DOI: 10.24996/ijs.2024.65.8.9

ISSN: 0067-2904

The Association of *TET2* Gene Polymorphisms (rs34402524 and rs2454206) and their Haplotypes with Response to Treatment in Chronic Myeloid Leukemia Patients

Noor Hashim Ismail[1,2]*, Ismail Abdulridha Abdulhassan [1], Abdul Hussein Moyet Al-Faisal[1]

[1]*Institute of Genetic Engineering and Biotechnology for Postgraduate Studies/ University of Baghdad/ Baghdad/ Iraq.*

[2] *Cancer Research Department, Iraqi Center for Cancer and Medical Genetics Research, Mustansiriyah University, Baghdad, Iraq.*

Received: 15/2/2023 Accepted: 13/7/2023 Published: 30/8/2024

Abstract

Genetic and epigenetic factors affect chronic myeloid leukemia (CML) treatment response. The aim of the present study was to investigate the association of two *TET2*single nucleotide polymorphisms (SNPs) (rs34402524 and rs2454206) with CML incidence and response to imatinib mesylate (IM) treatment. Blood samples were collected from fifty CML patients (25 responders and 25 non-responders) and 50 healthy controls.Genotyping of *TET2* gene rs34402524and rs2454206 was done by qualitative real-time *PCR*-based genotyping methods. The results of the present study revealed a significant increasein risk association of *TET2* rs34402524 and rs2454206 heterozygous genotypes (TG) and (AG) with CML incidence and significant increased risk association of *TET2* rs34402524 heterozygous genotype (TG) with non–responsiveness to IM treatment in CML patients.This study revealed a strong linkage disequilibrium (LD) of *TET2* gene rs34402524 and rs2454206 SNPs among CML patients and controls and the haplotype G-G was significantly associated with a 2.475fold increased risk for CML incidence.In conclusion, both *TET2* gene SNPs (rs34402524 and rs2454206) heterozygous genotypes(TG) and (AG) associated with increased risk of CML incidence and the heterozygous genotype (TG) of *TET2* gene rs34402524 SNP associated with increased risk of non –responsiveness to IM treatment in CML patients. Both studied *TET2* SNPs (rs34402524 and rs2454206) could be used as markers for predicting response to imatinib in CML patients.

Keywords: Chronic Myeloid Leukemia, *TET2* gene, Polymorphisms, Imatinib mesylate.

العلاقة بين تعدد الطرز الوراثية لجين *TET-2* (rs34402524وrs2454206) وانماطها الفردانية في الاستجابة للعلاج في مرضى ابيضاض الدم النقوي المزمن

نور هاشم اسماعيل[1,2]،عبد الحسين مويت الفيصل[1]، اسماعيل عبد الرضا عبد الحسن[1].

[1] معهد الهندسة الوراثية والتقنيات الاحيائية للدراسات العليا ، جامعة بغداد ، بغداد، العراق.

[2] المركز العراقي لبحوث السرطان والوراثة الطبية ،الجامعة المستنصرية، بغداد ، العراق.

*Email: noor.hi@uomustansiriyah.edu.iq

الخلاصة:

تؤثر العوامل الجينية و فوق الجينية على استجابة ابيضاض الدم النقوي المزمن للعلاج. هدفت هذه الدراسة إلى التحري عنالارتباط بين اثنين من تعدد الأشكاللجين (rs34402524 *TET-2* وrs2454206)مع حدوث ابيضاض الدم النقوي المزمنوالاستجابة لعلاج الايماتينيب. تم جمع عينات الدم من خمسين مريضا مصابا بابيضاض الدم النقوي المزمن (25 مستجيبا و 25 من غير المستجيبين) ، وخمسون من الأصحاء من نفس العمر والجنسكمجموعة سيطرة .تم إجراء دراسات التنميط الجيني عن طريق تفاعل البلمرة المتسلسل اللحظي النوعي. كشفت الدراسة الجزيئية عن ازدياد خطر الاصابة بحدوث ابيضاض الدم النقوي المزمن معالطرز الوراثية متغايرة الزيجة TG و AG لتعدد اشكال النيكليوتيدات المفردة لجين *TET*2 في rs34402524 وrs2454206 ، وازدياد خطر عدم الاستجابة للعلاج لدى مرضى ابيضاض الدم النقوي المزمن مع الطراز الوراثي متغاير الزيجة TG لجين *TET2* في rs34402524. اظهرت هذه الدراسة عن اختلال توازن قوي في الارتباط لتعدد اشكال النيكليوتيدات المفردة لجين *TET2*، rs34402524 و rs2454206 ، بين مرضى ابيضاض الدم النقوي المزمن والاصحاء ، وكان النمط الفرداني G–G مرتبطًا بشكل كبير معنويا بزيادة خطر الإصابة بابيضاض الدم النقوي المزمن بمقدار 2.475 ضعف.بالاستنتاج ، يرتبط كل من الطرز الوراثية متغايرة الزيجة TG و AG لتعدد اشكال النيكليوتيدات المفردة لجين *TET*2 في rs34402524 وrs2454206 بزيادة خطر الإصابة بسرطان الدم النقوي المزمن ، والطراز الوراثي متغاير الزيجة TG لتعدد اشكال النيكليوتيدات المفردة لجين *TET*2 في rs34402524 عدم الاستجابة للإيماتينيب لدى مرضى ابيضاض الدم النقوي المزمن. يمكن استخدام تعدد اشكال النيكليوتيدات المفردة لجين *TET*2 في rs34402524 وrs2454206 كعلامات للتنبؤ للاستجابة للإيماتينيب في مرضى سرطان الدم النقوي المزمن.

