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Influence of multi-drug transporter gene ABCG2 polymorphism (C421A) in clinical out care in some Iraqi chronic myeloid leukemia patients treated with imatinib mesylate

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Abstract: In CML patients, Imatinib Mesylate (IM) treatment is a good choice with an excellent efficacy outcome. But unfortunately, a significant obstacle of IM resistance has emerged relating to the genetic polymorphism in drug transporter genes such as ABCG2 which effects the metabolism and pharmacokinetic IM. This study investigate the influence of ABCG2 C421A SNP in IM response among some Iraqi CML Patients (71 patients : 43 Male , 28 Female) aged between (20-70) years in chronic phase with positive Philadelphia (Ph) chromosome, including 11 newly diagnosed , and 60 treated with standard dose IM (400mg) on frontline treatment , 30 of them were IM response and 30 resistance to IM drug , on the other hand 25 apparently healthy individuals were included as control group. After the patients were informed about the details of the research, they were approved to take samples of their blood for study , 3ml of peripheral blood was withdrawn from CML patients and control groups . The frequency of homozygous mutant genotype (AA) of SNP*abcg2* C421Awas significant higher in IM resistant CML patients as compared to IM good response CML patients with (O.R=1.309 and $p < 0.01$) . The SNP *abcg2* C421A was found to contribute to the genetic susceptibility of CML when evaluated with healthy control subjects. These reconnoitering results give a reasonable cause to explore such SNPs to be used as a biomarker in prediction the response to IM treatment before getting started.

Key words : *abcg2* C421A SNP, polymorphisms , Imatinib Mesylate , CML.

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Introduction

Chronic Myeloid Leukemia (CML), is a proliferative imbalance in myeloid cells, constitutes a proportion 14% of all leukemic types. Imatinib mesylate (IM), also called Glivec or Gleevec is a tyrosine kinase inhibitor used as a first choice treatment for CML. In spite of good results of the treatment with IM , many CML patients (in advanced phase) have no response for IM, while others who appeared a primary response to IM may ultimately develop resistance to it. Progress an IM resistance is multi-factor phenomenon in CML patients including many mechanisms; of these two main mechanisms of resistance, The breakpoint cluster region - Abelson murine leukemia viral oncogene homolog 1 (BCR-ABL) dependent mechanism and BCR-ABL independent

mechanism. Pathways of BCR-ABL dependent mechanism included ABL kinase mutations and amplification of BCR-ABL. Many of IM resistance CML patients didn't match with BCR-ABL dependent mechanism, they may fit with BCR-ABL independent mechanism of IM resistance which encompass several mechanisms (1).

Multidrug resistance (MDR) in cancer is a phenomenon that occurs when cancer cells become simultaneously resistant to structurally unrelated chemotherapeutic agents ,it is eventually lead to the failure of cancer treatment and remains one of the primary causes of suboptimal outcomes in cancer therapy (2). Several cellular mechanisms can be responsible for MDR, such as reduced apoptosis, advanced DNA damage repair mechanisms or altered drug metabolism. However, the most common mechanism of resistance is the active efflux of drugs by ATP-binding cassette (ABC) transporters (3, 4, 5, 6) . ATP-binding cassette (ABC) transporters are a family of transporter proteins that contribute to drug resistance via ATP-dependent drug efflux pumps. ATP-binding cassette efflux transporters that limit xenobiotics, including a large number of therapeutic drugs, from entering the cancer cells. P-glycoprotein (P-gp, *ABCB1* or *MDR1*) and breast cancer resistance protein (BCRP, ABCG2) are two prominent members of the ABC transporter superfamily. Both have broad and partly overlapping substrate specificities that include a variety of structurally diverse drugs currently used in the clinic (7 , 8 , 9) These two "gatekeeper" transporters constitute a vital part of the protective defence mechanism by limiting drugs from accessing the cells and thereby rendering them ineffective (10 ,11)

Genetic polymorphisms are considered as critical determinants of expression level or activity and subsequently response to selected drugs. Single nucleotide polymorphisms (SNPs) in ABCB1 and ABCG2 genes have the potential to alter protein function and could also influence the efficacy of absorption or elimination (12 ,13) . However, few researches have been conducted on impact of ABCB1 and ABCG2 SNPs on imatinib treatment outcome worldwide as well as in Iraq (1 ,14 ,15 , 16,17).

This study aimed to investigate the influence of allele and genotype frequencies of SNP ABCG2 C421A in CML patients undergoing IM treatment and to determine whether different genotype pattern of this SNP have any influence on the pharmacokinetic of IM in mediating good response and resistance to it and associated consequences on clinical outcome is discussed.

