



BCR-ABL KINASE DOMAIN MUTATIONS - E255K, Y253 H AND M351T AMONG SUDANESE POPULATION WITH CML

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ABSTRACT

This study aims to find the frequency of mutations occurring in the P-loop at three codons, 255 (E255V/K), 253 (Y253H), and 351 (M351T), in Sudanese CML patients. A hospital-based, cross-sectional, descriptive study was conducted on 99 CML patients, in 2018/05 - 2019/05. All were tested for BCR-ABL tyrosine kinase domain mutations using Nested PCR, followed by restriction enzyme digestion. Females had a higher frequency of the Y253H mutant allele over males (83.3% vs 16.7%), while males had a higher frequency of the M351T mutant allele (66.7% vs 33.3%), however, all these findings were statistically insignificant ($p=0.158$ and 0.258 respectively). On the other hand, the E255K mutation was distributed in 50% of each. Regarding clinical status, the mutant allele of Y253H was found in 3 out of 41 cases (6.1%) of patients undergoing Imatinib Mesylate treatment and in 3 out of 25 cases (12%) of treatment-resistant patients, but this was not significant ($p=0.389$). Similar results were observed for the M351T mutation, in which the mutant allele was only insignificantly higher in resistant patients than treated ($p=0.22$). The E255K mutant allele was detected only in patients with resistance. The T315I mutation is commoner in males, but Y253H in females, despite all mutant cases of E255K being TKI resistant and equal between sexes, with an even lower prevalence. The E255K mutation may play a larger role in TKI resistance aetiology than Y253H and M351T.

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Introduction

Chronic Myeloid Leukemia (CML) is a myeloproliferative neoplasm of hematopoietic stem cells, characterized by increased proliferation and accumulation of the granulocytic cell line, without loss of differentiation capability [1]. According to data published by the WHO in 2020, leukemia is ranked the 4th and 3rd cause of cancer and mortality respectively in Sudan, and CML represents the most frequent hematopoietic cancer in Sudanese adults as reported by Mohamed EM Saeed *et al.* [2] and Basabaen AA *et al.* [3]. More research regarding CML would be useful, to minimize its prevalence, alleviate its complications

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and in turn reduce the mortality rate of the disease. For this reason, recent studies have been instigated in Sudan regarding CML patients, to ascertain how susceptibility to the disease is affected by polymorphism of certain genes such as xenobiotic ones [4-6] and BCR-ABL kinase gene as in this study.

The Ph (Philadelphia) chromosome is considered a cytogenetic hallmark of CML, as it occurs in 90-95% of patients [7]. It is an abnormally short chromosome 22, created from a reciprocal translocation between the ABL gene on chromosome 9 on the q34 band and the BCR gene on chromosome 22 on the q11 band, t(9;22)(q34;q11), generating a fusion gene named "BCR-ABL". This oncogene is an active TK (tyrosine kinase) leading to CML pathogenesis that presents as increasing cell proliferation, change of cell adhesion, and increase in apoptosis resistance [8].

A TKI (Tyrosine Kinase Inhibitor), specifically IM (Imatinib Mesylate), is considered a frontline targeted therapy for Ph-positive CML. It works by binding to BCR-ABL tyrosine kinase at the ATP binding site, so inhibiting the enzyme's activity and preventing phosphorylation of the tyrosine substrate involved in the signal transduction that leads to leukemogenesis [9, 10]. Despite a tremendous clinical response to IM treatment, ~20-30% of patients have experienced resistance to it [11]. This is a setback for both patients and treating physicians, and therefore new strategies for overcoming drug resistance and existing treatment options have been developed [12].

The development of IM resistance is associated with many different mechanisms; point mutations in the kinase domain of the BCR-ABL gene have been reported as a major cause in patients with CML, and it had been detected in ~40-50% of all resistant cases [13]. These mutations can corrupt the binding of IM to tyrosine kinase resulting in drug resistance [14]. Currently, two theories have emerged to explain IM resistance in terms of point mutations: (1) direct inhibition by altering the amino acid located at the critical binding point between the inhibitor and the kinase, or (2) indirect inhibition by changing the protein conformation of the kinase domain, specifically in the key regions which constitute the ATP binding loop (P-loop), activation loop (A-loop) and catalytic cleft [15].

Many studies have investigated the frequency of different types of point mutation in CML patients, but few reports exist from Sudan. These few studies have looked at the prevalence of xenobiotic gene mutations in CML and AML patients 4-6. Here we focus on finding the frequency of mutations that occur in the P-loop, especially the mutation at codon 255 (E255V/K) and the one at codon 253 (Y253H). In addition to this, the one that occurs in the catalytic domain at codon 351 (M351T) in Sudanese CML patients is also studied.

