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Mutational analysis of T315I in patients with chronic myeloid leukemia who did not respond to second-generation tyrosine kinase inhibitors

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Abstract:

BACKGROUND: Chronic myelogenous leukemia (CML) is a hematological stem cell disorder that associates with mutations in BCR-ABL. Although mutations identified in more than 30 different amino acids, the highest degree of resistance was associated with single-point mutation T315I of the ABL gene.

OBJECTIVES: This study aims to identify the prevalence of T315I mutation among CML patients who lost their Major molecular response to second-generation tyrosine kinase inhibitor (TKI) at the time of the study and its relation to failure response.

SUBJECTS AND METHODS: A prospective cross-sectional study included 50 adult patients diagnosed with CML. We have used the Sanger sequencing polymerase chain reaction based methods for detection of T315I mutation.

RESULTS: Out of 50 patients, 28 were female (56%). Patients' age was ranged between 23 and 63 years with a mean age of 42.8 ± 11.46 years. There was no expression of T315I mutation in any of 50 CML patients, however, there was another mutation have been detected which is rs2070997 (response to drug) and was positive in 18 patients. There were 8 (16%) patients that have rs2070997 mutation have primary failure on first-generation TKIs, while the other 10 (20%) patients were those who started second-generation TKIs directly.

CONCLUSIONS: T315I mutation was not common in patients with CML-chronic phase. Other (BCR-ABL dependent or independent) mechanism could be responsible of TKIs resistance.

Keywords:

Chronic myelogenous leukemia, T315I mutation, tyrosine kinase inhibitor resistance

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Introduction

Chronic myelogenous leukemia (CML) is a clonal disorder of pluripotent steam cell that account for around 15% of leukemia and may occur at any age. It is characterized by increased and unregulated growth of myeloid cells in the bone marrow, and the accumulation of excessive white blood cells.[1] Despite CML had increased proliferation, but without the loss of their capacity to differentiate.[2]

However, 95% of CML patients have the ABL gene from chromosome 9 fused with the breakpoint cluster (BCR) gene from chromosome 22, resulting in a short chromosome known as the Philadelphia chromosome. Although mutations have been identified in more than 30 different amino

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acids, the highest degree of resistance was associated with single-point mutation T315I of the ABL gene in the BCR-ABL fusion transcript. This acquired point mutation results in amino acid substitution at position 315 in BCR-ABL1, from threonine (T) to an isoleucine (I). Early detection of T315I mutation in CML patients during therapy could allow alternative treatment before resistance is detected cytogenetically or before disease progression become evident.

Subjects and Methods

A prospective cross-sectional study conducted on 50 (according to size sample calculation)[3] adult patients who diagnosed as *de novo* CML according to the laboratory and FISH analysis. The collection period was from December 2018 to November 2019. The patients were seen in the Hematology Unit of Baghdad Teaching Hospital in Medical City. For each patient, a questionnaire was done included patients general information (past and recent medical history, drug history, type and duration of treatments, compliant, result of blood and polymerase chain reaction [PCR] test). The Ethical Committee of the College of Medicine, University of Baghdad, approved this study. Informed consents were taken from all patients included in the study.

Inclusion criteria

1. Patients more than 18 years old diagnosed as CML based on the presence of Ph Chromosome according to ELN 2013[4]
2. Received second-generation tyrosine kinase inhibitor (TKI) with failure to response
3. Patients compliant with second-generation TKIs
4. No hematological response after 3 months or no major molecular response (MMR) after 6–12 months (Bcr-Abl > 0.1%).[4]

Exclusion criteria

1. Patients younger than 18 years with primary or secondary resistance to first-generation TKI
2. Newly diagnosed CML patients
3. Patients noncomplaint on second-generation TKIs.

Blood sampling

Three milliliter of fresh peripheral blood were collected from the eligible patients into EDTA tubes. DNA extraction using the Geneaid Kit for DNA extraction (Lot No. FE16205-N) then DNA was directly frozen at −20 C in the teaching laboratories of medical city until the time of sequencing.

Methods

The analysis of data and determined sequence variation between samples of specific gene using geneious software after amplification, all the tests were done in the National Centre of Teaching Laboratories/Genetic department/ Medical City and in the Advance Scientific (ASCO) learning center.