1. Introduction

Leukemia ranked the fifth among the commonest ten major cancers in Iraq according to the 2019 and 2020 annual reports of Iraqi cancer registry [1, 2]. Chronic myeloid leukemia (CML) is a type of myeloproliferative malignant disorder defined by the hallmark pathognomonic presence of the Philadelphia (Ph) chromosome, (t(9;22)(q34;q11)), generating BCR-ABL1 oncoprotein, a constitutively active tyrosine kinase that causes CML and is the target of tyrosine kinase inhibitors (TKIs) [3]. Chronic myeloid leukemia has three phases with 85% - 90% of individuals presenting in a stable phase and the remaining in an accelerated phase or blast crisis. Chronic phase CML proceeds inexorably to accelerated phase/blast crisis without treatment, however TKIs drastically lowers the pace of progression to blast crisis [4].

Genetic and epigenetic alterations can cause treatment resistance in malignant disorders, including leukemia [5-10]. The TKIs resistance mechanisms in CML are usually classified into BCR-ABL1-dependent and independent mechanisms. Several mechanisms are associated with TKIs resistance in addition to BCR-ABL1 mutations and overexpression which includes abnormal drug transporter activity, alternative signaling pathway activation, genomic instability, leukemia stem cell (LSC) persistence, immune system dysfunction and epigenetic dysfunction [11-13].

DNA methylation of cytosine residues in CpG dinucleotides as epigenetic factor silences gene transcription and occurs where it is laid down by DNA methyltransferases (DNMTs) and gets removed passively through cell division or by the activity by methyl-cytosine dioxygenases (also known as ten-eleven translocation proteins; (*TET*s) [14]. Ten-eleven translocation 2 (*TET2*) is a member of the *TET*s family of proteins (*TET1–3*) that promotes site-specific DNA demethylation [15]and also plays an important role in normal and

malignant hematopoiesis, stem cell differentiation, immune regulation, DNA damage and repair response pathways [16]. Ten-eleven translocation 2 gene is muted with high frequency in patients with hematological malignancies and is also as considered one of the non-driver genetic change that may influence the development and outcome of myeloproliferative neoplasms in general [15].

Ten Eleven Translocation 2 gene mutations and polymorphisms are common in hematological diseases, including CML [17-19]. Studies have connected various *TET2* polymorphisms to acute leukemia. This may imply that *TET2* polymorphisms and its mutations play a role in the progression of CML from the chronic phase to the accelerated phase or blast crisis [20-23]
The aim of the present study was to investigate the association of two *TET2* single nucleotide polymorphisms (SNPs) (rs34402524 and rs2454206) with chronic myeloid leukemia incidence and CML patient's response to imatinib mesylate (IM) treatment, the first-generation TKI.

2. Materials and Methods

2.1. Ethical Considerations

The study protocol was approved by the Ethics Committee of the Iraqi Ministry of Health and Environment (No.7114 on February 23, 2021), and a written informed consent was obtained from all participants before entering the study.

2.2. Study Subjects

This is a case - control study which was conducted through the period from March 2021 to August 2022. Fifty cases of CML patients who were treated with IM therapy for at least one year as frontline therapy (25 responders CML patients and 25 non-responders CML patients to IM therapy), were collected from Baghdad Teaching Hospital/ Medical City and The National Center of Hematology/ Mustansiriyah University. In addition, 50 subjects as apparently healthy individuals with similar age and sex, were recruited as controls group. Patient's response to IM was based on molecular and hematological response results according to European Leukemia Net 2020 [24]. From each study subject, patients and controls volunteer, 4 ml peripheral blood (PB) was collected and dispensed into 2 tubes containing tripotassium ethylenediaminetetraacetic acid (k3EDTA), each with 2 ml, one for CBC while other one for DNA extraction.

2.3. Genomic DNA Extraction

Genomic DNA extraction was done using ReliaPrep™ Blood gDNAMini prep Systemkit (Promega, USA, Catalog number: A5081) according to the manufacturer’s guidelines. The concentration and purity of the purified DNA were measured by NanoDrop, Q5000 (Quawell, USA) microvolume UV-Vis spectrophotometer. The purity of DNA samples∼1.8 has been estimated to be with an acceptable 260/280 ratio.

2.4. Genotyping of TET2 exon 11rs34402524 and rs2454206

Genotyping of *TET2* gene rs34402524 (c.5162,leu1721Trp) was done by TaqMan allelic discrimination assay qualitative real time PCR (qPCR) using WizPure qPCR master (probe) kit, 5 µl Template DNA , 1.5 µl for each of forward and reverse primers (10µM) and 1 µl for each ROX labeled Probe (Wild) and Cy5 labeled Probe (Mutant). Primers and probes are listed in Table 1.The LM-2012 real-time PCR (ShanghaiFosun, China) (with LM-2012 real-time PCR Analyzer) was used to detect the fluorescence. The total reaction volume of qPCR mix was 25 µl. The primers and probes were used in TaqMan allelic discrimination assay qualitative real time PCR (qPCR) for genotyping of *TET2* rs34402524 designed by Beacon

