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Relation of common ABL kinase domain mutations with resistance to Tyrosine Kinase Inhibiters in patients with Chronic Myeloid Leukemia in Middle Euphrates of Iraq

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Background: Chronic myeloid leukemia (CML) is a hematopoietic stem cell disease, associated with a reciprocal translocation between chromosomes 9 and chromosome 22, lead to the formation of the BCR-ABL fusion gene (Philadelphia chromosome). This fusion gene is believed to play golden role in the initial development of CML with constitutive tyrosine kinase activation.

Successful use of tyrosine kinase inhibiters (TKIs) play a role in improve survival and increase prevalence of CML, but un fortunately mutations in the BCR-ABL kinase domain may cause, or contribute to increase, resistance to TKIs in CML patients. .

Objective: This study was designed to assess the association of five most common BCR-ABL kinase domain mutations (T315I, M351T, E255K, M244V and E255V) with resistance state of CML patients on TKIs in Iraqi Middle Euphrates region.

Patients and methods: A retrospective case-control study in which 85 patients with chronic myeloid leukemia in chronic phase (45 patients as cases group and 40 patient as control group) were selected from three hemato-oncology centers in middle Euphrates in Iraq during the period from January 2016 till October 2016 out of a total of 240 CML patients (108 male and 132 female) who were registered during this period in these three centers and all patients on TKI (Imatinib and Nilotinib). Venous blood sampling done for BCR-ABL kinase domain mutations screening.

Results: four patients from cases group (4/45) were carriers of one of five selected ABL kinase domain mutations and no one of control group. T315I mutation was detected in 3/45 (6.6 %) of resistant patients, with a significant risk association to develop resistance to TKI therapy (odd ratio and C. I.) (6.67, 0.3340 - 133.2255). E255V was detected in 1/45 (2.2 %) and also had significant risk association to develop resistance to TKIs (odd ratio, C.I.) (2.73, 0.1081 -68.9424). No one of these mutations had significance correlation with demographic or hematological features. M351T, E255K and M244V were not detected in any one of our study groups CML patients.

Conclusions: T315I and E255V among five ABL kinas domain mutations were detected in our CML patients with resistance to TKIs. All of them may play a role in development variable degree of resistance to first and second generation TKIs weather primary or secondary.T315I mutation is most common mutation within BCR-ABL domain kinase gene.

Key words: CML, TKIs, ABL domain kinase mutations

Introduction: Chronic Myeloid Leukemia (CML) is a hematopoietic stem cell disease, characterized by a reciprocal translocation between chromosomes 9 and 22, resulting in the formation of the Philadelphia chromosome (Ph.).[(1)] This translocation t(9;22) results in the head-to-tail fusion of the breakpoint cluster region (BCR) gene on chromosome 22 at band q11 and the Abelson murine leukemia (ABL) gene located on chromosome 9 at band q34. The product of the fusion gene (BCR-ABL) is believed to play a central role in the initial development of CML. [(1)] this genetic abnormality was first named at 1960 so it was one of the first malignancies to be linked to a clear genetic abnormality. [(2)]

In less than last 10 years, the prognosis of CML has changed from that of a fatal disease to a disorder amenable simply to lifelong oral medication and compatible with a normal lifespan.[(3)] This change has been made possible by a deep understanding of the molecular pathogenesis and a determination to develop targeted and selective drugs.[(4)]

Introduction of imatinib (Gleevic, Novartis) into clinical practice nearly one decade ago, has dramatically changed treatment and follow-up of CML. [(5)] Imatinib specifically targets tyrosine kinase activity of the oncogenic protein encoded by BCR/ABL gene. Then after, new other tyrosine kinase inhibitors (TKIs) were developed. [(6, 7)]

ABL kinase domain mutations are identified in about 30–50% of CML cases and it is variable as a consequence of different methods of detection, nature of resistance, and disease phase examined and are the most frequently identified mechanism of treatment resistance.[(8)]

More than 100 kinase domain mutations are known till now to cause varying degrees of resistance to the TKIs. These point mutations usually result in amino acid changes, which decrease binding affinity of TKIs, but not the usual substrates. [(9)]

Materials and methods: A retrospective case-control study in which 85 patients with chronic myeloid leukemia in chronic phase (45 patients as case group and 40 patient as control group) were selected from three hemato-oncology centers in middle Euphrates in Iraq (Karbala, Babylon and Al Najaf centers) during the period from January 2016 till October 2016 out of a total of 240 CML patients (108 male and 132 female) who were registered during this period in these three centers.

