outcome of the disease.

Patients and Methods

Study group

Between March 2005 and April 2011, 175 newly diagnosed adult patients with Philadelphia-positive (Ph⁺) CPCML were enrolled in this study. Seventy two patients; 44 males and 28 females were selected for *ABL* kinase domain mutational screening representing all resistant patients (28/175, 16%) in this study with >2 fold rising *BCR-ABL* level in addition to all suboptimal responders (18/175, 10%) and a fraction of patients representing optimal responders (n:30) with stable/decreasing transcript level. The study was conducted in accordance with the Declaration of Helsinki and was approved by the National Cancer Institute (NCI) Institutional Review Board. Median age of patients at diagnosis was 42 years (range 18-72 years). Median Follow up period was 41.6 month (7.5-49.5).

Patient eligibility

Inclusion criteria were (1) morphologic and cytogenetic evidence of Ph⁺ CML in early chronic phase (defined as less than 12 months from diagnosis); (2) age 18 years or older; (3) normal renal and hepatic functions (4) normal cardiac function. Women at childbearing age were required to have a negative pregnancy test before starting IM, and to use contraception during therapy. Exclusion criteria included previous treatment for CML (Busulfan, IFN- α , or Ara-C). Exceptions included hydroxyurea and anagrelide for the treatment of elevated WBC (>50 \times 10⁹/L) and platelet count (>700 \times 10⁹/L), respectively; usage was limited to 4 weeks before starting IM therapy.

Treatment

Patients received 400 mg IM orally once a day. The dose was reduced for any \geq Grade 3 drug-related hematologic toxicity. No dose adjustments were made for Grade 1 or 2 hematologic toxicity. No dose reductions below 300 mg/day were allowed. Any toxicity had to be resolved within 28 days.

Quantitation of *BCR-ABL* mRNA transcript levels by RQ-PCR

Patients were monitored by RQ-PCR for *BCR-ABL* transcript levels at diagnosis and at 3-month intervals during IM therapy. The *ABL* control gene and the *BCR-ABL* target gene transcript levels

were quantitated using TaqMan Universal PCR Master Mix (*Applied Biosystems, Foster City, California, USA*), the *BCR-ABL* Mbcr FusionQuant Kit (*Ipsogen, Luminy Biotech Entreprises, Marseille, France*) and the Smart Cycler detection system (*Cepheid, USA*). A set of reference RNA was provided from *Ipsogen* to convert results into international scale (IS).

Definition of treatment responses

Hematologic, molecular and cytogenetic responses were defined according to European Leukemia Net criteria. MMR was defined as a 3-log reduction of *BCR-ABL* transcripts level, corresponding to \leq 0.1% on international scale (IS) [11]. PFS was defined as loss of hematologic or cytogenetic response, death, or development of advanced CML; OS defined by the absence of death from any reason [12].

Abl Kinases Domain (AKD) mutations screening

Mutation screening was performed using allele specific oligonucleotide - polymerase chain reaction (ASO-PCR). Genomic DNA was extracted from peripheral blood using Gentra Puregene blood kit (*QIAGEN, Hilden, Germany*). ASO-PCR assay was established for the detection of 16 known mutations which were selected according to their frequency in IM-resistant CML patients. Mutation panel selected included M244V, L248V, G250E, Q252H (a, b) showing 2 different nucleotides substitution, Y253H, Y253F, E255K, E255V, V299L, F311L, T315I, F317L, M343T, M351T, E355G and F359V mutations. Mutated or wild-type sequences were specifically amplified in a PCR reaction to analyze the most frequently identified mutations in the AKD (amino acids 220 to 498). The strong specificity of the assay was demonstrated for each mutation by detection of wild *ABL* sequence in DNA from clinically-declared healthy controls. Sequence of forward and reverse primers for both wild and mutant types used for ASO-PCR was adapted from previous reports [13,14]. The amplified products were detected by electrophoresis on 2% ethidium bromide-stained agarose gel.

Statistical analysis

The chi-square test was used to determine the significance between variables. Kaplan-Meier survival curves were used to assess outcome of patients with and without mutations [15]. Log rank test was used to compare between groups. PFS was calculated from start of treatment until the first reported appearance of AP or ABC. OS was calculated from start of treatment until death. All analyses were performed using the statistical package for the social sciences (SPSS software 17; SPSS Inc., Chicago, USA) [16].