Recently, it has been recognized that not all mutations that originate in the P-loop have clinical implications, but the mutations which take place in codon 253 (Y253H) and codon 255 (E255K) are associated with drug resistance and poor prognosis [16, 17]. On the other hand, previous studies have reported that there is an association between the presence of the M351T mutation and the emergence of the T315I mutation, which is known to be the one that causes IM resistance [18]. Accordingly, early detection and characterization of BCR-ABL point mutations may have a significant role in tailoring the best therapeutic plan for patients with CML [19], hence this will help the physician in optimizing the management of the patients' clinical status. This study goals to detect the mutations occurring in codons, 255 (E255V/K), 253 (Y253H), and 351 (M351T), in Sudanese CML patients

Materials and Methods

Study Design

This is a hospital-based, cross-sectional, descriptive study. It was performed from May 2018 - May 2019 to determine the impact of BCR-ABL kinase domain mutations, specifically Y253H, E255K, and M351T in CML patients of Sudan.

Subjects

One hundred CML patients attending the Radiation and Isotopes Center of Khartoum (RICK), Sudan, were invited to take part. CML was identified by the presence of the BCR-ABL gene, detected by the RT-PCR (Reverse Transcriptase Polymerase Chain Reaction) technique performed following the CBC and bone marrow (BM) examination.

A total of 99 CML patients were tested for BCR-ABL tyrosine kinase domain mutations (a single patient being excluded owing to the failure of PCR amplification). From the questionnaire, 25 of the patients were new cases, the remaining 74 patients receiving 500 mg/day of imatinib mesylate therapy. Of the latter, 25 patients showed resistance to the treatment and accordingly were given a higher dose of imatinib mesylate (800 mg/day), based on the RIC hospital protocol. Patients negative for BCR-ABL fusion gene, or who tested positive for other types of leukemia were excluded. All subjects included were interviewed by questionnaire to ascertain age, gender, residence, occupational history, and family history of cancer.

Mutation Detection

For each subject, two milliliters of blood was collected in an EDTA container, RNA was extracted using a high pure RNA isolation kit (Roche Applied Science, Germany) according to the manufacturer's procedure, and the isolated RNA was preserved at -80°C until use. RNA was transformed into cDNA in a one-step process using RT-PCR with Oligo (dT) 15 primer in accordance with the manufacturer's recommendations (iNtRON Biotechnology, Inc., Korea).

Nested PCR was used for the detection of ABL-kinase domain mutations, followed by restriction enzyme digestion. In the first step, the common BCR-ABL allele was amplified using primers that annealed in BCR exon 13 and ABL exons 7, producing fragments of 675 bp containing different mutation sites encoding ABL amino acids from 207 - 414. In the second

step, amplification using specific primers for each mutation in turn (Y253H, E255K, and M351T) was performed separately. All primers and PCR protocols used were as previously described [20].

Following amplification, mutation-specific restriction enzymes were applied to reveal the proportion of mutant and wild-type alleles using RsaI enzyme for Y253H mutation, MnlI enzyme for E255K mutation, and NcoI enzyme for M351T mutation. For each reaction, 10µl of PCR product, 18µl of D.W, 2µl of each respective enzyme and 2µl of the corresponding buffer were incubated overnight at 37°C for complete digestion, followed by a step for enzyme inactivation by incubation for 20 min at 80°C for RsaI or at 65°C for MnlI and NcoI. Finally, the products were visualized using 3% ethidium bromide-stained agarose gel. The occurrence for all the studied mutations caused loss of restriction sites in the ABL tyrosine kinase domain, with banding patterns shown in **Table 1**.

Statistical Analysis

SPSS v25 (Statistical Package for Social Sciences) was used for statistical analysis. Data were expressed as both numbers and percentages of the total for categorical variables. Possible interactions of the kinase domain mutation with respect to gender and age were assessed using the chi-square test. The OR (odds ratio) with a CI (confidence interval) of 95% was calculated by logistic regression. P-values of less than 0.05 were considered statistically significant.

Results and Discussion

The cohort comprised 44 (44.4%) males and 55 (55.6%) females with a mean age of 44.77 ranging from 11 - 70 years. After categorizing by age into two groups, 23 (23.2%) of the patients were <40 years old while 76 (76.8%) were ≥40 years. According to the treatment status of the patients, 25 (25.3%) were new cases, 49 (49.3%) were under treatment and 25 (25.3%) showed treatment resistance. Analysis revealed that the frequencies of the wild-type alleles Y253H, E255K, and M351T were respectively 93 (93.3%), 97 (98%), and 93 (93.9%). Y253H and M351T mutant alleles were equally distributed among CML patients, comprising 6 (6.1%) for each, while only 2 (2%) of the patients had the E255K mutant allele as shown in **Table 2**. As regards gender difference, females had a higher frequency of the Y253H mutant allele than males (83.3% & 16.7% respectively), while the M351T mutant allele was more frequent among males (at 66.7%) than females (at 33.3%), all of these findings being statistically significant (p<0.05). Moreover, the E255K mutation was equally distributed among males and females, each accounting for 50%. The effect of age was also considered and analysis showed that the frequency of the Y253H mutant allele was only insignificantly increased among the patients ≥40 years (83.3%). However, mutant alleles of E255K and M351T were found only among patients ≥40 years (**Table 3**).

