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Relationship between IM Response and the C3435T SNP of *abcb1* Gene among Some Iraqi CML Patients.

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ABSTRACT

Although the striking efficacy of IM in treatment of CML, resistance develops in many patients has considered as important issue. Genetic variation revealed to be an important factor that can reduce or enhance the risk of developing a disease. The SNP have the potential to alter protein function and could also influence the efficiency of pharmacokinetic and metabolism of drug. In this study, the relationship between IM response and the single nucleotide polymorphisms (SNPs) of *abcb1* gene was investigated among some Iraqi CML patients undergoing IM treatment . 71 Philadelphia (Ph) chromosome positive CML patients (Chronic phase) (43 Male, 28 Female) aged 20-70 years , including 11 untreated which are newly diagnosed , 30 under-treatment of Imatinib mesylate (IM) which are IM response and 30 resistance to IM drug , treated with standard dose IM (400mg) on frontline treatment and 25 apparently healthy individuals. After obtaining written-informed consent, 3ml peripheral blood was collected from CML patients and normal healthy individuals . The results of *abcb1* gene exhibit homozygous mutant CC genotype of SNP C3435T with significantly increase ($p < 0.05$) in CML patients showing IM resistant compared with CML patients with IM good response (O.R=0.53) . These explorative results urge us to propose the prospect of exploration these SNPs as biomarkers to prediction the response to IM drug in CML patients before beginning the treatment.

Keywords: *abcb1* C3435T, polymorphisms, imatinib mesylate, chronic myeloid leukemia (CML).

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INTRODUCTION

Chronic myelogenous leukemia (CML) is a clonal bone marrow stem cell unrest in which a proliferation of mature granulocytes and their precursors is found . It is a type of myeloproliferative disease accompanied with a characteristic chromosomal translocation, the fusion gene generate a protein *bcr-abl* called tyrosine kinase [(1)].

Imatinib mesylate (IM), the synthetic tyrosine kinase inhibitor known as Gleevec or Glivec , has been well notarized as first line treatment for CML. Although the striking efficacy encounter with IM therapy, numerous CML patients in advanced phases do not be interrogated to IM , others who at first respond to IM may ultimately acquire resistance disease. In patients with CML the development of impedance to IM is a multi factorial phenomenon and it may be mediate by a multifarious mechanisms. Nevertheless, there are 2 spacious mechanisms of impedance, *bcr-abl* dependent and *bcr-abl* independent [(2)] . *bcr-abl* dependent pathways thought that this acquired impedance is due to gene (*bcr-abl*) amplification or somatic mutations in the drugs target genes ,while *bcr-abl* independent pathways suggesting that pharmacokinetic impedance may also play a definitive role in the ultimate impedance of patients on chronic imatinib [(3)] .

The multidrug resistance (MDR) is remain a major problem in chemotherapy treatment believes to be responsible for poor response of patients towards chemotherapy. The better-characterized resistance mechanism is the one mediated by permeability glycoprotein (P-GP) encoded by MDR1 gene, which is accountable for drug efflux [(4,5,6)] . MDR1 gene was detected in cancer cells where it is accountable for multiple resistances to anticancer agents [(7)] . Membrane bound efflux transporters has an important roles in mediation chemo sensitivity and impedance of tumor cells. There are seven subfamilies (ABCA to ABCG). Many of the ABC family are importance in the mechanism of multidrug resistance [(8)] . Important genes encoding ABC transporters correlating with multidrug resistance (MDR1) includes *abcb1* encoding permiblity – Glycoprotein (PGP) also named as *abcb1* with protein product MRP1 . *abcb1* are transporters involves in IM transport [(9,10)] . Variability in drug efficacy due to genetic factors called pharmacogenetics which is a part of pharmacogenomics , a term that involved all genes in the human genome that may affect drug effectiveness . With the last advancement in pharmacogenomics , genetic polymorphisms have been showed up as one of the most important factors affected the pharmacokinetics and pharmacodynamics of many drugs [(11,12)] . The ability to prediction a patients drug response based on their genetic information is emerging as a solved to reduce adverse events and/or improve therapeutic effectiveness [(13,14)] . This study aimed to determine the polymorphisms of multidrug resistance gene (MDR) *abcb1* correlate with imatinib response or resistance in chronic myeloid leukemia .