Designer 8.21 program, synthesized and lyophilized by Genewiz Ltd. (USA). The thermal profile was as follows: hold at 95°C for 600 seconds, then 5 cycle: denaturation at 95°C for 10 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 15 seconds. Finally, 40 cycles of denaturation at 95°C for 10 seconds, annealing at 57°C for 35 seconds (Fluorescent signal acquiring step on orange and red filters (ROX and CY5)), and extension at 72°C for 15 seconds. The genotyping of *TET2* gene rs2454206 (c.5284, Ile 1762 Val) was done by high resolution meting technique. A Rotor gene Q real-time PCR System, 5-plex with HRM (Qiagen) was used to perform an amplification, followed by an HRM analysis (qPCR-HRM) using *TransStart*®Tip Green qPCR Super Mix kit (TransGen Biotech Co, China), 5 µl Template DNA, 1 µl for each of forward and reverse primers (10µM), listed in Table 1, with a total reaction volume for qPCR mix 20 µl. The thermal profile was as follows: hold at 94°C for 60 seconds (1 cycle), then 40 cycle: denaturation at 94°C for 5 seconds, annealing at 52°C for 15 seconds, and extension at 72°C for 20 seconds, finally dissociation from 60°C to 90°C (0.1°C / 2sec). The primers used in genotyping of *TET2* rs2454206 were designed by the Primer 3plus, V4, synthesized and lyophilized by Alpha DNA Ltd (Canada). Each run for genotyping of of *TET2* exon 11rs34402524 and rs2454206 included positive controls (A synthetic DNA fragment for wild and mutant sequences of rs34402524 and rs2454206 cloned into a pUC-GW-Amp vector) synthesized by Genewiz Ltd. (USA) were used in genotyping studies as positive controls for wild and mutant rs34402524 and rs2454206.

Table 1: Primers and probes used in Genotyping study of *TET2*gene exon 11rs34402524 and rs2454206 SNPs.

Genotyping Primers and Probes				
***TET2* gene rs34402524 SNP**				
Primer / Hydrolysis Probes	**Sequence (5′ →3′ direction)**	**Length**	**Tm**	**Product Size**
Forward	**GCAGTTGTACCATTAGAC**	**18**	**52**	**164**
Reverse	**GTGAGAAGGTGAATGATG**	**18**	**52**	
ROXLabeled Probe (Sense Wild)	**ATGTAGGGAAATTGCCTCCTTATCC**	**25**	**67.5**	
CY5 labelled Probe (Sense Mutant)	**ATGTAGGGAAATGGCCTCCTTATCC**	**25**	**69.1**	
***TET2* gene rs2454206 SNP**				
Primer	**Sequence (5′ →3′ direction)**	**Length**	**Tm**	**Product Size**
Forward	**TGAACATCATTCACCTTCT**	**19**	**52**	**49**
Reverse	**CGGAGCTGCACTGTAGT**	**17**	**54**	

2.5. Statistical Analysis

The Statistical Package for Social Sciences (SPSS-version 22) was used to analyze data. After a normality testing, the quantitative data was presented by the median (interquartile range, IQR).Mann-Whitney U-test was used to compare between the studied groups. Qualitative data were presented as the frequency (percentage) and significant differences were assessed by Pearson's Chi-square (X^2) or Fisher's exact tests. The allelic and genotypic frequencies were calculated by direct gene counting method. Chi-square test of independence, Odds ratios (ORs) and its 95% confidence interval (CI) were estimated to identify the association strength of *TET2* gene SNPs with CML risk and response to treatment by using WINPEPI program for epidemiologists [25]. Hardy Weinberg equilibrium (HWE) analysis was assessed using Pearson Chi-square test (χ^2-test) goodness of fit test using the online HWE calculator. Haplotype frequencies and linkage disequilibrium (LD) between *TET2* SNPs

were estimated using SHESIS plus online –based platform. The LD coefficient (D′) and correlation coefficient (r^2) were used to define LD. The D′ value has a range between 0 (no LD) and 1.0 (complete LD) [26]. A probability (*p*) value ≤ 0.05 was considered statistically significant.

3. Results and Discussion

3.1. General Characteristics of the Studied Groups

The studied subjects included 50 patients with CML receiving IM therapy, 25 responders CML patients and 25 non-responder CML patients to IM therapy, with a median (IQR) treatment duration (months) of 48 (22.5-132) and 56 (22-96) respectively, without significant statistical differences (P>0.05). The effectiveness of IM, in addition to other generations of TKIs in CML treatment, has been shown by many studies by improvement in 5-year survival from 30 to 40% in the pre-TKI period to 96% following the drug's debut [27]. Additional 50 apparently healthy controls with similar age and sex were recruited as controls group. Demographic, hematological and molecular characteristics of study groups are listed in Table 2. The median (IQR) age of patients group was 46.5 (38-54.5) while that for controls group it was 46.50 (39-55), without significant differences (P>0.05). The median (IQR) age for responder and non-responder CML patients were 49 (38-56.5) and 45 (38-54) respectively, without significant differences (P>0.05). This is comparable to Ning *et al*. review [28] as younger age distribution among Asian population was younger than 50 years old compared to older than 50 years old in western countries, and it's almost same as other Iraqi studies [29, 30]. This study found that females had a higher significant (P<0.05) failure rate to respond to medication than males (64% vs. 36%) which may be attributed to different sex compliance and socioeconomic backgrounds of patients during IM shortage. In contrast, several recent researchers have found that women are more likely than men to have better molecular responses [31].

The results of this study showed that CML patients had a significantly lower (P<0.001) hemoglobin level in 56% of patients compared to controls group, significantly higher (P<0.05) WBC and platelets counts in 14% of patients compared to the controls. A comparison between responder and non-responder CML patients revealed that non–response to treatment is associated with a significant lower (P=0.001) hemoglobin level in 80% of non-responders CML patients, significantly (P=0.01) higher WBC and platelets counts in 28% of them. Patients who lose their IM response and progress to a more severe form of the disease experience bone marrow suppression attributable to an increase in cloned leukemic cells [32]. The responders CML patients also exhibited a lower hemoglobin level in 32% of them which may be related to their long-term IM therapy [33]. The median (IQR) of BCR-ABL % (IS) was significantly higher (P<0.001) for non-responders compared to the responder CML patients (0.84 (0.34-6.255) and 0.0032 (0.0001-0.0245) respectively). This difference served to highlight the characteristics of the patient groups that were examined in this study. These results defined the CML patients' response to TKI according to European Leukemia Net 2020 [24].