Among the total of 25 new cases, kinase domain mutation was reported in only one patient who was found to have the mutant allele of M351T comprising 4% while Y253H and E255K mutations were not detected, as shown in **Table 4**.

The odds ratio was calculated to assess the association between kinase domain mutations and the treatment status of patients. Patients under treatment, and that showing treatment resistance, were analyzed and the new cases were excluded. The mutant allele of Y253H was found in 3 (6.1%) of the patients under imatinib mesylate treatment and in 3 (12%) of patients who showed resistance to the treatment, but these results did not differ statistically (p. value= 0.389, OR= 0.48, CI= 0.09-2.56). Similar results were observed for M351T mutation in which the mutant allele was only insignificantly higher among resistant patients than in those under treatment (12% & 4.1 % respectively, p. value = 0.22, OR= 0.30, CI= 0.05-2.00). It was not possible to calculate a p-value for E255K mutation on account of collinearity as the E255K mutant allele was detected only among patients with resistance to the treatment (given in **Table 5**).

Table 1. Restriction enzymes and digestion products for tyrosine kinase mutations

Mutations	Restriction enzymes	Digestion products	
		Wild type (bp)	Mutant (bp)
Y253H	RsaI	138, 30	168
E255K	MnlI	136, 31	167
M351T	NcoI	218, 123	341

Key: bp= base pair.

Table 2. Frequency of demographic data, clinical status and kinase domain mutations among CML patients

Variable	Frequency	Percent	
Gender	Male	44	44.4%
	Female	55	55.6%
Age (Year)	< 40	23	23.2%
	≥ 40	76	76.8%
Treatment status	New cases	25	25.3%
	Under-treatment	49	49.5%
	Resistant	25	25.3%
Kinase domain mutation			

Y253H	Wild type (Y)	93	93.9 %
	Mutant (H)	6	6.1%
E255K	Wild type (E)	97	98%
	Mutant (K)	2	2%
M351T	Wild type (M)	93	93.9 %
	Mutant (T)	6	6.1%

Table 3. Distribution of kinase domain mutations according to the gender and age of the patients.

Kinase domain mutation	Gender N (%)		Age N (%)		
	Male	Female	<40 year	≥40 year	
Y253H	Wild type (Y)	43(46.2%)	50 (53.8%)	22(23.7%)	71(76.3%)
	Mutant (H)	1(16.7%)	5(83.3%)	1(16.7%)	5(83.3%)
	P. value	0.158		0.694	
E255K	Wild type (E)	43(44.3%)	54(55.7%)	23(23.7%)	74(76.3%)
	Mutant (K)	1(50.0%)	1(50.0%)	0 (0%)	2(100%)
	P. value	0.873		0.432	
M351T	Wild type (M)	40(43.0%)	53(57.0%)	23(24.7%)	70(75.3%)
	Mutant (T)	4(66.7%)	2(33.3%)	0 (0%)	6(100%)
	P. value	0.258		0.164	

Key: N= total number.

Table 4. Distribution of kinase domain mutations among new cases.

Kinase domain mutation	N (%)	
Y253H	Wild type (Y)	25 (100)
	Mutant (H)	0 (0)
E255K	Wild type (E)	25 (100)
	Mutant (K)	0 (0)
M351T	Wild type (M)	24 (96)
	Mutant (T)	1 (4)

Table 5. Association between kinase domain mutations and the treatment status of the patients.

Kinase domain mutation	Resistance N (%)	Under treatment N (%)	P. value	OR	95% CI
Y253H	Wild type (Y)	22(88%)	46(93.9%)	0.389	Reference
	Mutant (H)	3(12%)	3(6.1%)		
E255K ^a	Wild type (E)	23(92%)	49 (100%)	-	-
	Mutant (K)	2(8%)	0(0%)		
M351T	Wild type (M)	22(88%)	47(95.9%)	0.30	0.05-2.00
	Mutant (T)	3(12%)	2(4.1%)		

Key: N= total number; OR= Odd Ratio; CI= Confidence Interval; a = E255K omitted because of collinearity.