MATERIALS AND METHODS

Subjects and Sampling

The study is carried out on 71 CML Iraqi patients (43 Male,28 Female) aged 20-70 years , including 11 untreated which are newly diagnosed , 30 under-treatment of Imatinib mesylate (IM) which are IM response and 30 resistance to IM drug and 25 apparently healthy individuals (Control Group) were selected to be matched with the patients ones in terms of age, gender and ethnicity (Iraqi, Arab) .The diagnosis of CML cases was based on the clinical examination of consultant physicians in The National Center of Haematology / The University of Mustansyria and Baghdad Teaching Hospital in Medical City . Three millileter of blood sample was taken by vein from all subjects were under study.

Analysis of Genes

Genomic DNA was extracted from the whole blood of CML patients and control using Wizard Genomic DNA Purification Kit (Promega) then, the extracted DNA was used for amplification of targeted fragments by using PCR. Specific primers were used after inspecting with Graphic program available on the web site of NCBI to check both the specify and the size of the product [(15)] . All primers were supplied by Alpha DNA company as a lyophilized product of different picomol concentrations, they were dissolved in free nuclease water to give a final concentration of (100 pmol /µl) as stock solution ,to prepare 10 pmol concentration of primer solution that is utilized in PCR 10 µl of stock solution was added to 90 µl of free nuclease distilled water to reach a final concentration 10 pmol/ µl. The sequences of these primers are listed in

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Table (1).The PCR condition for amplification of *abcb1* gene fragment was as, 95 °C for 3 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 64 °C for 30 seconds and extension at 72 °C for 30 seconds with a final 5 minutes cycle at 72 °C. PCR-RFLP was applied for identification of this genes by using restriction enzyme *MboI* for *abcb1* . Incubation for 4 hours at 37 °C.

Table (1): Sequences of primers used in this study.

Gene	Primers	Sequence of primers	PCR product size	Reference Of primers
abcb1	F primer	5'-- TGCTGGTCCT GAAGTTGATC TGTGAAC -3'	248bp	(Ameyaw *et al*.,2001)
	R primer	5'-ACATTAGGCA GTGACTCGAT GAAGGCA -3'		

PCR Products Sequencing

The PCR products of analyzed *abcb1* gene regions and primers were sent to Macrogen Company (U.S.A) for sequencing . The sequencing data was analyzed using the National Center for Biotechnology Information (NCBI) site and Bio edit system.

Statistical Analysis

The program of Statistical Analysis System (SAS) [16] was dependable in studying the difference in the parameters of study groups. Chi square was used to compare the importance of proportion in this study.

RESULTS AND DISCUSSION

Genomic DNA extraction

Genomic DNA was extracted from fresh blood by using Wizard Genomic DNA Kit (Promega , USA) to obtain a pure DNA for PCR amplification. This method involves lysing of red blood cells and dissolving the undesirable contaminants such as protein and RNA, in addition to degrade the cell membrane of white blood cells. The results of DNA extraction showed that fresh blood samples yielded enough DNA concentrations for PCR amplification Figure (1) .The quantification of DNA by nanodrop revealed that the DNA concentration ranged between 80 – 120 ng /µl and purity range was between 1.8 – 1.9.

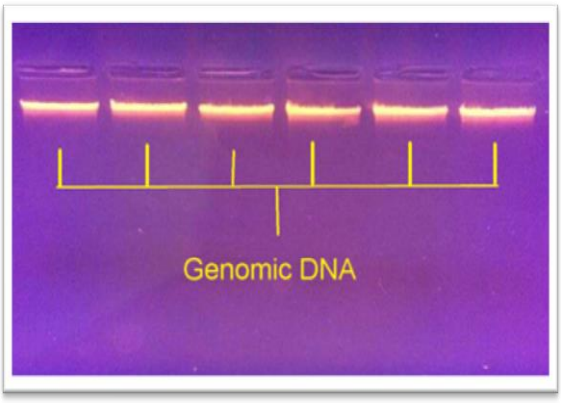

Figure (1) Gel electrophoresis of genomic DNA visualized under UV after staining with ethidium bromide on 1% agarose gel at 70 volt /cm for 30 minutes .