Results

RQ-PCR results and mutational status

Twenty eight resistant patients (18 with 1ry resistance and 10 with 2ry resistance) and all 18 suboptimal responders in addition to a group of 30 patients with optimal response to IM randomly selected (15 in CMoIR and 15 in MMoIR) were submitted for mutation screening. Sixteen resistant patients (16/28, 57%) showed a mutation event while none of the optimal (0/30, 0%) or suboptimal responders (0/18, 0%) with stable or decreasing transcript level showed any detectable mutation ($p < 0.001$). Baseline characteristics, mutational status and disease outcome of the 28 IM-resistant patients are listed in table 1.

Frequency of AKD mutations in IM-resistant CML patients

Nine different AKD mutations were found in 16/28 (57%) patients. Mutations of the P-loop (amino acids 248-255) were detected in 9/16 (56%) patients while non-P loop mutations were detected in 7/16 (44%)

UPN	Sex	Age	Therapeutic response	Mutational status	Disease outcome
1	M	48	Primary resistance	Q252Hb (P)	AP, died
2	M	47	Primary resistance	M351 T (NP)	CP, alive
3	M	29	Primary resistance	Q252Hb (P)	CP, alive
4	F	60	Primary resistance	F359V (NP)	CP, alive
5	M	40	Primary resistance	M351T (NP)	AP, alive
6	M	49	Primary resistance	Y253H (P)	AP, alive
7	F	47	Primary resistance	T315I (G)	ABC, died
8	F	39	Primary resistance	Y253F (P)	ABC, died
9	F	65	Primary resistance	Y253F (P)	ABC, died
10	F	40	Primary resistance	E255K (P)	ABC, died
11	M	39	Primary resistance	V299Lb (NP)	CP, alive
12	M	49	Primary resistance	Y253H (P), E255K (P)	ABC, died
13	M	63	Secondary resistance	Q252Ha (P)	ABC, died
14	M	52	Secondary resistance	F359V (NP)	CP, alive
15	F	25	Secondary resistance	M351 T (NP)	CP, alive
16	M	46	Secondary resistance	M351T (NP)	ABC, alive
17	F	30	Primary resistance	NM	ABC, died
18	M	45	Primary resistance	NM	CP, alive
19	M	46	Primary resistance	NM	CP, alive
20	M	47	Primary resistance	NM	CP, alive
21	M	49	Primary resistance	NM	CP, alive
22	M	51	Primary resistance	NM	CP, alive
23	M	57	Primary resistance	NM	CP, alive
24	F	65	Primary resistance	NM	CP, alive
25	F	67	Primary resistance	NM	CP, alive
26	F	43	Secondary resistance	NM	CP, alive
27	M	44	Secondary resistance	NM	CP, alive
28	M	66	Secondary resistance	NM	CP, alive

UPN- Unique Patient Number; CP- Chronic Phase; AP- Accelerated Phase; ABC- Acute Blast Crisis; P- P Loop; NP- Non-P-Loop; G- Gatekeeper; SG-TKI- Second Generation Tyrosine Kinase Inhibitor; NM- No Mutation

Table 1: Patient Characteristics, mutational status and disease outcomes of IM-resistant patients.

patients. Q252H was detected in 3/16 patients (19%), Y253H in 2/16 patients (12%), Y253F in 2/17 (12%) and E255K in 2/17 (12%) patients. From seven non-P-loop mutations, 1/16 patient (6%) showed V299L, 4/17 patients (25%) showed M351T, and 2/16 patients (12%) showed F359V (Figure 1). One patient had both Y253H and E255K mutations (more than one mutant clone per patient). T315I was detected in 1/16 (6%) patients only.

Mutation site, frequency, and disease phase

Mutations were detected in 6/17 (35%) patients in CP and in 3/3 (100%) patients in AP and in 7/8 (87%) patients in ABC at time of rise of BCR-ABL level (Table 2). Frequency of mutations was higher in advanced phases (AP and ABC) than in CP-CML (Table 3, $p=0.01$). Among 6 mutations found in CP-CML patients; 5 mutations (83%) were non-P-loop and 1 mutation (17%) was located in the P-loop region. Two/3 mutations (67%) of AP were in the P-loop region. Among 7 detected mutations in ABC patients, 5 mutations (71%) were located in the P-loop region, 1 mutation (14%) non-P-loop and 1 mutation (14%) was located in gatekeeper region T315I.