Patients:

Case group includes 45 CML patients resistant to TKI treatment out of 67 resistant patients eligible for the study, who complete ≥6 months of treatment with TKI, 43 patients were in primary resistant state and 2 patient with secondary resistance. The other 22 patients refuse to participate in this study.

- ***Primary resistance patients*:**

CML patients who complete 6 months of TKIs treatment and their molecular response after treatment is either under warning category (BCR-ABL level 1-10 % IS after 6 months or 0.1-1 % IS after 12 months of treatment) or under failure category (BCR-ABL level >10 % IS after 6 months or >1 % IS after 12 months) according to the ELN guideline for the molecular monitoring of CML patients on TKI. [(10-12)]

- ***Secondary resistance patients*:**

CML patients who complete ≥ 6 months of TKIs treatment and their molecular response to treatment was optimal (BCR-ABL level <1 % IS after 6 months or < 0.1 % IS after 12 months) according to the ELN guideline but lose their response at any time during the period of treatment, that does not fit for the optimal response criteria. [(11, 12)]

Exclusion Criteria:

In this study we excluded all patients with newly diagnosed, lost their data and poor compliance to treatment.

Follow up adherence of patients to treatment was approved as possible by oral or phone communications with each patient to exclude the possibility of poor compliance as a cause for response failure.

Control group definition:
Control group includes 40 CML patients out of 195 CML patient of good response to TKI treatment randomly selected, according age and gander, who complete ≥6 months of treatment with TKI and their molecular response to treatment was at optimal category (BCR-ABL level ≤1 % IS after 6 months or ≤ 0.1 % IS after 12 months) according to the ELN guideline without any evidence of secondary resistance. [11, 12]

Ethical issue:
The study protocol was approved by the Ethics Committee of the College of Medicine, University of Kufa. In addition to an oral permission for blood sampling and do laboratory analysis was attained from all patients included in this study.

Assessment of exposure:
After categorization of CML patients eligible for the study into study and control groups, the presence or absence of most common 7 ABL kinase domain mutations and their correlation with the differences in the response criteria of patients to TKIs according to ELN guideline were assessed. These mutations identified from important and published articles in CML field.

Potential Confounders:
Other causes of resistance to TKIs treatment (like other less common Kinase Domain mutations and ineffective GIT absorption of drug) were not assessed in this study due to limitation in the cost and time.

ASO-PCR:
Blood samples were collected from all participated patients. First of all DNA was extracted from blood samples using commercial available DNA extraction Kit (Promega, USA) following manufacture instructions. ASO-PCR was performed in 30µl reaction (Kang et al., 2006; Wongboonma et al., 2012). Briefly, five master mixes were prepared, for T315I, M351T, E255K, M244V and E255V mutations detection. PCR master mixes were prepared according to the stander procedure as the manufacture company advice. We design 13 different ASO-PCR primer sets according to the frequency of known *ABL* gene mutations (5 mutations) for normal allele and mutant allele (primers synthesis by Macrogen Korea). The sequences of mutant primers were adapted from a previously published article and sequences of normal primers (wild type primers) were designed using BLAST search and Primer 3. The sequences of forward and reverse primers used for ASO-PCR are shown in below.[13]

A. Normal forward Primers for normal alleles:

N/T315I-F: gcccccgttctatatcatcac, N/M351T-F: ccactcagatctcgtcagccat, N/E255K-F: gcgggggccagtacgggg, N/M244V-F: gaacgcacggacatcacca and N/E255V-F: gcgggggccagtacgggga for T315I, M351T, E255K, M244V and E255V respectively.

B. Mutant forward Primers for mutant alleles:

M/T315I-F: gcccccgttctatatcatcat, m/M351T-F:ccactcagatctcgtcagccac, m/E255V-F: gcgggggccagtacggggt, m/M244V-F: gaacgcacggacatcaccg and m/E255K-F: gcgggggccagtacggga for T315I, M351T, E255K, M244V and E255V respectively.

C. Reverse primers

244-R: gccaatgaagccctcggac (This primer was used for mutations: M244V, E255K, E255V), 315-R: ggatgaagtttttcttctccag (This primer was used for mutation T315I) and 351-R1: gccctgagacctcctaggct (This primer was used for mutation M351T).