Standard sequencing

PCR product was send for Sanger sequencing using ABI3730XL, automated DNA sequences, by Macrogen Corporation – Korea. The results were received by E-mail then analyzed using geneious software [Figure 1a and b].

This method based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during *in vitro* DNA replication. This requires a single-stranded DNA template, a DNA primer, a DNA polymerase, normal deoxynucleotide triphosphates (dNTPs), and modified di-deoxynucleotide triphosphates (ddNTPs), the latter of which terminate DNA strand elongation. These chain-terminating nucleotides lack a 3′-OH group required for the formation of a phosphodiester bond between two nucleotides, causing DNA polymerase to cease extension of DNA when a modified ddNTP is incorporated. The ddNTPs may be radioactively or fluorescently labeled for detection in automated sequencing machines.

The DNA sample is divided into four separate sequencing reactions, containing all four of the standard deoxynucleotides (dATP, dGTP, dCTP and dTTP) and the DNA polymerase. To each reaction is added only one of the four dideoxynucleotides chain terminator that labeled with fluorescent dyes, each of which emit light at different wavelength (ddATP, ddGTP, ddCTP, or ddTTP), while the other added nucleotides are ordinary ones. Following rounds of template DNA extension from the bound primer, the resulting DNA fragments are heat denatured and separated by size (or length) using capillary electrophoresis. Then detecting and recording the fluorescence dye, and output the data as fluorescent peak trace chromatograms [Figure 2].[5]

Results

Out of 50 patients diagnosed as CML, 28 were female (56%) and 22 were male (44%) with 1.2/1 ratio. Patients' age was ranged between 23 and 63 years with a mean age of 42.8 ± 11.46 years. The mean age for female patients was 40.36 ± 10.82 years while that for male was 45.91 ± 11.75 years. No significant difference was found between the mean age of male and that for female patients ($P = 0.89$). The mean of disease duration was 72.9 ± 45.8 months. Twenty patients (40%) were treated with second-generation TKIs from the beginning without receiving first-generation TKIs while 30 (60%) of patients failed on first-generation TKIs then initiate second-generation TKIs.