Impact of mutational status on OS and PFS

Median OS of the 56 patients without detectable mutations was 42.6 months (13.5- 49.5) versus of 37.5 months (7.5-45) in the mutation positive group. The estimated OS and PFS at 49 months in the mutation positive group were 56.3% and 37.5%, respectively versus 98.2% in non-mutation group ($p<0.001$) (Figures 3a and 3b).

Median PFS of the mutation positive group was 13.5 months (3-45) versus 42.6 months (13.5- 49.5) in the non mutation group ($p<0.001$). Seven/9 (78%) patients with P-loop mutation (Q252Ha, Q252Hb, Y253F, Y253H, E255K) and one patient with T315I mutation died as a result of disease progression to either AP or ABC. Median OS of P-loop/ T315I group was 14 months (7.5-39) versus 42 months (40-45) in patients with non P-loop mutations (Table 1; Figure 2). No disease related death occurred in patients with non P-loop mutations. The estimated rates of OS (Figure 3a) and PFS (Figure 3b) at 48 months for patients with P-loop/T315I mutations were 22.2% and 11% versus 100% and 71% in patients with non-P loop mutations, respectively ($P=0.003$, $p=0.017$) (Figures 3c and 3d). Estimated OS of resistant patients with mutations detected in CP was 100% versus 30% for patients with mutations detected in AP/ABC ($p=0.001$) (Figure 3e).

Discussion

Measurement of *BCR-ABL* transcript levels by RQ-PCR proved to be the gold standard of identifying patients at risk of IM failure [17,18]. More recently studies focused on other predictive factors of response to IM therapy as the 10% *BCR-ABL*^{IS} transcript level at 3 months and the pretreatment transcript level [19-21]. However, point mutations within the AKD have been reported as the most common mechanism for IM resistance or progression in CML patients. Identification of molecular basis of IM resistance is valuable as it can provide prognostic information and contribute to determining appropriate therapy to prevent or overcome resistance [3]. It was suggested that a 2-fold increase in transcript level can be an indication of AKD mutation testing [18]. However, it is still difficult to define the extent of transcript increase that justifies mutation screening [22-24].

The choice of therapy should be guided by multiple factors including mutational analysis, disease phase, patients' characteristics and safety profile [25]. The ELN recommendations consider any rise in transcripts level a warning element requiring more stringent and careful monitoring [11] whereas fluctuations in PCR results in MMolR patients may be due to sampling effect. Since the ELN included confirmed loss of MMolR among events defining a suboptimal response to IM, in patients showing an increase in *BCR-ABL* transcript level, a confirmed loss of MMolR should trigger a mutation analysis as this could be a quite reproducible predictor of loss of CCyR [26].

In the present study among 28 refractory patients showing >2 fold

RQ-PCR	Consecutive <i>BCR-ABL</i> transcript levels (n=72)			
	Rise of > 2-fold (n=28, 39%)		Stable or decreasing (n=44, 61%)	
Mutational status	Mutation Positive	No mutation	Mutation Positive	No mutation
	16 (57.1%)	12 (42.9%)	0 (0%)	44 (100%)
No. of resistant patients	16 (100%)	12 (100%)	0 (0%)	0 (0%)

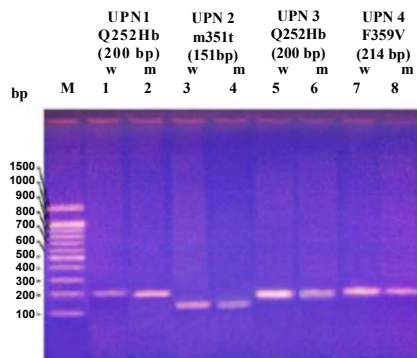
Table 2: Mutational status of patients with rising and stable/decreasing *BCR-ABL* transcripts level.