Statistical Analysis:
Statistical analysis was performed using SPSS 22 (statistical package for social sciences) and Excel 2010 programs. Data analysis was done using t- test, analysis of variance (ANOVA) & chi –square test for tables with frequencies, percentages, range mean &standard deviation.

Results: Five ABL domain kinase mutations had been evaluated by using ASO – PCR which were T315I, M351T, E255K, and M244F AND E255V in both control and study group .No mutations within control group and only 5 patients (nearly 11%) of study group had mutations.

T315I had the highest frequency as it recorded 60 % (3 out 5 mutations) of total mutations and one mutation (20 %) for E255V. **Tables 1, 2 ande3 with Figures 1and 2.**

Table 1. General characters of cases and control groups

General characters		Control group	Cases group	P. Value
Gender	Female	23 (57.5 %)	26 (57.8 %)	0.979
	Male	17 (42.5 %)	19 (43.2%)	
Age (Mean in years ± S.D.)		46.25 ± 10.37	43.9 ± 12.95	0.506
Type of treatment	Imitinib	36 (90%)	33 (73%)	0.050
	Nilotinib	4 (4%)	12 (27%)	
Duration of treatment (Mean in months ± S.D.)		21.4 ± 9.3	18.9 ± 12.6	0.319
Geographical distribution	Babylon	13 (32%)	17 (38%)	0.326
	Al Najaf	11 (28%)	11 (24%)	
	Karbala	10 (25%)	12 (27%)	
	Al Qadisiyah	4 (10%)	3 (7%)	
	Al Muthana	2 (5%)	2 (4%)	

Table 2: Type of ABL domain kinase mutations in study and control group

Mutations		Cases group	Control group	Total	P value	Odd ratio and 95% C.I
T315I	**Positive**	3/45	0/40	3/85	0.096	6.67 0.334 -133.225
	Negative	42/45	40/40	82/85		
M351T	**Positive**	0/45	0/40	0/85	-	
	Negative	45/45	40/40	85/85		
E255K	**Positive**	0/45	0/40	0/85	-	
	Negative	45/45	40/40	85/85		
M244F	**Positive**	0/45	0/40	0/85	-	
	Negative	45/45	40/40	85/85		
E255V	**Positive**	1/45	0/40	1/85	0.343	2.73 0.108-68.942
	Negative	44/45	40/40	84/85		
Total	**Positive**	5/45	0/40	5/85	-	
	Negative	40/45	40/40	80/85		