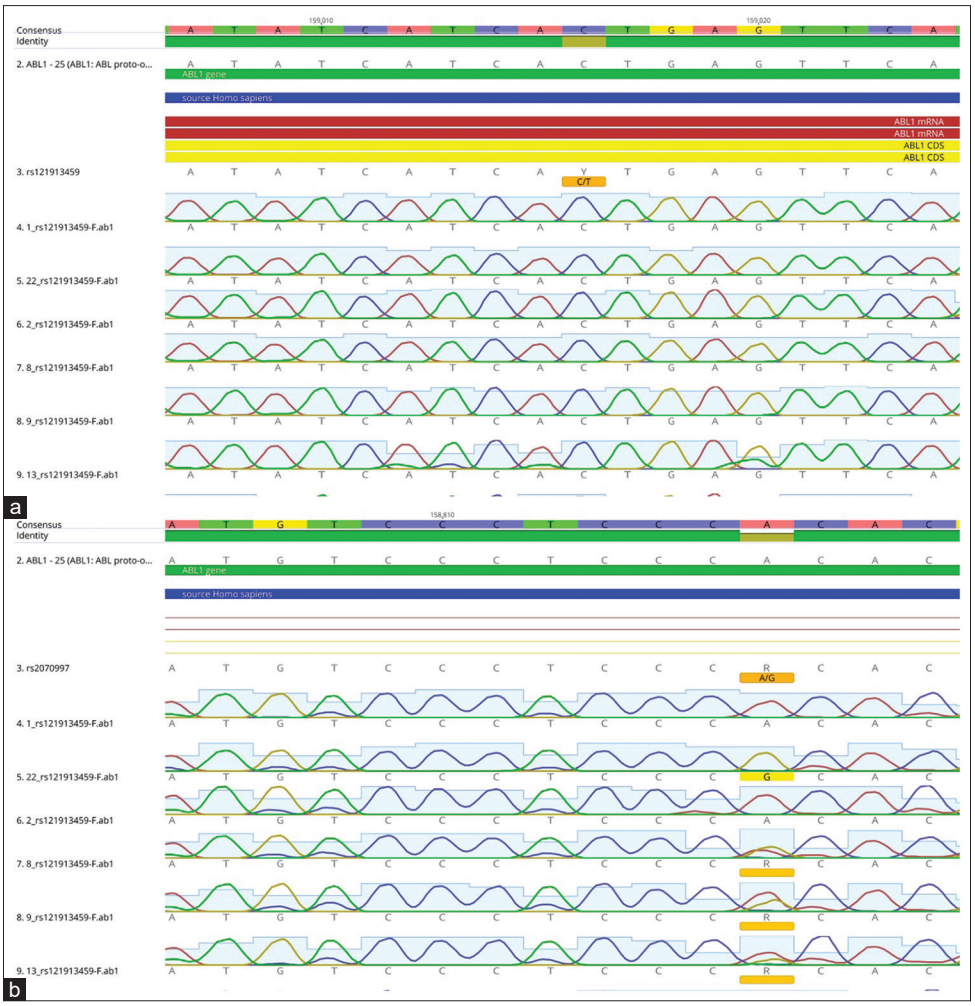

Figure 1: (a) DNA sequencing for T315I mutation (no mutation C/T = Y was not detected). (b) DNA sequencing for rs2070997 show normal (A/A = A), homogeneous (G/G = G) and heterogeneous (A/G = R) mutation

All the patients were compliant with treatment except one, who showed noncompliance on first generation TKI. All patients lost their response to Nilotinib by either MMR or hematological and (MMR). The mean PCR level of Ph chromosome after loss of response was 15.8% ± 18.5%, with minimum level of 0.18% and maximum level of 70%. There were no significant statistical difference in PCR level between both genders ($P = 0.567$). The white blood cell (WBC), Hb, and platelet counts of all patients in there last visit are described in Table 1 which show significant statistical difference in both gender regarding WBC and PLT count.

There was no expression of T315I mutation in any of 50 CML patients that were included in this study, however, through the analysis of Sanger sequencing, there was another mutation that have been expressed in some patients, this mutation is rs2070997 and result from substitution of G instead of A. This rs2070997 mutation was found in 18 (36%) patients (11 females and 7 males) but no significant statistical difference was found when both genders were compared ($P = 0.585$). The mean age of patients with rs2070997 mutation was 44 ± 12 years. There was no statistical significance difference between the mean of age of patients with mutation as compare to patients without mutation ($P = 0.91$).

Eight (16%) of the patients who had the rs2070997 mutation had primary failure on first-generation TKIs, six of them were found to have heterogeneous mutation (AG) and two of them were found to have homogenous mutation (GG).

The remaining 10 (20%) patients who had the rs2070997 mutation had been started second generation TKIs directly, nine of them were found to have heterogeneous mutation (AG) and one of them was found to have homogeneous mutation (GG). The progression-free survival (PFS) on first-generation TKIs for those patients with mutation was 35 ± 21 months, and it was statistically significant lower than the PFS for those without mutation ($P = 0.007$), [Figure 3].