Materials and Methods:

Subjects and Sampling:

The study is carried out on 71 CML Iraqi patients (43 Male,28 Female) aged between (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) . The extracted DNA was used for amplification of targeted fragments by using PCR. Specific primers (table 1) , 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 . Primers were supplied by Alpha DNA company.

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

Gene	Primers	Sequence of primers	PCR product size	Reference of Primers
abcg2	F primer	5′- GTTGTGATGGGCACTCTGATGGT -3′	289bp	(Kobayashi *et al.*,2004)
	R primer	5′- CAAGCCACTTTTCTCATTGTT -3′		

Polymerase Chain Reaction Components:

The components of PCR reaction with volume amounts are shown in table(2) under optional conditions listed in table(3) (was used for amplification of *abcg2* gene fragment).

Table (2): PCR reaction components for amplification of *abcg2* genes.

Component	Component of one sample (µl)
PCR pre Mix (Ready-to-se):*Taq* DNA polymerase , dNTPs, MgCl2 and reaction buffer (PH 8.5)	12.5
Forward primer	0.5
Reverse primer	0.5
DNA template	5
D.W	6.5
Final volume	25

Polymerase Chain Reaction Programs:

Optimization of PCR reaction was accomplished after several trials; thus the following programs were adopted .

Table (3): PCR amplification program of *abcg2* gene.

Steps	Temperature (C^{o})	Time	No. of cycles
Initial denaturation	95	3minutes	1
Denaturation	95	30 seconds	35
Annealing	58	30 seconds	
Extension	72	30 seconds	
Final extension	72	5 minutes	1

Analysis of PCR Products:

The PCR products and the DNA (50bp) ladder marker have been resolved by electrophoresis, 3 µl of loading buffer plus 7µl of the product were loaded on 2 % agarose gel (2g agarose/100 ml1X TBE buffer) and run at 70 volt for one hr. The gel was stained with (0.5 µg/ml) ethidium bromide. In addition, bands were visualized on UV transilluminator and then photographed. DNA ladder (50bp) has been used to estimate the molecular size of the bands.

PCR-RFLP of *abcg2* gene:

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was applied for identification of target gene by using restriction enzyme *HpyCH4III* for ***abcg2*** (Table 4) enzyme . The product with HPYCH4III was incubated for 4 hours in 37°C . The digested product was separated by electrophoresis in 3 % agarose gel (3 gm agarose dissolved with 100 ml 1XTBE) along with 25-300bp ladder firstly at 25 Volt for 30 mint., then increase the Volt to 70 volt for 1 hour, in 1XTBE buffer. After that it was visualized under UV light using an ultraviolet transilluminator.

Table (4) : RFLP Programs for *abcg2* gene.

Component	Component ofone sample (µl)
PCR product	10
Enzyme	1
Buffer	2
D.w	7
Total volume	20

PCR Products Sequencing:

The PCR products of analyzed *abcg2* gene 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) (19) was dependable in studying the difference between parameters of study groups. Chi square was used to compare the importance of proportion in this study.

Results and Discussion:

Genomic DNA extraction:

The results of DNA extraction showed high purity (1.8 – 1.9) and good yield of DNA concentration (80-120 ng) using nanodrop for PCR amplification Figure(1) .

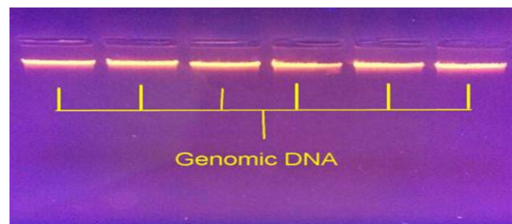

Figure (1): Gel electrophoresis of genomic DNA visualized under UV after staining with ethidium bromide on 1% agarose gel at 70 volt /cm for 30 minutes.

MDR1 *abcg2* gene analysis:

As noted in the figure (2) , to determine *abcg2* gene polymorphism (rs2231142 SNP) **,** a 289 bp fragment in exon 5 region from base (105116 – 105405 , *NG_032067.2*) was targeted for amplification by the forward and reverse primers that were designed by Kobayashi *et al.* (2005), using PCR technique (Figure 2). The rs2231142 SNP is located within this fragment at 105152 position (C). The polymorphisms of this SNP were detected using PCR-RFLP. The conversion of C allele to A allele at this position (ACNGT to AANGT) prevents the restriction enzyme *HpyCH4III* for restriction at this site. Therefore, after digestion of PCR product with this enzyme, the fragments that were obtained polymorphism at rs2231142 SNP were as follows: 251bp , 21bp and 17 bp for CC genotype; 268bp, 251bp,21bp and 17bp for CA genotype; 268bp and 21bp for AA genotype (Figure 3). The other names for this SNP are C421A and g.105152C >A. This SNP resulting substitutes a glutamine for a lysine. For checking the PCR-RFLP results of rs2231142C>A, Figure 4 shows the results of sequencing.