A

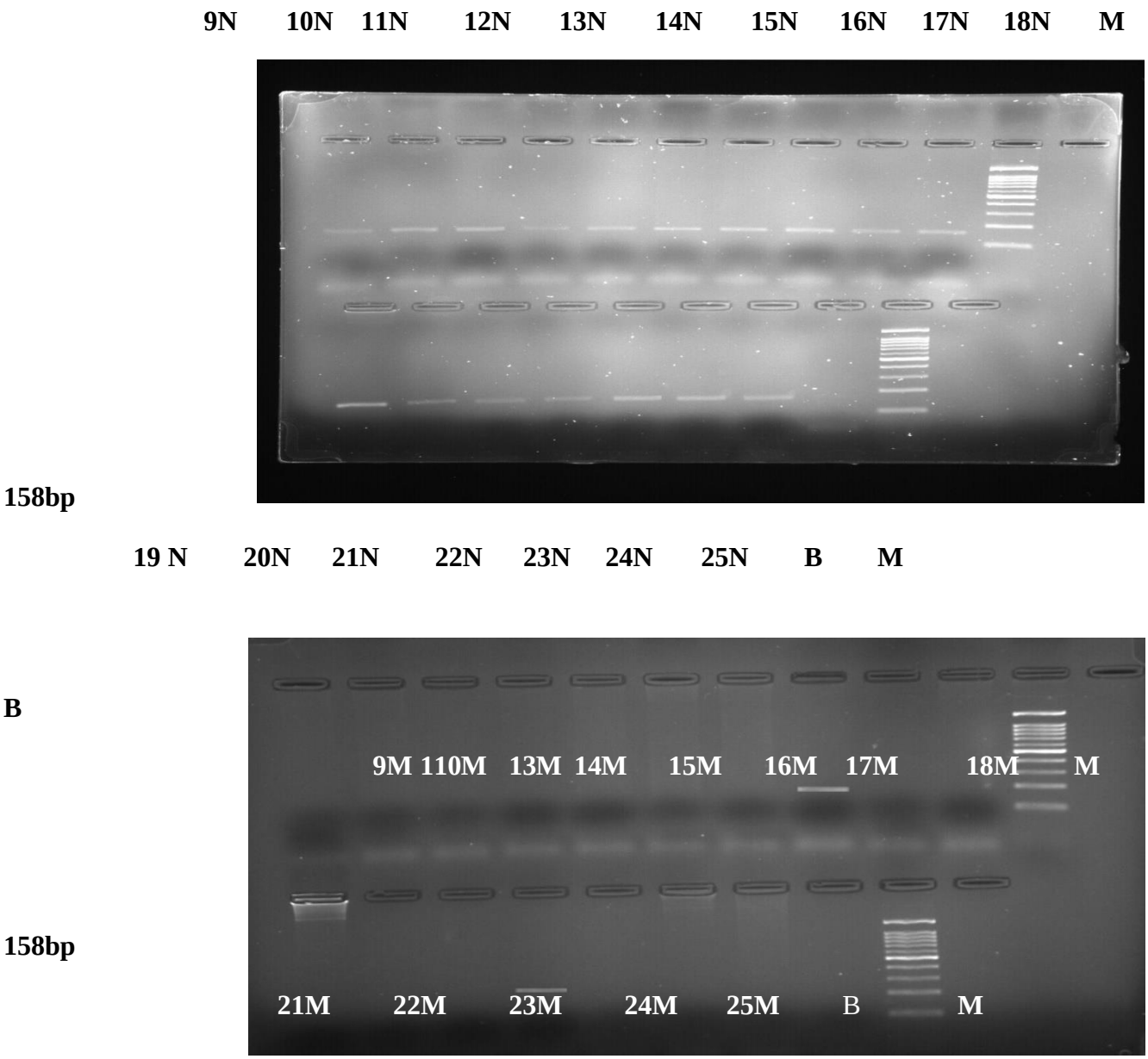

Figure 1. A and B: Electrophoresis on agarose gel 2%. 9-25 numbers are sample from cases group patients for T315I mutation. A show 9N-25N is amplification of normal allele and B show 9M-25M indicate amplification of mutant allele. **M** (DNA ladder 100 bp) B blank or negative control. In this figure show amplification of normal allele and only 16 M and 22M are amplificated allele other are not.

Association of positive mutations with general patients finding like age, gender, type and duration of TKI management and type of resistant if present. Table 3.

Table 3: Association of T315I and E255V mutations with general patients finding in cases group

General Features for mutations		Positive mutation	Negative mutation	P value
T315I				
Mean of Age (years)		44.6	44.7	0.981
Gender	Male	2	16	0.375
	Female	1	26	
Type TKI	First generation	1	32	0.105

treatment	Second generation	2	10	
Mean treatment duration (months)		24.3	19.5	0.455
Primary resistance		3	40	0.699
Secondary resistance		0	2	
E255V				
Mean of Age (years)		28	44.8	0.220
Gender	Male	0	18	0.387
	Female	1	26	
Type TKI treatment	First generation	1	32	0.542
	Second generation	0	12	
Mean treatment duration (months)		16	19	0.815
Primary resistance		1	42	0.827
Secondary resistance		0	2	

Association of positive mutations with hematological findings:

A. **T315I:** In positive cases the mean hemoglobin concentration was 11.67g/l + 1.15 (Mean ±SD), while in negative cases mean hemoglobin concentration was 11.99 g/l $\pm$ 1.78. The mean total white blood cells count in positive cases was 10.2 $\pm$ 3.2 x10^9\L, while in negative cases 8.24 $\pm$ 3.52 x10^9\L. WBC deferential mean recorded about 54 , 36 , 4 , 2 and 1 % for neutrophil , lymphocytes , monocytes , eosinophil and basophil respectively eight cases (17.7 %) of them had basophilia within this resistant cases (positive and negative for all studied mutations). Also within cases group there were six cases (13.3 %) recorded immature cells ranged from myeloblast to band form neutrophil. The mean platelet count in positive cases was 270$\pm$ 11.3 x10^9/L and in negative cases group was 211$\pm$ 79.4 x10^9/L.