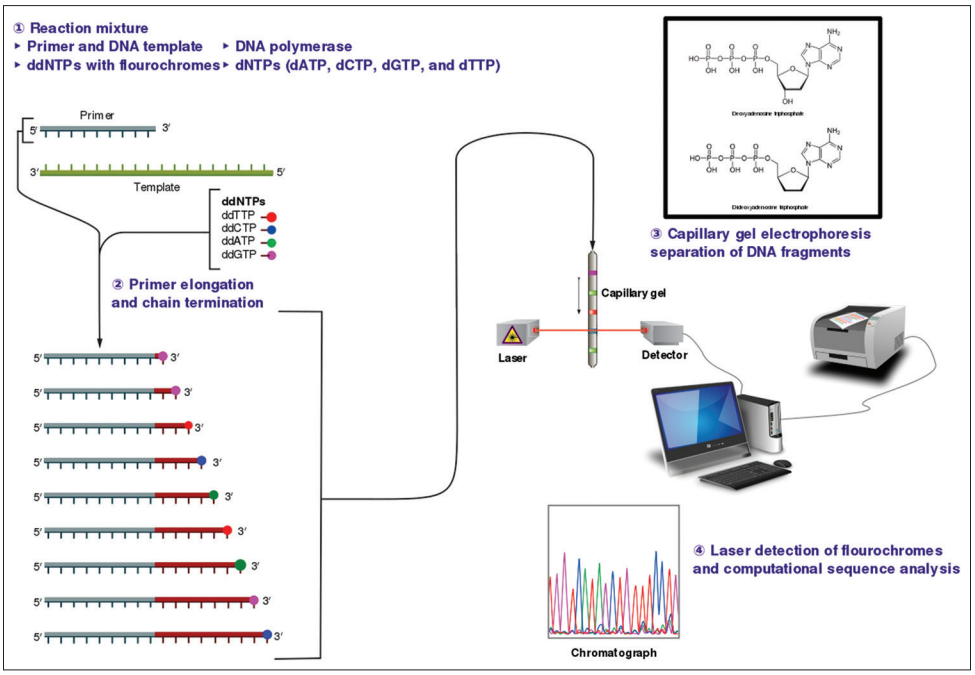

Figure 2: The Sanger (chain-termination) method for DNA sequencing

The PFS on second-generation TKIs for both mutated and nonmutate groups were 38.06 ± 8.88 and 41.16 ± 21.58 months, respectively which is statistically significant ($P = 0.001$) [Figure 4]. In addition, the difference in mean regarding age, disease duration, WBC, Hb, PLT, resistance duration, and PCR level is summarized in Table 2.

For the mutant group, 8 (16%) patients lost their molecular response only while the other 10 (20%) patients lost both hematological and molecular response. There was no significant statistical difference observed between both groups, $\chi^2 = 0.42$.

Discussion

The introduction of TKIs dramatically changed the progression of disease in CML patients and provided a powerful treatment for them, still there are some patients did not respond to these treatment option and showed resistance to it. Many researches are directed toward identifying the causes of such resistance and try to capture mutations associated with it.[6]

The aim of our research is to identify the prevalence of T315I mutation among CML patients who did not achieve MMR to second-generation TKI.

Based on sample size calculation, we recruited 50 CML patients who lost their MMR to second generation TKI at the time of the study.

Out of 50 patients diagnosed as CML, 28 were female (56%) and 22 were male (44%) with 1/1.2 male to female ratio, this was in line with other regional studies,[7] and this it was in reverse to other global studies.[8-10]

Patients' age was ranged between 23 and 63 years with a mean age of 42.8 ± 11.46 years, and this was lower than other studies that showed higher mean of age.[11,12] This disparity may related to fact that, in Iraq, high hazardous

Table 1: The correlation between gender and hematological parameters

	Gender				*P*
	Female		Male		
	Mean±SD	Range	Mean±SD	Range	
WBC	10.4±5.0	4.8-23.9	14.0±6.7	6.3-29.3	0.038
Hb	11.9±3.0	7.5-17.3	12.1±3.4	6.8-17.3	0.82
PLT	220±117	81-451	335±175	81-620	0.008

SD=Standard deviation, WBC=White blood cell, HB=Hemoglobin, PLT=Platelet

Table 2: The comparison between mutated and non-mutated group.

	Rs2070997 Mean±SD		*P*
	AA (*n*=32)	AG (*n*=18)	
PFS on 2nd TKI	41.16±21.58	38.06±8.88	0.002
Disease duration	80.31±50.79	59.83±32.63	0.049
Resistance duration	11.78±6.76	14.83±5.52	0.41
WBC	12.62±6.97	10.85±3.64	0.031
Hb	11.57±3.04	12.68±3.25	0.71
PLT	252±140	302±176	0.11
Age	42.06±11.53	44.11±11.56	0.9
PCR	20.55±20	7.57±8.9	0.001

TKI=Tyrosine kinase inhibitor, PFS=Progression-free survival, PCR=Polymerase chain reaction, SD=Standard deviation, WBC=White blood cell, HB=Hemoglobin, PLT=Platelet, AA=Adenine/Adenine, AG=Adenine/Guanine