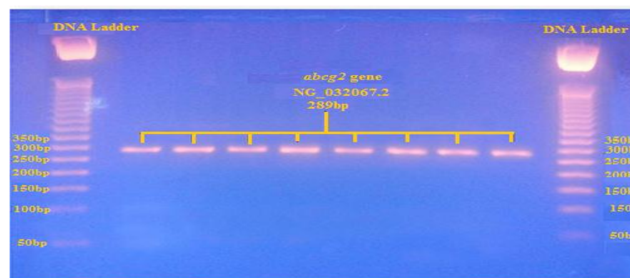

Figure (2): PCR product (289bp) of targeted fragment flanking the rs2231142C >A SNP (*NG_032067.2*) in exon 5 visualized under UV light after staining with ethidium bromide. The electrophoresis was on 2% agarose gel at 70 volt / cm for one hours.

Figure (3): PCR product (289bp fragment) digested with *HpyCH4III* restriction enzyme and electrophoreses on 3% agarose. The genotypes at 105152 position (*NG_032067.2*) are: CC(251bp,21bp and 17 bp), CA (268bp, 251bp,21bp and 17bp) and AA (268bp and 21bp).

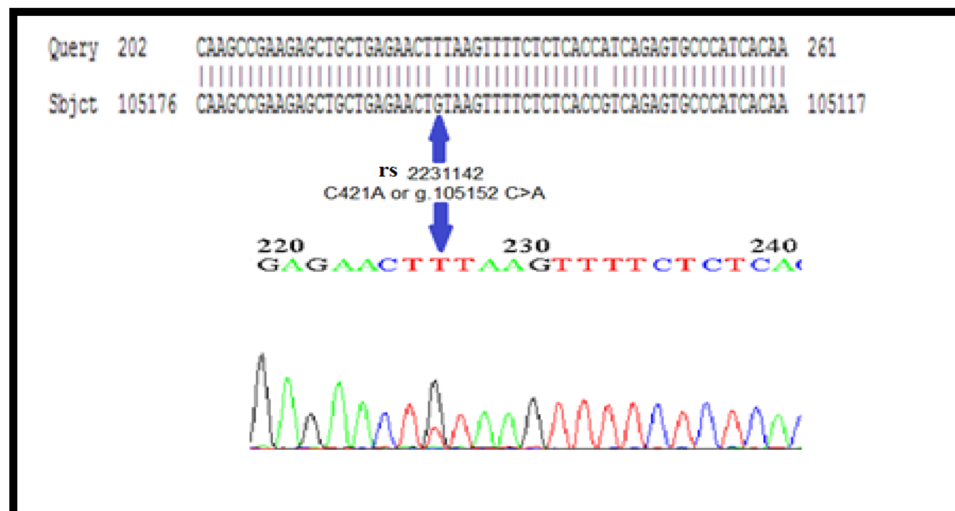

Figure (4): Electropherogramdeicting the g.105152C>A (rs2231142) SNP position and its flanks,C421A mutation code for reveres mutation.

With regard to *abcg2*C421A polymorphism, the distribution of genotype and allele frequency at105152 site (rs2231142) were presented in table (5). The AA genotype frequency was significantly higher with O.R=1.066 and ($p<0.01$) in CML patients when compared with apparently healthy subjects (52.11% *versus* 20.00% , respectively) , while AC genotype frequency was statistically insignificant in CML patients. Despite that there was a high percentage of AC genotype in CML patient as compared with apparently healthy subjects (4.23% versus 0.00%, respectively). This study suggest increasing the samples size to obtain more accuracy results. The frequency of CC genotype was highly significant with O.R=1.285 and ($p<0.01$) in apparently healthy subjects when compared with CML patients (80.00% versus 43.66%, respectively).

Comparison of the allele frequencies of A and C of *abcg2* C421A between CML patients showed that the variable allele A was higher than C allele, it was (0.54 and 0.46, respectively). As a conclusion, there were a significant differences in the genotype and allele frequencies of *abcg2* C421A between apparently healthy subject group and CML patients group ,referring to the present relation between these variants and the risk for CML (p<0.01).

Table (5) : Genotype and allele frequencies of C421A SNP in MDR1 *abcg2*gene.