Table 2: Demographic, Hematological and Molecular Characteristics of Study Groups.

Variable	Group		*P*-value	Group		*P*-value
	Controls (n=50)	CML Patients (n=50)		Responders CML Patients	Non-responders CML Patients	
Age (Years)	46.50 (39-55)	46.5 (38-54.5)	0.95 NS	49 (38-56.5)	45 (38-54)	0.627 NS
Sex n (%)						
Male	26(52.00%)	26(52.00%)	1 NS	17(68.00%)	9(36.00%)	0.024^{*}
Female	24 (48%)	24 (48%)		8 (32.00%)	16(64.00%)	
Hemoglobin (g/dL)						
≥12 (g/dL)	50 (100%)	22 (44%)	$<0.001^{**}$	17 (68%)	5 (20%)	0.001^{**}
< 12(g/dL)	0 (0%)	28 (56%)		8(32%)	20(80%)	
White blood cells ($\times 10^3$/mm^3)						
≤ 10 ($\times 10^3$/mm^3)	50 (100%)	43 (86%)	0.012^{*}	25(100%)	18 (72%)	0.01^{*}
>10 ($\times 10^3$/mm^3)	0 (0%)	7(14%)		0(0%)	7 (28%)	
Platelets ($\times 10^3$/mm^3)						
<450 ($\times 10^3$/mm^3)	50 (100%)	43 (86%)	0.012^{*}	25(100%)	18 (72%)	0.01^{*}
>450 ($\times 10^3$/mm^3)	0 (0%)	7 (14%)		0(0%)	7 (28%)	
BCR-ABL %(IS)	-	-	-	0.0032 (0.0001-0.025)	0.84 (0.34-6.255)	$<0.001^{**}$
Treatment Duration (Months)	-	-	-	48 (22.5-132)	56 (22-96)	0.68 NS

NS: Non- significant, * and ** means significant at 0.05 and 0.01 levels respectively.

3.2. Genotypes and Allele frequencies of TET2 gene rs34402524 and rs2454206 SNPs

The resulting output of real-time PCR machine of the analysis process for *TET2* gene rs34402524 SNP by TaqMan allelic discrimination assay real time PCR and rs2454206 SNP of by HRM-qPCR are shown in Figures 1 and 2. The genotype and allele frequencies of *TET2* gene SNPs, rs34402524 and rs2454206, are shown in Tables 3 and 4 among study groups. Genotype frequencies of both *TET2* SNPs in controls and genotype frequencies of *TET2* gene rs2454206 SNP in total CML patients**,** responders and non-responders CML patients, were in good agreement with Hardy Weinberg Equilibrium (HWE) (P>0.05). Three exceptions were found: *TET2* gene rs34402524 SNP in total CML patients, *TET2* gene rs34402524 SNP in responder CML patients group and *TET2* gene rs34402524 SNP in non-responders CML patients group, in which a significant departure from the equilibrium was recorded (P<0.001, P<0.05 and P<0.001 respectively). Testing for deviations from HWE is a fundamental requirement in population genetic research and it should be given special attention for the controls group [34]. The deviation of controls individuals from HWE might be caused by a variety of causes, the most important of which being mistakes in genotyping, population stratification and racial or ethnic heterogeneity, or, in samples of diseased individuals, an association with the disease [35, 36].

The results of distribution of *TET*2 rs34402524 and rs2454206 genotypes and allele frequencies in CML patients group compared to controls group are shown in Table 3. The

results showed that the frequency of wild genotypes (TT) and (AA) was lower in CML patients (26% for both) as compared to 58% and 44% respectively of controls. Whereas, heterozygous genotypes TG and AG were higher in CML patients (74% and 56% respectively) as compared to controls(42% and 38% respectively). The homozygous mutant of *TET*2 rs34402524 genotype (GG) was not detected in study groups. However, the homozygous mutant genotype frequencies of rs2454206 were similar in CML patients and controls (18%). These results implied that heterozygous genotypes of both *TET*2 SNPs associated with a significant increased risk of CML incidence ($P=0.001$, with odd ratio (95% CI) =3.93 (1.69-9.15) and ($P<0.05$, with odd ratio (95% CI) =2.49 (1.01-6.13) respectively. The frequency of the T and A alleles of *TET*2 rs34402524 and rs2454206 was lower in CML patients compared to controls (63% versus 79%, and 54% versus 63% respectively). Whereas, the frequency of the G alleles was higher in CML patients compared to controls, (37% versus 21% and 46% versus 37% respectively).

Table 4 shows the distribution of genotype and allele frequencies of *TET2* rs34402524 and rs2454206 among responder and non-responder CML patients. The frequencies of wild genotypes (TT) and (AA) were lower in non-responders CML patients, 12% and 24% respectively, as compared to 40% and 28%respectively of responder CML patients. While heterozygous genotypes (TG) and (AG) were higher in non-responder CML patients 88% and 60% respectively as compared to the responder CML patients (60% and 52% respectively). The homozygous mutant of *TET*2 rs34402524 genotype (GG) was not detected in study groups, whereas the homozygous mutant of rs2454206 was lower in non-responder CML patients (16% as compared to 20% in responder CML patients). The results indicated that the heterozygous genotype (TG) in rs34402524SNP of *TET2* gene in this study associated with significant increased risk of CML patient's non-responsiveness to IM treatment ($P<0.05$), with odd ratio (95% CI) =4.89 (1.15-20.79). The frequency of the T allele of *TET*2 rs34402524 was lower in non-responder CML patients compared to responder CML patients (56% versus 70%), whereas the frequency of the G allele was higher in non-responder CML patients compared to responder CML patients (44% versus 30%). The frequency of the A and G alleles of *TET*2 rs2454206 were similar in both responder and non-responder CML patients, 54% and 46% respectively.