MDR1 *abcb1* gene analysis

For MDR1 *abcb1* gene polymorphism (rs1045642 SNP ; g. 208920T>C) detection study ; a fragment 248bp in exon 26 (208729 – 208976 , NG_011513.1) was targeted for amplification by the forward and reverse primers that used by Ameyaw *et al*. [15] , using PCR- RFLP technique (Figure 2).

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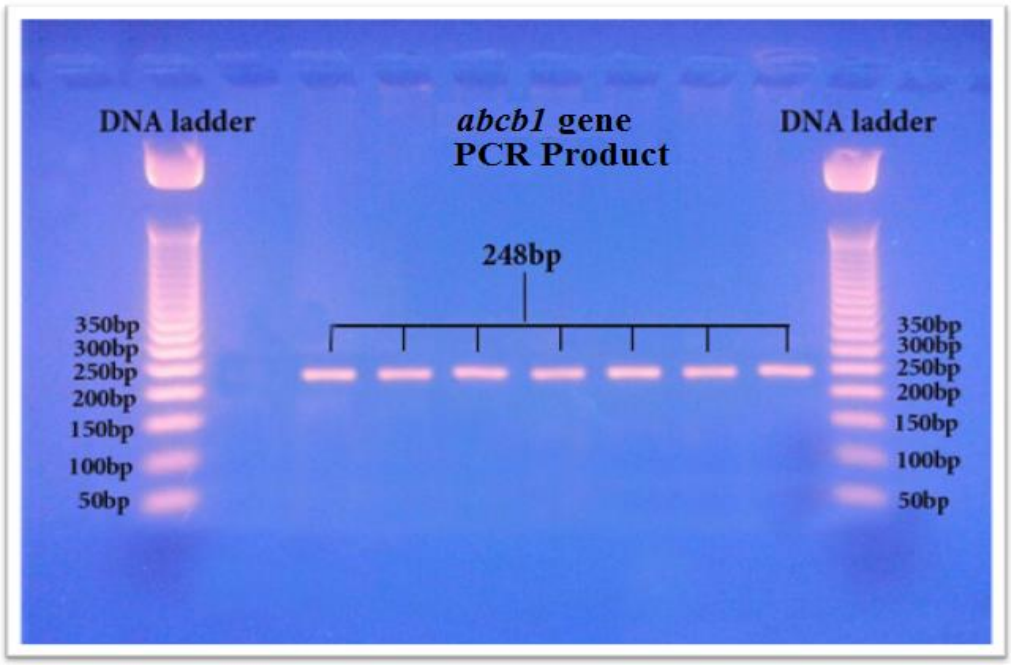

Figure 2. PCR product (248 bp) of targeted fragment flanking the rs1045642T>C SNP (NG_011513.1) in exon 26 of *abcb1*visualized under UV light after staining with ethidium bromide. The electrophoresis was on 2% agarose gel at 70 volt / cm for one hour.

The rs1045642 SNP (C3435T SNP) is located within this fragment at 208920 position (T) . The conversion of T allele to C allele at this position create a site for *MboI* restriction enzyme (GATT to GATC). Therefore, after digestion of PCR product with this enzyme, the fragments that were obtained were as follows: 232bp and 16bp for TT genotype; 232 , 172 , 60 and 16bp for TC genotype ; 172 , 60 and 16 bp for CC genotype (Figure 3). This SNP is silent and did not change the amino acid. For checking the PCR-RFLP results of rs1045642 T>C SNP (Figure 4) show the results of sequencing.

