Pharmacogenetics: Influence of *CYP2C9*2* and *3 Alleles Polymorphisms on Iraqi Type 2 Diabetic Patients

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Abstract

Background: Sulfonylureas (SUs) are the most prescribed anti-diabetic drugs. The enzyme responsible for metabolizing of SUs is hepatic cytochrome P_{450}2C9 (CYP2C9). The *CYP2C9* gene has numerous allelic variations; among those, the *CYP2C9*2 and CYP2C9*3 are the most common and clinically significant allelic variations. The pharmacokinetics of SUs are dramatically impacted by *CYP2C9* genotype. **Objective:** Evaluation of the association of genetic polymorphisms in *CYP2C9* gene with the efficacy of glibenclamide (GB), second-generation SUs, by investigating two *CYP2C9* allelic variants. **Materials and Methods:** Blood samples were collected from 113 type 2 diabetes patients. Allele specific amplification-polymerase chain reaction was used to genotype the *CYP2C9* gene. Fasting serum glucose, fasting insulin, and glycated hemoglobin (HbA1c) levels were measured as part of the biochemical analysis. **Results:** The CYP2C9 gene variants were analyzed in a study group. The results exposed that 75 patients carried the wild (*CYP2C9*1/*1*) genotype, 25 were heterozygote allele (*CYP2C9*1/*2*) for *CYP2C9*2* gene, 4 were homozygous for the variant *CYP2C9*2* allele (*CYP2C9*2/*2*), and 9 were heterozygous for the variant *CYP2C9*3* allele (*CYP2C9*1/*3*). Statistically significant difference was found in mean HbA1c between the mutant and wild alleles group ($P = 0.044$). The mean HbA1c for those carrying the *CYP2C9*2* and*3 alleles ($n = 38$) was 8.4750 compared to 9.3177 for those carrying the CYP2C9*1 allele ($n = 75$), which indicate better glycemic control. **Conclusion:** The accordance of *CYP2C9*2* and*3 was found to be associated with severe hypoglycemia (odd ratio [OR] = 2.045). The OR suggests a strong association between CYP2C9*2 and*3 alleles and hypoglycemia. Our findings imply that the diabetic patients with *CYP2C9* polymorphism are more likely to suffer hypoglycemia than those with wild type alleles when treated with GB.

Keywords: CYP2C9, diabetes mellitus type 2, glibenclamide, pharmacogenetic, sulphonylurea

Introduction

More than a million Iraqis suffer from diabetes.[1] Diabetes mellitus 2 (DMT2) occurrence in Iraq was described to range from 8.5% to 13.9%. Diabetes was found to be prevalent in 19.7% of adults aged 19–94 in a local study of approximately 5400 people in Basrah, Southern Iraq.[2] Really, there are inadequate epidemiological research and randomized clinical trials linked to diabetes in Iraq, making it challenging to fully comprehend the prevalence of diabetes in the country and the most appropriate treatments for Iraqis.[3] Anti-diabetic medications are extensively used class of drugs with a wide range of functions. Sulfonylureas (SUs) are a type of oral diabetes medication that is widely used to treat type 2 diabetes mellitus (T2DM). SUs lower blood glucose levels by stimulating the release of insulin from pancreatic beta cells. As a result, patients with T2DM who are taking SUs are at risk of hypoglycemia, which can be fatal.[4] In Iraq, SUs remain the most commonly used anti diabetes agent. This could be due to their lower cost, the option of once-daily dosing, and the presence of an association with metformin in the same tablet.[3] Their main influence is to raise insulin levels in the blood. The cytochrome P450 2C9

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(CYP2C9) enzyme metabolizes the most commonly used SU, including the second generation agents glyburide, glipizide, and glimepiride. CYP2C9 polymorphisms have been shown to influence the pharmacokinetics of SUs in many clinical trials, as an anti-diabetic agent.[5] However, nearly 60 *CYP2C9* alleles located in the coding region of the gene have been identified.[6] The most common allele is designated as *CYP2C9*1* and is considered the wild-type allele.[7] Among the genetic variants reported in the human CYP2C9 gene CYP2C9*2 (rs1799853) 430 C > T cytosine trans-version to thiamine, encodes for a cysteine substitution at amino acid residue 144, producing the Arg144Cys, CYP2C9*2 was reported to be clinically significance that result in decrease of enzyme activity and lower intrinsic clearance.[8,9] The second single nucleotide polymorphism (SNP) is CYP2C9*3, an adenine to cytosine substitution at position 1075 (rs1057910) 1075A > C, 1075 encodes for a leucine substitution at amino acid residue 359, producing the Ile359Leu.[7] The main aim of the current study is to determine the influence of genetic polymorphisms in a drug metabolizing enzyme CYP2C9 gene on the medical state of T2DM patients and to investigate the effects of these allelic variants on glibenclamide (GB) effectiveness and efficiency.