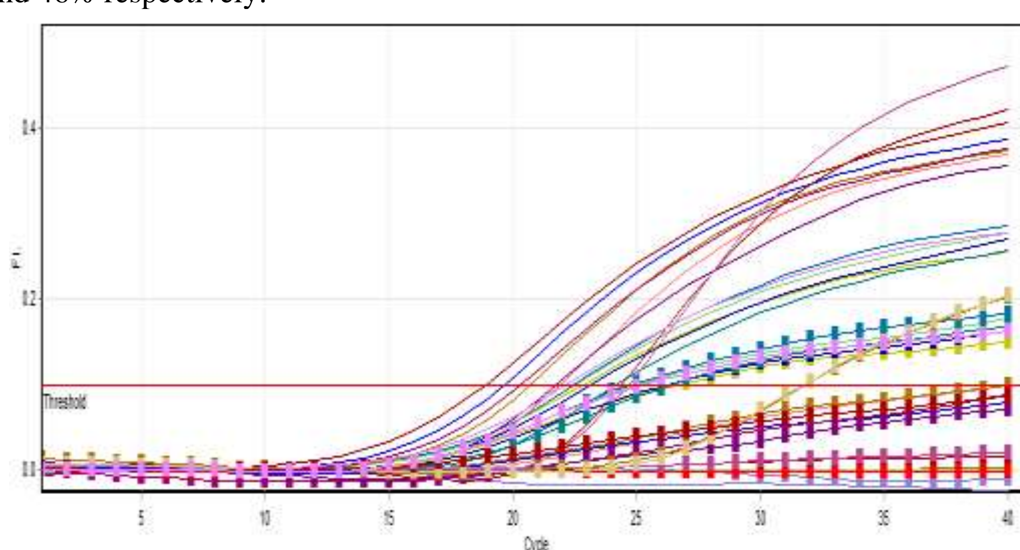

Figure 1: The result output of TaqMan allelic discrimination assay real time PCR for CML patients and controls inrs34402524SNP genotypes with *TET2* rs34402524 primers and probes. Images captured using LM-2012 real-time PCR machine with LM-2012 real-time PCR analyzer**.**

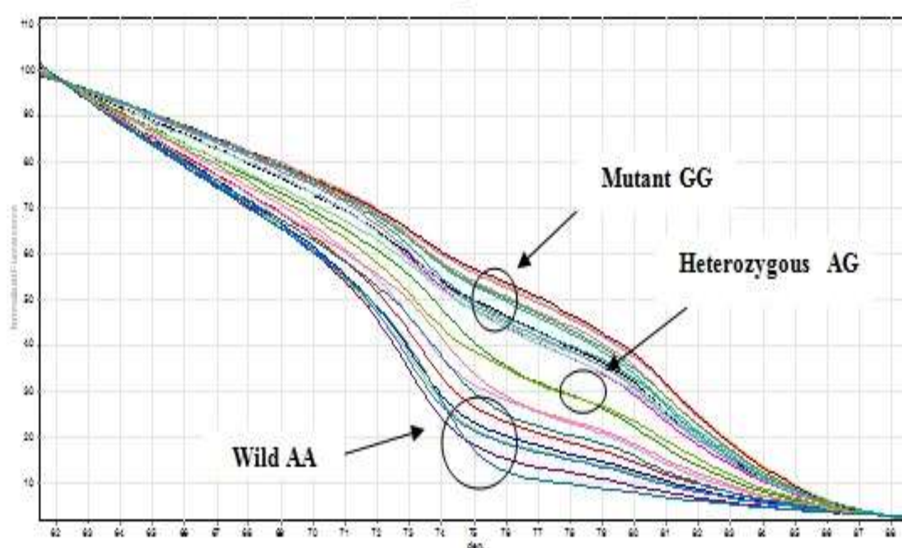

Figure 2: The result output of HRM-qPCR for the three genotypes (Wild, Heterozygous and Mutant) of rs2454206 SNP of *TET2* gene. Images captured using Qiagen Rotor Gene Q qPCR Machine.

Table 3: Distribution of genotype and allele frequencies of *TET2* SNPs (rs34402524 and rs2454206) and Hardy Weinberg Equilibrium (HWE) analysis among CML patients and the controls groups

TET2 **SNPs Genotype and allele frequency**	**Group**		**OR (95 % CI)[##]**	**χ^2**	***P* value**
	Controls (N=50)	**CML patients (N=50)**			
***TET2* rs34402524 Genotype frequency**					
TT	29 (58.00%)	13 (26.00%)	Reference	-	-
TG	21 (42.00%)	37 (74.00%)	3.93 (1.69-9.15)	10.509	0.001**
GG	0 (0.00%)	0 (0.00%)	-	-	-
HWE[#] *P* value	0.06 NS	<0.001**			
***TET2* rs34402524 allele frequency**					
T	79 (79.00%)	63 (63.00%)	-	-	-
G	21 (21.00%)	37 (37.00%)	-	-	-
***TET2* rs2454206Genotype frequency**					
AA	22 (44.00%)	13(26.00%)	Reference	-	-
AG	19 (38.00%)	28(56.00%)	2.49 (1.01-6.13)	4.038	0.044*
GG	9 (18.00%)	9 (18.00%)	1.69 (0.54-5.35)	0.809	0.368 NS
HWE[#] *P* value	0.19 NS	0.37 NS			
***TET2* rs2454206 Allele Frequency**					
A	63 (63.00%)	54(54.00%)	-	-	-
G	37 (37.00%)	46(46.00%)	-	-	-

[#] HWE: Hardy Weinberg Equilibrium, [##]OR (95% CI): odd ratio (95% confidence interval), X^2: chi square, NS: Non- significant, * and ** means significant at 0.05 and 0.01 levels respectively.