Resistant patients (n=28)	Disease phase			p value
	CP (n = 17)	(AP and ABC) (n = 11)		
Patients with mutation (n=16)	6/17 (35.3%)	10/11 (90.9%)		0.01
Location in AKD	P-loop +T315I (n=9)	1/6 (16.7%)	8/10 (80%)	0.05
	Non-P-loop (n=7)	5/6 (83.3%)	2/10 (20%)	0.01

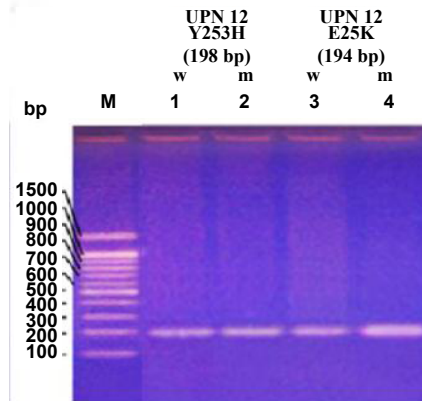
CP- chronic phase; AP- accelerated phase; ABC- Acute Blast Crisis; AKD- ABL Kinase Domain

Table 3: Correlation between Frequency and site of mutations and disease phase.

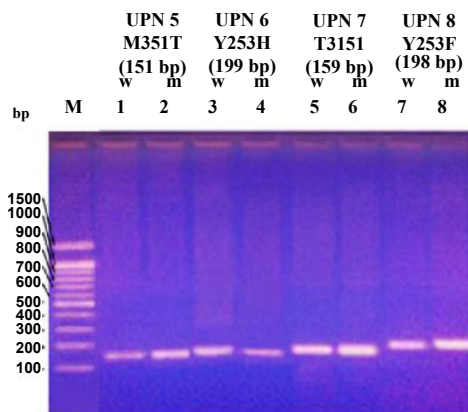
a. Patients' no.1, 2, 3 and 4.



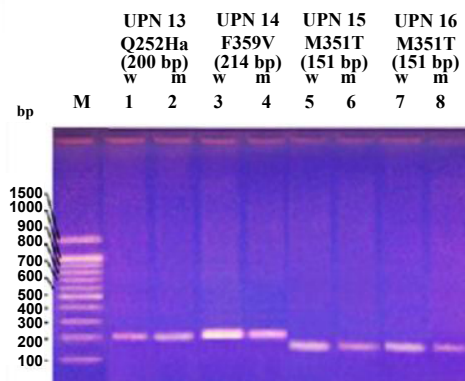
d) Patient no.12.



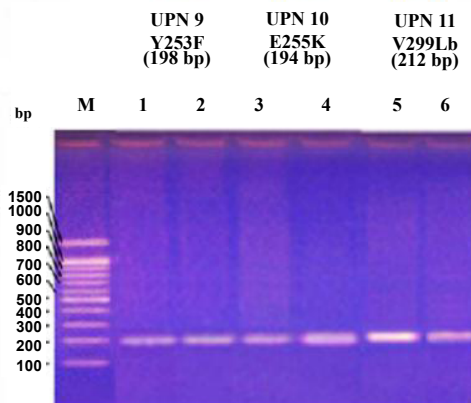
b) Patients' no.5, 6, 7 and 8.



e) Patients' no.13, 14, 15 and 16.
M: DNA marker (ladder; 100 bp).