Materials and Methods

Research design and inclusion criteria

Diabetic patients were enrolled from the Al-Wafaa Diabetes Clinic in Mosul, Iraq, for the treatment of diabetes mellitus. Blood samples were collected after overnight fast (2 mL venous blood) were collected in ethylene diamine tetra acetic acid tube for the DNA extraction. Furthermore, 3 mL venous blood was obtained in a gel tube for the determination of fasting blood sugar (FBS), fasting insulin (FI), and glycated hemoglobin (HbA1c). The Iraqi Ministry of Health's Ethics Committee approved the research protocol at the Nineveh health department. All participants were given written informed consent before participating.

Inclusion criteria require that the participating patient had to be on a treatment plan that comprised a consistent dose of glibenclamide and metformin medicine for at least three months to be eligible. No congeniality between the patient who has been enlisted and the other participants in the study. The following exclusion criteria were used to determine whether to recruit a patient in this study: (1) prolonged alcoholism; (2) earlier pancreatic pathology; (3) renal failure; (4) hypoglycemic treatment with insulin or insulin equivalents; (5) poor medical records or insufficient history data; (6) refusal to sign a written consent document. Homeostasis model assessment 2 as an index of insulin resistance (HOMA2IR) was applied to evaluate the natural course of diabetes and the impact of treatment by assessing long-term changes in beta-cell function and insulin resistance in diabetic patients. The homeostasis model assessment 2 was downloaded from university of oxford to calculate the HOMA2-IR (www.dtu.ox.ac.uk/homacalculator/) using the result of fasting glucose, FI tests.

Genotyping

The DNA was extracted from frozen blood by Gene JET Whole blood genomic DNA Purification Mini Kit (Thermo Fisher Scientific, USA). The polymerase chain reaction (PCR) mix to the final volume of 20 µL consisted of 1 µL (0.2 µM) of outer forward primer, 1 µL (0.2 µM) of outer reverse primer, 1 µL (0.2 µM) of inner forward primer, 1 µL (0.2 µM) of inner reverse primer, 4 µL of DNA template, and 11 µL of nuclease free water PCR grade. The components are added to AccuPower®PCR PreMix (Bioneer, Korea) tube that contains the freeze-dried pellet. Primer sequences are listed in Table 1. Only if the normal type allele is found, the PCR amplifies the area between the wild form primer and the reverse external primer. In contrast, only if the mutant allele is found the area between the internal mutation specific primer and the external forward primer be amplified. A fragment size can be used to distinguish between wild type and mutant amplicons. A third fragment will also create as an internal control, representing the region between the two exterior primers.

The following PCR-thermal conditions were used: 95°C for 5 min for initial denaturation, 35 cycles at 95ºC for 30 s (62ºC regarding CYP2C9*2 and 58ºC analyzing CYP2C9*3) for 45 s and 72ºC for 45 s, and final extension at 72ºC for 5 min. Polyacrylamide gel electrophoresis

Table 1: Primers for detection of CYP2C9 polymorphisms and their sequences

Target single nucleotide polymorphism	Oligonucleotides	Primer Sequence 5′→ 3′	Product size (bp)
CYP2C9*2	Outer forward	AATAGTAACTTCGTTTGCTGTTATCTC	581
	Outer reverse	CAGTAGAGAAGATAGTAGTCCAGTAAGGT	
	Inner forward-M	GGAAGAGGAGCATTGAGGACT	127
	Inner reverse-W	GGGCTTCCTCTTGAACACG	493
CYP2C9*3	Outer forward	GCCATTTTTCTCCTTTTCCAT	
	Outer reverse	GGAGAACACACACTGCCAGA	434
	Inner forward-W	GCACGAGGTCCAGAGATACA	259
	Inner reverse-M	TGGTGGGGAGAAGGTCAAG	177

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was used to separate the PCR products, and ethidium bromide was used to visualize them. The length of the amplification refractory mutation system-PCR products was determined by comparing them to a 50–1000 DNA ladder.