Table 4: Distribution of genotype and allele frequencies of *TET2*SNPs (rs34402524 and rs2454206) and Hardy Weinberg Equilibrium (HWE) analysis among responders and non-responders CML patients groups.

TET2 SNPs Genotype and Allele Frequency	Group		OR (95 % CI)[##]	χ^2	*P* value
	Responders CML (n=25)	Non-Responders CML Patients (n=25)			
***TET2* rs34402524 Genotype frequency**					
TT	10 (40%)	3 (12%)	Reference	-	-
TG	15 (60%)	22 (88%)	4.89 (1.15-20.79)	5.094	0.024*
GG	0 (0 %)	0 (0%)	-	-	-
HWE[#]*P* value	0.03*	<0.001**			
***TET2* rs34402524 AlleleFfrequency**					
T	35 (70%)	28 (56%)	-	-	-
G	15 (30%)	22 (44%)	-	-	-
***TET2* rs2454206 Genotype Frequency**					
AA	7 (28%)	6 (24%)	Reference	-	-
AG	13 (52%)	15 (60%)	1.35 (0.36-5.04)	0.196	0.658 NS
GG	5 (20%)	4 (16%)	0.93 (0.17-5.15)	0.006	0.937 NS
HWE[#]*P* value	0.81NS	0.30 NS			
***TET2* rs2454206 Allele Frequency**					
A	27 (54%)	27 (54%)	-	-	-
G	23 (46%)	23 (46%)	-	-	-

[#] HWE: Hardy Weinberg Equilibrium, [##]OR (95% CI): odd ratio (95% confidence interval), X^2: chi square, NS: Non- significant, * and ** means significant at 0.05 and 0.01 levels respectively.

The results of the present study revealed a significant increased risk association of *TET2* gene rs34402524 and rs2454206 SNPs heterozygous genotypes with CML incidence and significant increased risk association of *TET2* rs34402524 heterozygous genotype for non–responsiveness to treatment (IM) in CML patients. Epigenetics modification of DNA by *TET2* via promotion of site-specific DNA demethylation which is an important factor for stem cells and progenitor cells' self -renewal and leukemia prevention [37]. Mutations and polymorphisms of *TET2* gene have been associated with a wide variety of myeloid malignancies which raises the possibility that they play a pleiotropic role in myeloid transformation [38, 39]. Ten Eleven Translocation 2 gene polymorphisms and mutations are common in hematological diseases, including CML [40]. Studies have connected various *TET2* polymorphisms to higher BCR-ABL1 levels, acute leukemia and response to treatment, implying that *TET2* gene polymorphisms and its mutations play a role in the progression of CML from the chronic phase to the accelerated phase or blast crisis [17-23].

Comparison of the differences in present study results regarding *TET2* gene SNPs, rs34402524 and rs2454206 genotypes frequencies distribution in patients and controls with previous studies results in CML may be related to racial and ethnic differences in *TET2* gene polymorphisms and in exposure to mutagens that increase or decrease leukemia risk as well as interaction of *TET2* gene with additional genomic mutations to induce hematopoietic

malignancies [41]. Ten Eleven Translocation 2 gene polymorphisms are influenced by racial variation. The *TET2* gene SNPs under study may be part of a racially-influenced germline phenotype. Differences in *TET2* SNPs between healthy individuals and those with CML patients imply that these germline phenotypes may have been absent or lost in CML patients during leukemogenesis [19]. Hirsch, *et al.* [42] revealed that several germline *TET2* gene SNPs or their combination may represent complex, most likely low penetrance predisposing factors for myeloid malignancies that also interplay with somatic variations and lead to cancer in later life.

3.3. Haplotype and Linkage Disequilibrium Analysis of *TET2* SNPs.

Haplotype and linkage disequilibrium (LD) analysis of *TET2* SNPs, rs34402524 (c.5162T>G) and rs2454206 (c.5284A>G) was performed by using SHESIS plus software to investigate their association with CML risk and response to treatment, due to their adjacent location on Chromosome 4 (4q24), exon11. Tables 5 and 6 summarize *TET2* SNPs haplotype frequencies and risk association to CML and its response to IM. haplotype analysis revealed a significant increased frequency ($P<0.05$) of the haplotype G-G in CML patients compared to controls (0.27 versus 0.13) with odd ratio (95%CI) =2.475 (1.191-5.142). Non-significant differences ($P>0.05$) in other haplotypes (T-A, G-A and T-G) were detected in CML patients in comparison to controls group. Analysis of haplotypes association in CML according to their IM response revealed no significant association ($P>0.05$) with any haplotypes (T-A, G-A, G-G and T-G) with CML response to IM.

Linkage disequilibrium (LD) analysis of *TET2* SNPs, rs34402524 and rs2454206, depicted in Figures 3 and 4, revealed a strong LD with (D' = 0.7 and $r^2 = 0.14$) among CML patients and controls, as shown in Figure 1 and eminently stronger among CML patients according to their IM response (D' = 0.76 and $r^2 = 0.29$) (Figure 2).