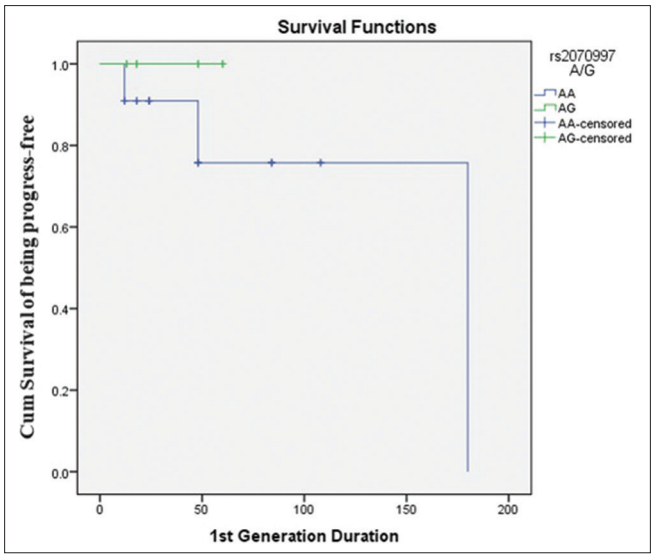

Figure 3: The progress-free survival (in months) on first-generation tyrosine kinase inhibitors in mutated and nonmutated group

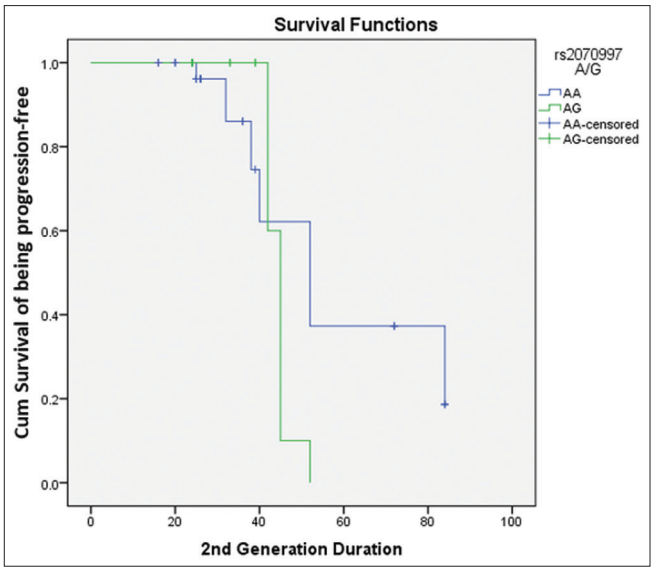

Figure 4: The progress-free survival on second-generation tyrosine kinase inhibitors in mutated and nonmutated group

ratio that increase the risk of malignancies in Iraqi people not only CML, but other hematological malignancies.[13]

Twenty patients (40%) were treated with second-generation TKIs from the beginning without receiving first-generation TKIs while 30 (60%) of patients failed on first-generation TKIs then initiate second-generation TKIs, there is no clear reason to why 20 patients initiated with second-generation TKIs, however, some countries assess patients age and the required response so they start with second-generation TKIs to achieve earlier and deeper molecular response.[14-16]

The compliance of patients on treatment play an important role in response to it, all of our patients in study were compliant with treatment except one, who showed noncompliance on first-generation TKI.[17]

The mean level of BCR-ABL1 fusion gene by PCR after loss of response was 15.8% ± 18.5%, it was comparable to other studies that assessed the PCR level after loss of response.[18,19]

The mean WBC, PLT, and Hb were higher in male in compare to female, and there was significant statistical difference between male and female in both WBC and PLT count. This significance higher mean level of WBC and PLT in male is explained by that, most of female patients did not lost their hematological but only molecular response and most of male patients lost both hematological and molecular response.[20]

In our study, there was no expression of T315I mutation in any of 50 CML patients that were included in our research, which is incomparable to all other researches that showed a prevalence of T315I range between 2.5% and 8%.[21-23]

Our explanation to that is, most of the global studies concluded that, the T315I mutations is associated with Advanced CML stage (i.e., accelerated and blastic phase),[21] while, most of our patients were in chronic phase of CML. The other explanation could rely on the random selection of patients from one hematology center in Baghdad.