Statistical analysis

The information was entered into a digital database. The database was analyzed using IBM SPSS Statistics for windows software package version 26 (2018) by International Business Machines Statistical Package for the Social Sciences (IBM SPSS) (SPSS Inc.).

One-way analysis of variance table was used to compare the means of the numerical variables between the different groups of the study. Chi-square test of independence was used to investigate the relationship between the patients and the control group. A *P* value of 0.05 was considered significant in all statistical analyses.

Pearson's Chi-square test detects substantial divergences between observed and predicted genotype frequencies.

Ethical approval

The research related to human use has been complied with all the relevant national regulations, institutional policies, and in accordance the tenets of the Helsinki Declaration and has been approved by the authors' institutional ethical committee in September 28, 2022, project No. B220905.

Results

For detection the C/T SNP (rs1799853) at codon 430 in exon 3 of gene that resulted in the *CYP2C9**2 allele, this SNP result in a shift in amino acid residue from arginine to cysteine at codon position 144 (Arg144Cys).[10] The location of this residue is on the surface of CYP2C9 enzyme.[11] Allele specific amplification (ASA-PCR) was conducted, utilizing specific primers under optimal amplification conditions. The amplified products appeared as clear bands following electrophoresis in the presence of a 50 bp ladder marker, as shown in Figure 1.

A PCR product was 581 bp (the complete fragment) amplified with external forward and external reverse primers. Amplification with inner forward and external reverse primer yielded a 127-bp product indicating the presence of a T allele (the mutant allele), while amplification with inner reverse and external forward primers yielded a 493-bp product indicating the presence of a C allele (the wild allele that is designate as *CYP2C9**1).

Another SNP A/C (rs1057910) located at codon 1075 in exon 7, resulted in the *CYP2C9**3 allele. This coding SNP leads to a shift in amino acid residue from isoleucine to leucine at codon position 359 (Ile359Leu), the position of this residue is inside *CYP2C9* enzyme.[11] The results of *CYP2C9* gene amplification with external forward and reverse primers yielded 434 bp product (the complete fragment), 295 bp product with inner forward and reverse primers presenting the A allele (the wild allele fragment), and 177 bp product with inner reverse and external forward primers showing the C allele (the mutant allele fragment *CYP2C9**3) [Figure 2].

Out of the 113 patients with DMT2, 25 patients (22.1%) were found to be heterozygous for the CYP2C9*2 polymorphism (*1/*2), whereas the homozygosity (*2/*2) for the CYP2C9*2 frequency in DMT2 was four patients out of 113 patients (3.5%). Nine patients (8%) were found to be heterozygous (*1/*3) in DMT2 patients group for the CYP2C9*3 polymorphism.

The genotype group's characteristics in terms of FBS, FI, HbA1c, neuropathy, nephropathy, and hypoglycemia were displayed in Table 2. In all of those parameters, there was no statistically significant association between the four groups tested. The mean HbA1c was 9.23, 8.27, 9, 37, and 8.91 for the genotypes 1*/*1, *1/*2, *2/*2, and *1/*3, respectively. The mean FBS was found to be 11.08, 10.15, 10.77 and 12.10 for the genotypes 1*/*1, *1/*2, *2/*2, and *1/*3 respectively. The mean FI was found to be