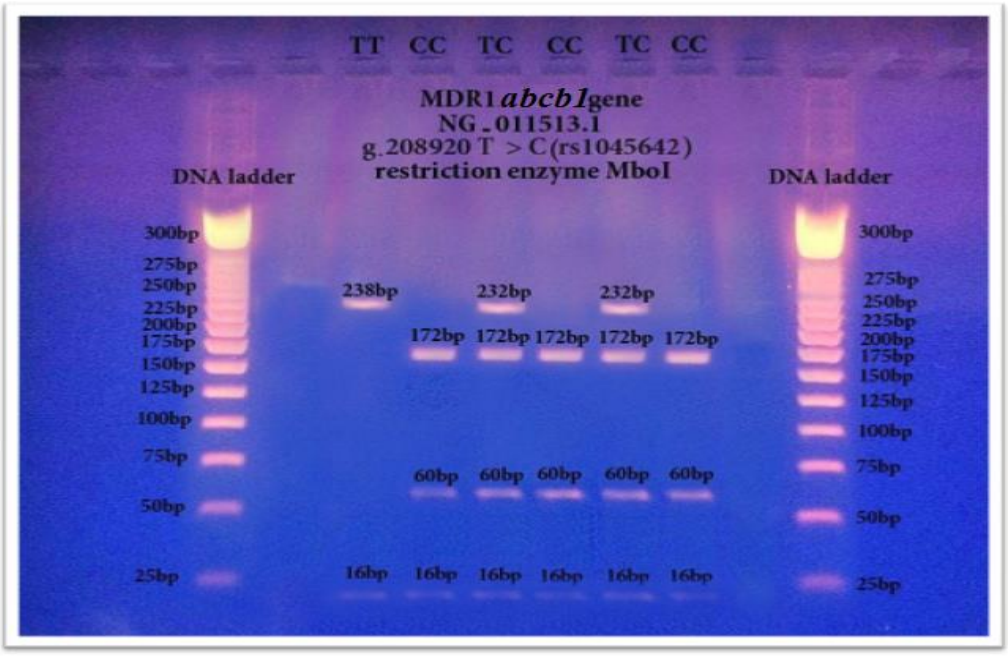

Figure 3. PCR product (248 bp fragment) of *abcb1*gene digested with *MboI* restriction enzyme and electrophoresed on 3% agarose. The genotypes at 208920 position (NG_011513.1) are: TT (232bp and 16bp), TC (232 bp ,172 bp ,60 bp and 16 bp) and CC (172bp , 60bp and 16 bp).

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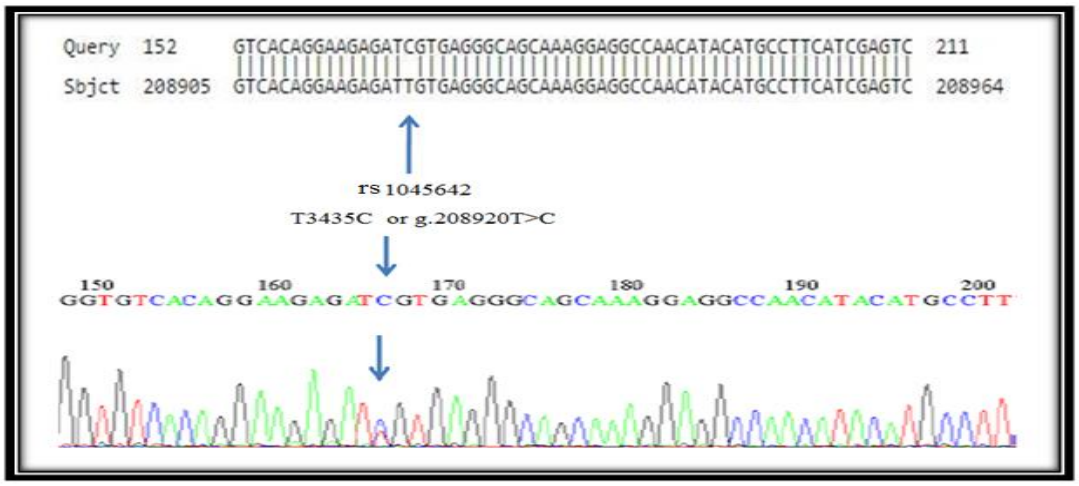

Figure 4. Electropherogram deicting the g.208920T>C rs1045642 SNP position and its flanks .

Table -2 shows the frequency of C3435T SNP in CML patients and control subjects. In patient group variant homozygous CC and heterozygous TC genotypes (12.68%), (66.20%) respectively were noted *versus* (21.13%) of normal TT genotype and in control group normal TT genotypes (20.0%) *versus* variant homozygous CC and heterozygous TC genotypes (12.00%) , (68.00%) respectively were noted .T allele was more frequent than C allele in both control and patients group ,it was 0.54 and 0.46 respectively. Our results had shown that no significant difference in the genotype and allele frequencies of *abcb1*C3435T between apparently healthy subject group and CML patients group , refer to a lack of relationship between these variants and the risk for CML (p>0.05).