Linkage disequilibrium (LD) is the measurement of non-random association of alleles at two loci within haplotypes. This study revealed a strong LD of *TET2* SNPs, rs34402524 and rs2454206, among CML patients and controls, and eminently stronger LD among CML patients according to their IM response and the haplotype G-G was significantly associated with a 2.475-fold increased risk for CML incidence. This haplotype could be used as biomarker in the Iraqi population to estimate CML incidence risk. Haplotypes, rather than genotypes at a single locus, might be regarded the primary units of heredity and may allow us to better understand the function of polymorphic traits (i.e., SNPs) in disease susceptibility [43, 44].

Table 5: *TET2* SNPs Haplotypes Frequencies and Risk Association to CML.

Haplotype	CML Patients (Freq)	Controls (Freq)	OR (95 % CI) #	X^2	*P* value
T-A	44(0.44)	55(0.55)	0.642 (0.368-1.122)	2.42	0.119 NS
G-A	10(0.1)	8(0.08)	1.277 (0.482-3.384)	0.244	0.621 NS
G-G	27(0.27)	13(0.13)	2.475 (1.191-5.142)	6.125	0.013*
T-G	19(0.19)	24(0.24)	0.742 (0.376-1.463)	0.74	0.389 NS

#OR (95% CI): odd ratio (95% confidence interval), NS: Non- significant, * and ** means significant at 0.05 and 0.01 levels respectively.

Table 6: *TET2* SNPs Haplotypes Frequencies and Risk Association to CML Response to IM.

Haplotype	Responders CML Patients (Freq)	Non-Responders CML Patients (Freq)	OR (95 % CI)#	X^2	*P*-value
T-A	14(0.28)	7(0.14)	0.418 (0.152-1.148)	2.953	0.085 NS
G-A	13(0.26)	20(0.4)	1.897 (0.812~4.431)	2.216	0.136 NS
G-G	2(0.04)	2(0.04)	1 (0.135~7.392)	0	1 NS
T-G	21(0.42)	21(0.42)	1 (0.451~2.212)	0	1 NS

#OR (95% CI): odd ratio (95% confidence interval), NS: Non- significant, * and ** means significant at 0.05 and 0.01 levels respectively.

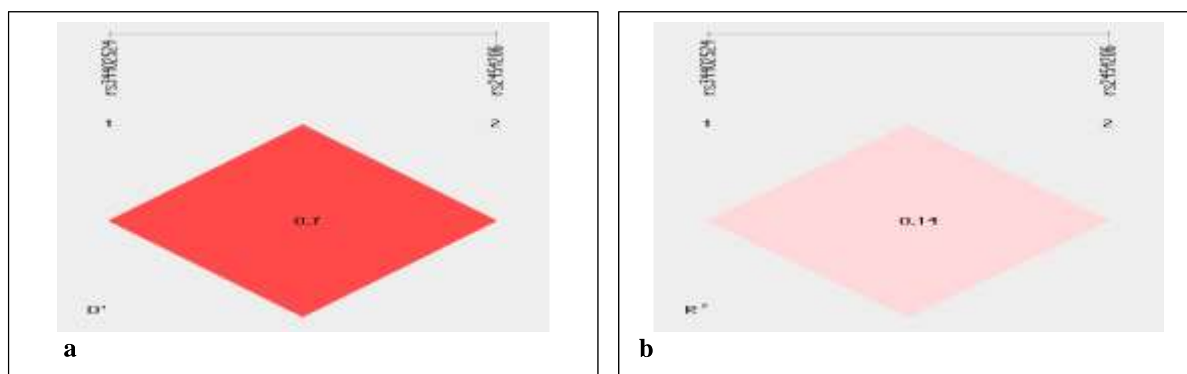

Figure 3: Linkage disequilibrium map of *TET2* gene SNPs (rs34402524 and rs2454206) among CML patients and controls genotyped using SHEsis Plus (a) Value in the LD block indicated the D' (b) Value in the LD block represented the r^2.

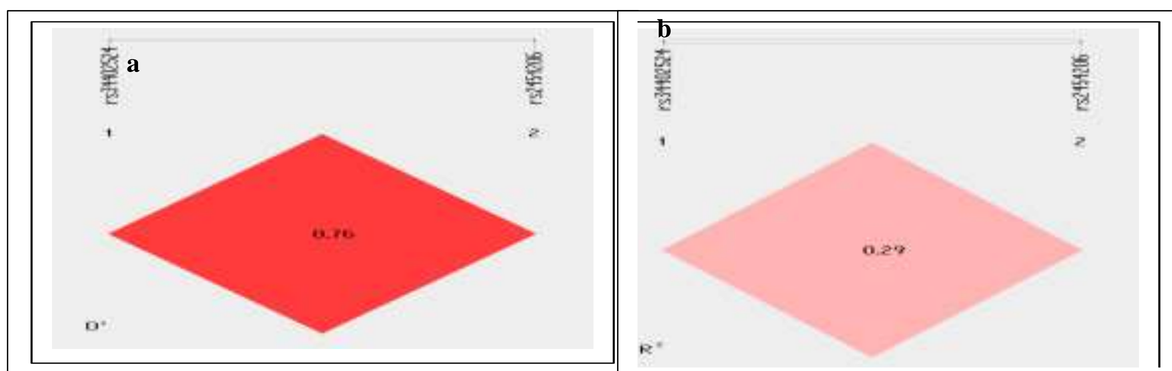

Figure 4: Linkage disequilibrium map of *TET2* gene SNPs (rs34402524 and rs2454206) among responder and non-responder CML patients genotyped using SHEsis Plus (a) Value in the LD block indicated the D' (b) Value in the LD block represented the r^2 .