Hematological malignancy is the second commonest type of cancer in Sudan with a high mortality rate, based on the recent report in 2020 of the WHO international agency on cancer, Globocan [21], with prevalence differing from that of more developed countries, especially occurring far more frequently than in the United States [22]. Recently, Amar A and co-workers found that about the third of leukemias in Sudan were CML (32.3%), accounting for the commonest hematological malignancy [23]. These studies reflected that leukemia is highly prevalent in Sudan, but the availability of data regarding the genetics and epigenetics of the disease is small due to limited funding. Hence, funding and supporting the research for this community would be crucial to conducting advanced research.

Kinase Domain (KD) mutations of ABL1 are the commonest mechanisms of resistance to Tyrosine Kinase Inhibitors (TKI) in CML, accounting for 36 - 48% of patients with Imatinib resistance due to BCR-ABL P-loop mutations [24, 25]. Thus, the purpose of the current research is to ascertain the prevalence of mutations of ABL P-loop (Y253H and E255K) and the mutation of the catalytic domain, M351T in CML patients.

To detect the prevalence of KD, we used customized RT-QPCR, nested PCR, and RLFP. We believe this method to be straightforward, quantitative, and rapid, with good sensitivity and specificity. Therefore, this method has been used to find the frequency of the ABL P-loop mutations (Y253H and E255K) and catalytic domain (M351T) mutations, correlated with gender, age, and clinical status in Sudanese CML patients.

Our study discovered that females outnumbered males, respectively at 55.5% and 44.4%. It also revealed that few cases of CML occur in young people - those under 40 - but that incidence increases with age, which is in agreement with previous studies. Cases over 40 years old accounted for 76.8%, while those under 40 were 23.2%, a finding close to that of Phukan A and co-workers, who found the median age of BCR-ABL transcript patients was 36 [26]. In contrast, the median age of Asian

and African patients with CML is 50 years at diagnosis [27]. In Western countries, the median age of CML patients is around 57 years, reflecting the higher median age. Patients over 70 account for more than 20% of the total, while children and adolescents account for 5%. Caucasian CML patients have a median age of 60 years. Other studies found that the incidence rate of CML was higher in men, with a peak at age 75-79 years [28, 29].

Our findings are consistent with those of Gharote and co-workers, who identified BCR-ABL in 33 men and 17 women with an average age of 40.75 years. Another study, by Pajiep *et al.*, also discovered that patients were 56-60 years old at the time of diagnosis, with a male/female ratio of 1.2:1.7. Little is known about the reason for these differences in the history of the disease, whether because of the African people's age pyramid people or unknown environmental and/or genetic factors.

Our focus is on the M351T mutation, where the particularly hydrophobic amino acid methionine is replaced with the less hydrophobic threonine at position 351. According to our research, M351T is found in a small percentage (6.1%) when compared with data from another study by Srivastava S and co-workers, who reported an M351T incidence of 10% [30]. In contrast, two different studies reported that the incidence of the M351T mutation was 32% and 10% [31, 32]. Moreover, Babu G discovered that M351T and T315I were the most common mutations [33] and Elnahass YH *et al.* reported M351T in 4/17 patients and E255K in 2/17 [34].

Regarding the E255K gene, its mutation was determined in two patients (2% of all mutations), including resistance treatment patients, and was not detectable in any new cases under treatment. This is consistent with a study of Mitra P and co-workers who discovered 4 cases of IM resistance in 100 CML [35], and one of Waggas A and co-workers who also discovered only a single patient (2%) positive for E255K but five (10%) for T315I [36]. However, other studies revealed that E255K was the most common mutation in CML patients [37].

Our final results showed the Y253H Mutation was present in both resistant and treating patients, but was not present in any new cases; the prevalence was 6.1%. This is in agreement with other studies such as one of Mir A *et al.*, who found a high prevalence after a long period of treatment and resistance [38].

Our findings are consistent with those of Hughes and co-workers, who reported that 4% of people have E255K and 4% have Y253H. Another study by Elias *et al.* discovered E255K in 4 patients (3.20%), M351T in 2 (1.6%), and Y253H in 1 (0.8%). Moreover, Tadesse F detected that of non-P-loop mutations, M351T was in 1 (3.2%). Among the P-loop mutations Y253H was in 1 (3.2%) [39].

Conclusion

Firstly, multiple mutations can play a role in the etiology of resistance development against TKI therapy, but the T315I mutation is significantly more common in CML patients. Secondly, Y253H and M315T had similar prevalence, even though E255K had a lower prevalence but played a significant role in resistance development against TKI. As a result, we recommend that genetic testing be performed to detect KD mutations using molecular techniques. This will aid in the selection of appropriate treatment strategies to prevent disease progression in CML patients. Because the number of samples used in the study was small, we will need to conduct additional research into the ABL-BCR mutations that occur in CML patients. Our data show that there are no risk factors associated with BCR-ABL mutations regarding age, gender, or race.

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