Table (2): Genotype and allele frequencies of C3435T SNP in MDR1 *abcb1* gene .

Genotype	Control No= 25	Patients No.= 71	O.R.	Chi-square
TT	5 (20.00%)	15 (21.13%)	0.073	0.349 NS
TC	17 (68.00%)	47 (66.20%)	0.166	0.382 NS
CC	3 (12.00%)	9 (12.68%)	0.004	0.009 NS
Allele frequency				
T	0.54	0.54	--	--
C	0.46	0.46	--	--

Odds ratio and their 95% confidence interval (ORs and 95%CIs) analysis was used to detect the MDR1 C3435T genotype risk factors carriers to developing CML between populations. Odds ratio test listed in (Table 3) revealed that the CML patients observed increased risk to developing CML related with 3435TC carriers in 1.378 fold than CC carriers (ORs: 1.378; 95% CI 0.92-1.63). Odds ratio analysis indicates that the MDR1 3435TC genotype is possible to be a risk factor for the development of CML, but we suggest increasing the samples size to obtain more accurate in confidence interval.

Table. 3 Analysis of MDR1 C3435T Genotype Risk Factors of CML and Control.

Genotype	Control : No= 25	Patients : No.= 71	odd ratios	ORs (95%CI)
TT	5 (20.00%)	15 (21.13%)	TT vs TC	1.208 0.92-1.61
TC	17 (68.00%)	47 (66.20%)	TC vs CC	1.378 0.92-1.63
CC	3 (12.00%)	9 (12.68%)	TT vs CC	0.502 0.86-1.62

The *abcb1*C3435T genotype and allele frequencies between IM good responses and IM non-responses CML patients observed in Table (4) . the homozygous CC genotype with a frequency of 16.67 %, was found significantly to be higher among IM resistant group with (O.R.=0.53) and (P<0.05) as compared to IM good responder CML patients with a frequency of only 6.67 % .While normal homozygous TT genotype and variant heterozygous TC having non- significant effect with O.R=0.07and 0.29, respectively. the allele frequency of C was found higher in IM resistance CML patients as compared to IM responder CML patients it was (0.47 and 0.40, respectively). The results had shown that carriers of the C3435C allele of *abcb1* had more resistance to IM, suggesting that the increase of risk to resistance to IM could be related to the presence of the C allele . Likewise, Deenik *et al*. [17] who reported that patients with variant homozygous showed lower probabilities to obtain a Major Molecular Response (MMR) and Complete Molecular Response. Study by Duluq *et al*. [18] has reported that *abcb1*/MDR1polymorphisms are associated with major molecular responses to standard dose IM and the SNP 3435 C>T has been repeatedly shown to predict changes in the function of P-Gp. The same result was reported with Ni *et al.* [19] , *abcb1* SNP C3435T that the variant homozygous allele showed higher resistance rate. Recently, Egyptian study about MDR1 Gene Polymorphism and Outcome in Egyptian Chronic Myeloid Leukemia Patients by Ghallab *et al*. [20] they were denoted that genotyping of MDR1 gene polymorphism (C3435T and G2677T) may help in early identification of CML patients not responding optimally to therapy and in planning CML individualized therapy. On the other side study was done by Kim *et al.* [21] on CML patients, did not find an association between *abcb1* polymorphisms and MMR . Difference lifestyles and difference levels of exposure to various risk factors may cause inter-individuals heterogeneity. Frequencies with which these polymorphisms exist in a population have also been shown to be ethnically related Cropp *et al*. [22] . This could be a reason for the dicrepancy in result.

Table 4. Frequency of *abcb1* C3435T Polymorphism in CML Patient with Good Response or Resistance to IM Treatment.

Genotype	IM Response No. = 30	IM Resistance No. = 30	O.R.	Chi-square
TT	8 (26.67%)	7 (23.33%)	0.07	0.824 NS
TC	20 (66.67%)	18 (60.00%)	0.29	1.02 NS
CC	2 (6.67%)	5 (16.67%)	0.53	4.279 *
Allele frequency				
T	0.60	0.53	--	--
C	0.40	0.47	--	--
*** (P<0.05), NS: Non-significant**				

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