3.4. Genotypes Combination Analysis of *TET2* SNPs

Genotypes analysis of *TET2* SNPs rs34402524 and rs2454206 was combined to study the interaction of various *TET2* SNPs genotypes and their effects on CML risk and CML response to IM treatment. The genotypes combinations frequencies of *TET2* SNPs, rs34402524 and rs2454206, and the odd ratio (95% CIs) in study groups are summarized in Tables 7 and 8. The TT/AA genotypes were considered as reference genotypes. As shown in Table 7, the genotypes GG/AA, GG/AG, and GG/GG were absent in all study groups. The frequencies of genotypes combination TG/AA and TG/AG were higher in CML patients group compared to

controls group, 20% versus 16% and 46% versus 20% respectively. A statistical significance for the increased association of these genotypes combinations with CML incidence (*P*<0.05, with odd ratio (95% CI) =5.83 (1.23 - 27.63)) and (*P*<0.001, with odd ratio (95% CI) =10.73 (2.51-45.81)) was detected. No significant statistical association of genotype combinations TT/AG, TT/GG and TG/GG with risk of CML incidence was recorded. Genotype combination risk association analysis for *TET2* gene SNPs in CML patients according to their response to treatment revealed that the genotypes GG/AA, GG/AG, and GG/GG were absent in all study groups (Table 8). The frequencies of genotypes combination TG/AA and TG/AG were higher in non-responder CML patients group compared to responder CML patients group (24% versus 16% and 56% versus 36% respectively). No significant statistical association for the co-presence of genotypes combinations TT/AG, TT/GG, TG/AA, TG/AG and TG/GG with CML non- responsiveness to treatment were found.

Alleles, genotypes, haplotypes, haplotype combinations and genotype combinations have been widely applied in gene-disease association studies [45, 46]. Combined analysis of *TET2* studied SNPs genotypes indicated that the synergistic effects of the presence of both heterozygous genotypes, TG and AG at rs34402524 and rs2454206 SNPs of *TET2* gene were more strongly associated with increased risk of CML incidence (OR=10.73 (2.51-45.81), *p*<0.001) than the presence of one of these genotypes of *TET2* SNPs, rs34402524 and rs2454206, as a result of strong linkage disequilibrium (LD) among CML patients and controls, and eminently stronger among CML patients according to their IM response.

Table 7: Genotypes Combination Analysis of *TET2* SNPs rs34402524 and rs2454206 in CML Patients and Controls

Genotype Combination	Group		OR (95%CI) #	X^2	*P*value
	Controls	CML Patients			
TT/AA	14 (28%)	3(6%)	Reference	-	-
TT/AG	9(18%)	5(10%)	2.59 (0.49 - 13.61)	1.309	0.253 NS
TT/GG	6(12)	5(10%)	3.89 (0.70 – 21.75)	2.530	0.112 NS
TG/AA	8(16%)	10(20%)	5.83 (1.23 - 27.63)	5.381	**0.020***
TG/AG	10(20%)	23(46%)	10.73 (2.51-45.81)	12.178	**<0.001****
TG/GG	3(6%)	4(8%)	6.22 (0.89 -43.66)	3.744	0.053 NS
GG/AA	0(0%)	0(0%)	-	-	-
GG/AG	0(0%)	0(0%)	-	-	-
GG/GG	0(0%)	0(0%)	-	-	-

#OR (95% CI): odd ratio (95% confidence interval), NS: Non- significant, * and ** means significant at 0.05 and 0.01 levels respectively.

Table 8: Genotypes Combination Analysis of *TET2* SNPs rs34402524 and rs2454206 in Responders and Non-Responders CML Patient

Genotype Combination	Group		OR (95%CI) #	X^2	*P*-value
	Responders CML Patients[1]	Non-responders CML Patients[2]			
TT/AA	3 (12%)	0(0%)	Reference	-	-
TT/AG	4(16%)	1(4%)	2.33 (0.07 -76.67)	0.226	0.634NS
TT/GG	3(12%)	2(8%)	5 (0.17 -146.64)	0.872	0.350 NS
TG/AA	4(16%)	6(24%)	10.11(0.41 -247.48)	2.011	0.156NS
TG/AG	9(36%)	14(56%)	10.68 (0.49 - 231.09)	2.281	0.131NS
TG/GG	2(8%)	2(8%)	7 (0.22 - 218.95)	1.227	0.268NS
GG/AA	0(0%)	0(0%)	-	-	-
GG/AG	0(0%)	0(0%)	-	-	-
GG/GG	0(0%)	0(0%)	-	-	-

#OR (95% CI):Adjusted (zero cell); adjusted odd ratio (95% confidence interval) After adding 0.5 to each cell, NS: Non- significant, * and ** means significant at 0.05 and 0.01 levels respectively.

4. Conclusions

In conclusion, significant increased risk association of heterozygous genotypes of both studied *TET2* SNPs (rs34402524 and rs2454206) with CML incidence, and significant increased risk association with non –responsiveness to imatinib for *TET2* rs34402524 SNP heterozygous genotype was recorded. As a result of strong linkage disequilibrium between both studied *TET2* SNPs, the synergistic effect of the presence of both heterozygous genotypes of both studied *TET2* SNPs rs34402524 and rs2454206 were found to be more strongly associated with an increased risk of CML incidence. Both the haplotype and genotype combination analysis were informative. Both studied *TET2* SNPs rs34402524 and rs2454206 could be used as markers for predicting CML incidence and response to Imatinib treatment in CML patients.

Conflict of Interest: The authors declare that they have no conflicts of interest.

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