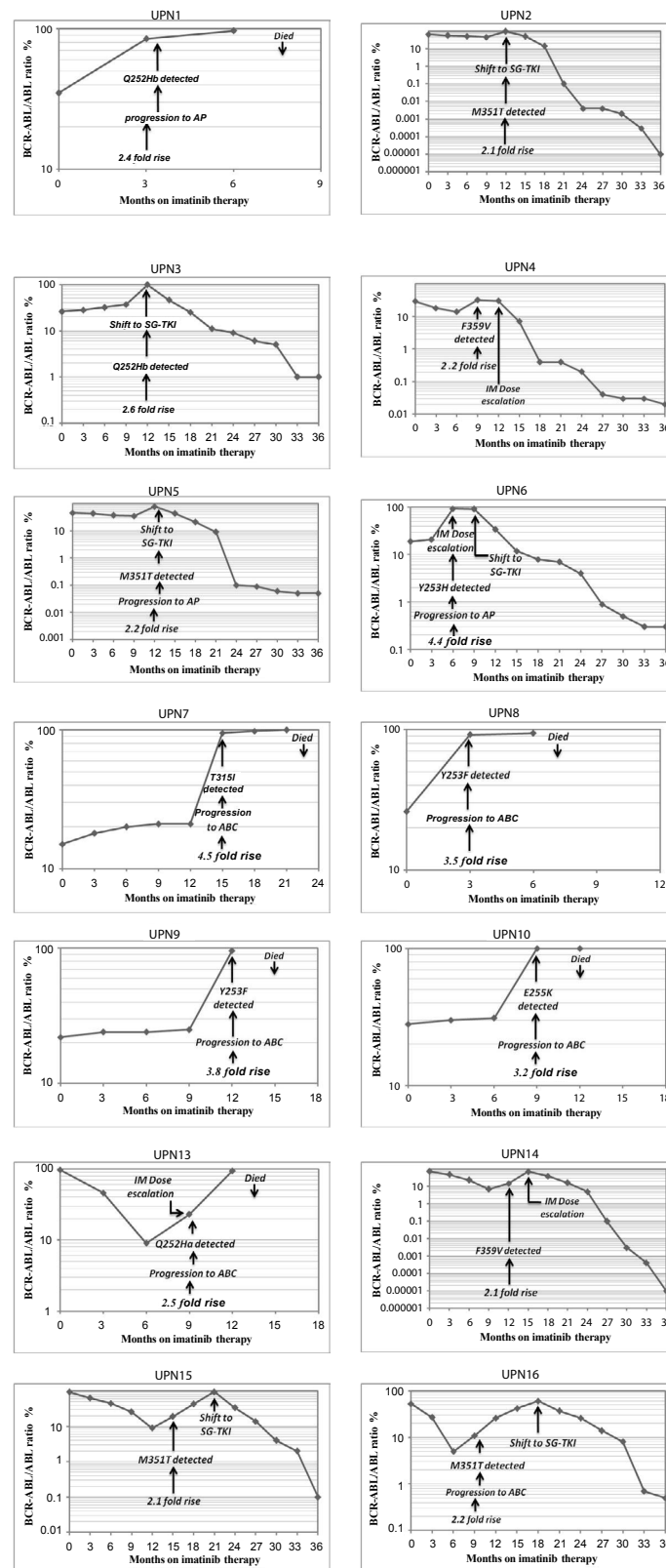


c) Patients' no.9, 10, and 11.



UPN: unique patient number; wt: wild type; mt: mutant type

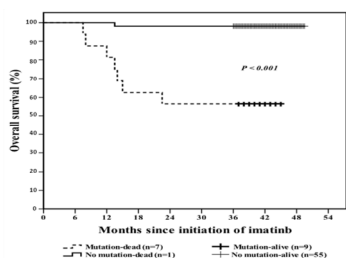
Figure 1: ASO-PCR products on ethidium bromide-stained agarose gel.



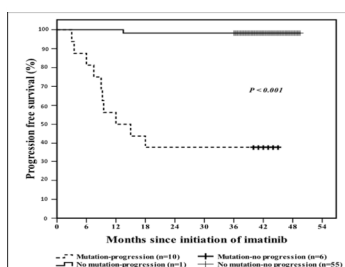
UPN, unique patient number; AP, accelerated phase; ABC, acute blast crisis; SG-TKI, second generation - tyrosine kinase inhibitor; NM, no mutation.

Figure 2: Individual patterns of BCR-ABL transcript levels in some of IM-resistant patients with mutations with > 2-fold rise in BCR-ABL transcript levels and their outcome.

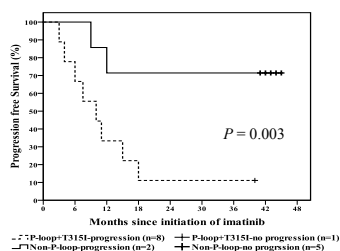
(a) OS



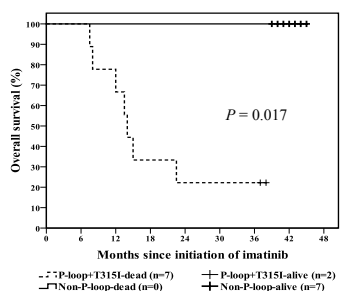
(b) PFS of mutation-screened patients according to presence (n=16) or absence (n=56) of mutations.