Genotype	Control No= 25	Patients No.= 71	O.R.	Chi-square
CC	20 (80.00%)	31 (43.66%)	1.285	9.305 **
CA	0 (0.00%)	3 (4.23%)	0.393	0.973 NS
AA	5 (20.00%)	37 (52.11%)	1.066	9.189 **
Allele frequency				
C	0.80	0.46		
A	0.20	0.54		
** (P<0.01), NS: Non-significant.				

Odds ratio and 95% confidence interval analyses were used to detect the MDR1 C421A genotype risk factors carriers to developing CML between populations. Odds ratio test listed in (Table 6) revealed that the CML patients observed increased risk to developing CML related with 421CA carriers in fold 1.407 than AA carriers (ORs:1.407; 95% CI 0.93-1.62). The analysis has indicated that the MDR1 421CA genotype is possible to be a risk factor for the development of CML.

Table 6: Analysis of MDR1C421A Genotype Risk Factors of CML and Control

Genotype	Control No= 25	Patients No.= 71	O.R.	O.Rs	ORS (95% CI)
CC	20 (80.00%)	31 (43.66%)	1.285	CCvsCA	1.278 (0.89-1.63)
CA	0 (0.00%)	3 (4.23%)	0.393	CAvsAA	1.407 (0.93-1.62)
AA	5 (20.00%)	37 (52.11%)	1.066	AA vs CC	0.593 (0.92-1.60)

The *abcg2* C421A genotype and allele frequencies between IM good responses and IM non-responses CML patients were observed in Table (7).The

frequency of homozygous CC genotype was found significantly higher among IM good responder CML patients as compared to IM resistant group with a frequency of 73.33% and 13.33% , respectively with O.R=1.502 and (P < 0.01).While , heterozygous CA genotype was found higher frequency in IM resistance CML patients as compared with IM good responder CML patients with a frequency of 10% and 0.00%, respectively. The variable heterozygous CA genotype has a significant effect with O.R=0.566 and (p < 0.05) in IM resistance CML patients. Also, the variant homozygous AA genotype that showed a higher frequency in IM resistance CML patients as compared with IM responder with a frequency of (76.66% and 26.66% respectively) with O.R=1.309 and P value (p < 0.01). The allele frequency of C allele was higher in IM response CML patients group than IM resistance, it was (0.73 and 0.18 , respectively) , while A allele frequency was higher in IM resistance CML patients than IM response, as it was (0.82 and 0.27 , respectively).

Odds ratio taste listed in (table 7) revealed that the CML patient observed increased risk in developing IM resistance related with 421CA carriers in 1.563 fold than AA carriers (ORs : 1. 563; 95% CI 0.92- 1.63), also CML patient observed increased risk in developing IM resistance related with 421AA carriers in 1.584 fold than CC carriers (ORs : 1. 584; 95% CI 0.92- 1.61) . Odds ratio analysis has indicated that the MDR1 421CA and 421AA genotypes are possible to be a risk factor for development of IM resistance for CML patients who were treated with IM drug .

Table (7): Frequency of *abcg2* C421A 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	O.Rs	ORS (95% CI)
CC	22(73.33%)	4 (13.33%)	1.502	12.563 **	CC vs CA	0.983 (0.91-1.62)
CA	0 (0.00%)	3 (10%)	0.566	4.425 *	CA vs AA	1.563 (0.92-1.63)
AA	8 (26.66%)	23 (76.66%)	1.309	11.297 **	AA vs CC	1.584 (0.92-1.61)
Allele frequency						
C	0.73	0.18				
A	0.27	0.82				
*** (P<0.05), ** (P<0.01).**						

These results agreed with Ghafouri *et al.* (20) who reported that breast cancer patients with AA genotype of ABCG2 C421A were at higher risk of progressing breast cancer. Patients with AA genotype of ABCG2 C421A were at higher risk of progressing breast cancer, but disagree in that breast cancer patients with A allele of ABCG2 had complete response to chemotherapeutic agents while in this study A allele frequency was higher in IM resistance CML patients than IM response. In the study by Takahashi *et al*. (16) it was reported that *abcg2* C421A genotyping done in Japanese population had shown correlation with respect to MMR and IM trough concentration, a lower IM clearance and a higher dose-adjusted IM trough concentration were found in CML patients with the C421A variant. The preliminary results of Au *et al*. , (1) demonstrated a significant correlation of the SNP ABCG2 C421A with IM efficacy and a lack of significant association of SNP ABCB1 C3435T with IM response in Malaysian CML patients.

This study conclude that in the abcg2 C421A (rs2231142) SNP conceders as a predicted factor for progression of CML cancer in Iraqi CML patients, and the A allele of ABCG2 C421A is associated with IM bad progresses in those patients.

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