B. **E255V:** In positive case the mean hemoglobin concentration was 10.40g/l, while in negative cases mean hemoglobin concentration was 12.01 g/l $\pm$ 1.74. The mean total white blood cells count in positive case was 8.40 x10^9\L, while in negative cases 8.38 $\pm$ 3.54 x10^9\L. The mean platelet count in positive case was 2.70x109/L and in negative cases group was 214$\pm$ 82.2 x10^9/L.

Discussion:

Mutations within the kinase domain of genetic abnormalities may contribute and lead to failure of management and loss of therapy effects. These mutations are the most commonly investigated mechanism of resistance to TKIs, but they are not the only one.[14] Really, the frequency by which mutations have been associated with TKIs resistance is variable top variation to the stage of CML often from twenty five percent to thirty percent of early chronic phase patients on specially first-line imatinib to approximately 70% to 80% of blastic crisis patients.[14,15]

The current study was conducted to investigate most frequent genetic abnormalities in published articles to provide the clinicians on how to best integrate the mutations analysis in the routine management of CML patients.

A single amino acid substitution by point mutation at the abnormal gene development is the commonest way of acquired resistance to TKIs, which inhibit drug binding by main drug activities on certain pathway lead to t loss drug targeting. [16]

BCR –ABL kinase domain mutations have a considerable effect on TKIs primary and secondary resistance in some patients with CML and such resistance can appear at any time during TKIs treatment, so they have important roles in treatment failure.[16]

Till now there are more than 100 known BCR-ABL point mutation.[13] The European Leukemia Net proposal recommend analysis of these mutations in cases of CML at time of diagnosis in patient showing accelerated or blastic phase or in cases ranked under warning or failure response, as it play important role in treatment choice.[10,11]

By ASO – PCR this study focused on five most common mutations according to prevalence of mutations published by different studies. [13] Four patients carried ABL domain kinase mutations, all of them from cases group .T315I was most common one recorded 6.6% of total study group cases, while E255V record 2.2% of total study grou

In related to T315I mutation a threonine amino acid to isoleucine amino acid replacement at position 315 site (T315I) of BCR-ABL protein results in clinical resistance to first and second generation of TKIS.[17] This mutation is located on kinase domain exon 6 and its frequency in BCR-ABL1-mutated CML was 20.2%. [13]

In this study T315I was 6.6% of total resistant cases (cases group), this results were in agreement with other workers in Iran [Chahardouli B, et al][17], it was higher than that recorded by other Iraqi workers [Maysaa A. R. Dhahi, et al 2013][18], less than [Elias Jabbour , et al 2008] who recorded 11% of CML resistant cases to TKIs were carrier to this mutation.[19(

Even there were no statistical differences between groups of sample size, which is mostly related to small sample size in both groups, but this mutation had good odds ratio (OR) 6.67 because odds ratio reflect the strength of association between presence or absence of certain properties in the presence or absence of specific diseases in a given population, An odds ratio more than 1 indicates that certain condition or event is most likely to occur in the specific group. [20(

Treatment duration of mutated cases ranged between 7-42 months since diagnosis and one of them was on Imitinib, while the other started with Imitinib and then after a while changed to Nilotinib .

In related to other demographic features there is no statistical significance between mutated cases and non-mutated one may be because small sample size and low prevalence of mutations, while in related to hematological feature also there is no statistical significance except that related to eosinophil present from white blood cells deferential as it was statistically significance, but no certain explanation, so it might occurred by chance due to other underline cause.

E255V mutation results in an a glutamic acid (E) amino acid replacement at site 255 in BCR-ABL from to a valine (V) amino acid. It is located on P-loop site of the KD exon 4. Incidence of this mutation in CML was 2.9 %.[13]

In the current study E255V mutation recorded 2.2 % of total study group and no one in control group, which in agreement with one of Indian studies in this field [24] and less than that recoded in Italy and Argentinean studies, where recorded 8% and 17% respectively.[23(Mutated case was categorized as primary resistance case, ranked under failure response and 16 months on Imitinib. Also there was no statistical difference between groups of sample size in this study and this mutation had good odds ratio (OR) 2.73 and it reflect the association of presence of this mutation with TKIs resistance among resistance CML patients.

There is no statistical difference between both sample size group in related to demographic and hematological findings.

Conclusions:

T315I and E255V among five ABL kinas domain mutations were detected in our CML patients with resistance to TKIs. All of them may play a role in development variable degree of resistance to first and second generation TKIs weather primary or secondary.T315I mutation is most common mutation within BCR-ABL domain kinase gene.

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