(c) OS of the 16 resistant patients with mutations according to the type of mutation.



(d) PFS of the 16 resistant patients with mutations according to the type of mutation.



(e) OS of 16 resistant patients with mutations according to disease phase at which a mutation was detected.

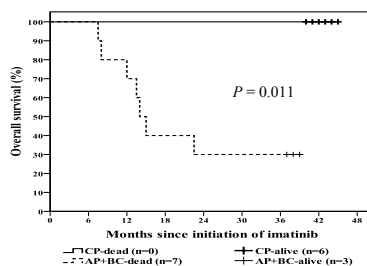


Figure 3: OS and PFS of patients.

BCR-ABL transcripts rise, 16 (57.1%) showed detectable mutations whereas no mutation was detected in optimal responders with stable or decreasing transcript level which add proof to Soverini et al. [26]. Strikingly, mutations were also undetected in suboptimal responders which raise the issue of other mechanisms of resistance as low OCT1 activity resulting in an inadequate intracellular concentration of IM [27]. All refractory patients did not achieve MMolR which explains the high mutation incidence at the time of rise of BCR-ABL. Mutation rate in this cohort was 16/28 (57%) which is relatively higher than that observed by others [28,29]. One study reported AKD mutations at a lower frequency (2 of 44 patients with relapsed or refractory disease) [30]. Differences in the frequency of mutation detection may be attributed to several factors; differences in the sensitivities of the techniques, in the time point of analysis, in phase of the disease and eventually due to differences in the genetic make-up of patient populations [31]. The frequency of BCR-ABL mutations in the present study was attributed to advanced phase of the disease at which mutations were detected [28]. Ten out of 16 patients with detectable mutation were in ABC (n: 7) and in AP (n:3). Seven/10 patients with detectable mutations in advanced phase died. Mutations were also detected in 6 CP patients, suggesting that this event is not restricted to patients in AP or ABC. However, none of them died due to shift to 2nd generation TKI, as early intervention that prevented progression of the disease. However, frequency of mutations was higher in advanced phases (AP and ABC) (10/11, 90.9%) compared to CP (6/17, 35.3%) indicating a cause – effect relation between disease progression and development of mutation.

OS and PFS were significantly decreased in patients harboring AKD mutations, a finding that has been stressed by others [18]. More importantly was the impact of the site of mutation on prognosis since different mutations have been associated with different degrees of resistance to IM as 7/8 patients with P loop and T315I mutations experienced disease progression. The increased kinase activity and transformation potency of several ATP binding loop mutations explain their association with poor prognosis leading to enhanced transforming capacity and associated genomic instability [30,32,33].

P-loop mutations conferred a true resistant phenotype and were not amenable to IM dose increase and implied alternative therapeutic strategies as second generation TKIs [34]. Two patients with P-loop mutations (Q252H and Y253H) were shifted to nilotinib and achieved CCyR in 1 year. T315I was detected in only one patient in the studied cohort who had no HLA identical donor for allogeneic stem cell transplantation and died in ABC. This rate was somewhat lower than that reported by Jabbour et al. [28] but higher than reported in two European studies [30].

Within our series there was also one patient harboring two known drug-resistant mutations, conferring increased oncogenic potency. On the other hand, patients with mutations that confer partial IM resistance as F359V regained clinical response with higher IM doses if the mutation was the sole abnormality [8,24]. In the present study, 2 patients with F359V mutation regained MMolR by increasing IM dose to 600 mg. The 4 patients harboring M351T mutation were shifted to dasatinib and 3 of them achieved MMolR within 12 months while one patient achieved CCyR. In this issue a stress on the choice of intervention with respect to the site and type of mutation should be considered [10].

In conclusion, more than 2 fold rise in BCR-ABL transcripts ratio in resistant CML patients who demonstrate molecular refractoriness to IM triggers mutation analysis as an early predictor of progression.

At this time, an early shift of therapy to appropriate 2nd generation TKI is beneficial when a positive mutation is detected in order to prevent disease acceleration. However, screening MMR patients with stable or decreasing *BCR-ABL* level for mutations is not warranted. ASO PCR is a specific and sensitive technique for mutation screening. M351T seems to be the commonest mutation in this CML Egyptian cohort. P-loop mutations are strongly associated with poor prognosis and mainly detected in advanced phases.

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