Despite there was no T315I mutation in our patients, there are other mechanisms could be responsible for resistance in CML patients. Theses mechanism are as follows:[24,25]

BCR-ABL-dependent resistance

Is reliant on mechanisms that subvert effective BCR-ABL1 kinase inhibition, such as point mutations in the kinase domain other than T315I (L248V, F317 L, G250E, H396R, M244V, T277A, F311I, M318T, Q252H, F359A, F359I, and Y326H) that impair drug binding or cellular/biological processes that interfere with TKI availability and result in suboptimal drug concentrations at the target. It is more likely responsible for acquired resistance and can be overcome using alternative TKIs therapy.

BCR-ABL independent resistance

Which occurs in the setting of effective BCR-ABL1 inhibition and become increasingly recognized as a major contributor to minimal residual disease negativity and primary resistance. Diverse pathways implicated in BCR-ABL independent resistance include growth factors, epigenetic regulators and apoptotic mechanism. Efforts to eradicate persistent leukemic stem cells (LSCs) have largely focused on combination therapy with TKIs and drugs targeting these diverse pathways.

This BCR-ABL independent resistance is classified to:

1. Leukemic cell intrinsic: CML cell survival can be mediated through cell-autonomous
2. Leukemic cell-extrinsic: micro environmental factors provided by the bone marrow niche.

The BCR-ABL-dependent resistances have many mutations; one of them is rs2070997 mutation of ABL1 gene that located on chromosome 9, which is single nucleotide variant result from substitution of G instead of A in position 130872696.[26,27]

This mutation was associated with Response To Drug (R). It is reported to association with response to amphetamines.[28]

The analysis of Sanger sequencing showed that there were 18 (36%) patients who had this mutation included 11 females and 7 males but no significant statistical difference was found when both genders were compared. We could not find any reports for this mutation in the papers we reviewed so we need to emphasis more about it and its potential impact on disease activity, severity and progression among CML patients.

The mean age of patients with rs2070997 mutation was 44 ± 12 years. There was no statistical significance difference between the mean of age of patients with mutation as compare to patients without mutation (P = 0.91). However, this need more analyses with controlled normal individuals.

There were 8 (16%) patients that have rs2070997 mutation have primary failure on first-generation TKIs (out of 8, 2 patients were have homogeneous mutation (GG) and the other 6 patients had heterogeneous mutation (AG) for rs2070997), while the other 10 (20%) patients were those who started second-generation TKIs directly (out of 10, one patient was had homogeneous mutation (GG) and the other 9 patients had heterogeneous mutation (AG) for rs2070997).

The mean duration of treatment with first-generation TKIs for the patients with mutation was 35 ± 21 months, and it was statistically significant lower than mean duration of the treatment for the patients without mutation (P = 0.007).

The mean of 2nd TKIs duration of both mutated and non-mutate groups were 38.06 ± 8.88 and 41.16 ± 21.58 months respectively which is statistically significant. This means that mutant group developed resistance for first and second-generation TKIs earlier than nonmutant group and this may be an indicator of disease severity. In addition, the difference in mean regarding age, disease duration, WBC, Hb, PLT, resistance duration, and PCR level we assess and only disease duration, WBC, and PCR level showing statistical significant lower value in compare to mutant group.

The molecular loss of response were observed in 8 (16%) patients with mutation, while 10 (20%) have loss of response for both hematological and molecular. There was no significant statistical difference observed between both groups.

Conclusions

1. T315I mutation analysis by sequencing was not detected in any of the patients included in this study
2. The rs2070997 mutation was detected in 18 (36%) patients included in this study, this mutation named (Response to drug)
3. Patients who carried the rs2070997 mutation had lower PFS (on first- or second-generation TKIs) than those without mutation
4. Other mechanism of resistance could be responsible for TKIs resistance and should be investigated (ABL1-dependent, ABL1-Independent mechanism).

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Conflicts of interest
There are no conflicts of interest.

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