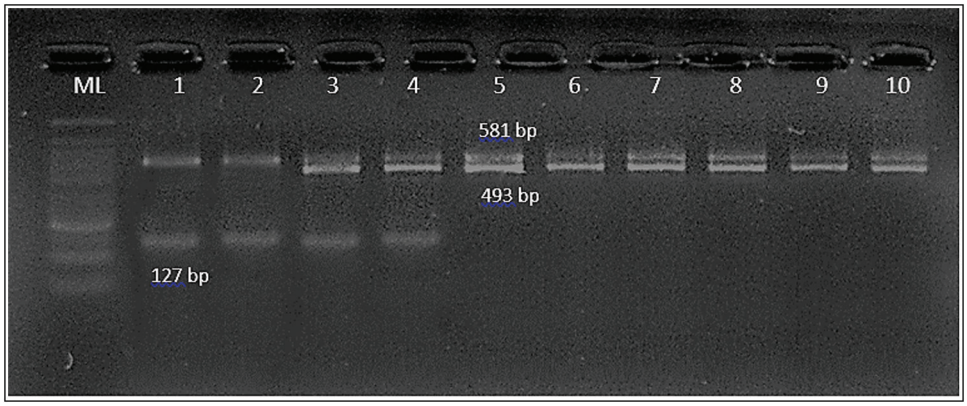

Figure 1: Amplification of exon 3 in CYP2C9 gene by ASA-PCR: where lanes 1–2 homozygote genotype for CYP2C9 *2/*2, lanes 3–4 heterozygous genotype *2/*1, lanes 5–10 the wild type CYP2C9 *1/*1. ML: molecular ladder (50–1000 bp). Agarose 1%, 7 V/cm for 45 min

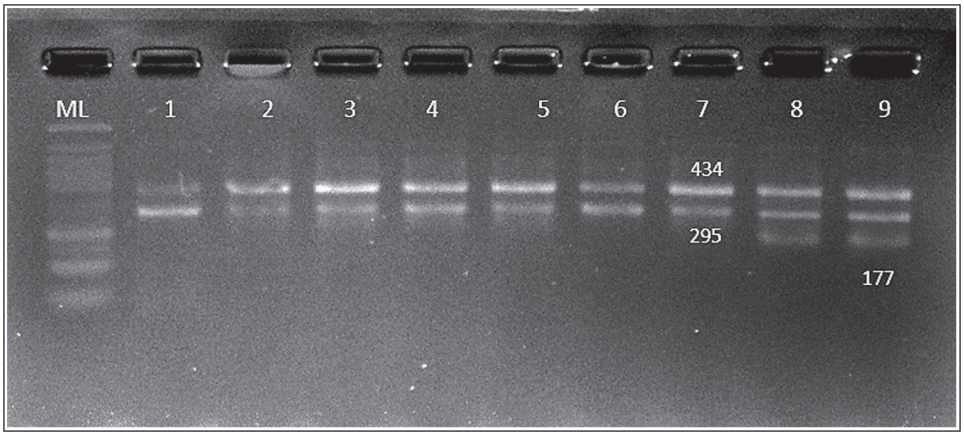

Figure 2: Amplification of exon 7 in *CYP2C9* gene by ASA-PCR using two sets of primer: where lanes 1–7 the wild type *CYP2C9* *1/*1, lanes 8 and 9 heterozygous genotype *3/*1. ML: molecular ladder (50–1000 bp). Agarose 1%, 7 V/cm for 45 min

Table 2: Diabetes indices, cardiovascular risk factors and hypoglycemic events in the four genotype groups

Genotype	*1/*1 (n = 75)	*1/*2 (n = 25)	*2/*2 (n = 4)	*1/*3 (n = 9)	*P*-value
Mean HbA1c	9.23	8.27	9.37	8.91	0.186
Mean fasting blood sugar, mmol/L	11.08	10.15	10.77	12.10	0.742
Mean fasting insulin, uIU/mL	12.6	12.22	9.02	14.1	0.861
Mean HOMA2-IR	1.89	2.14	1.47	2.35	0.680
Neuropathy	58.67%	76%	75%	66.66%	0.975
Nephropathy	58.67%	21.33%	50%	6.2	0.485
Retinopathy	44%	44%	50%	44.44%	0.414
Severe hypoglycemia	28%	16%	50%	22.22%	
Mild hypoglycemia	12.28%	20%	0	22.22%	0.341

One-way analysis of variance table was used to compare the means. Chi-square test of independence was used to investigate the relationship between each genotype and the categorical variable

Table 3. Diabetes indices, cardiovascular risk factors and hypoglycemic events according to *CYP2C9* genotypes

Genotype	CYP2C9*2 and *3 (n = 36)	CYP2C9*1 (n = 75)	*P*-value	OR
Mean HbA1c	8.4750	9.3177	*0.044	
Mean fasting blood sugar, mmol/L	10.511	11.040	0.489	
Mean fasting insulin, uIU/ml	13.553	11.863	0.406	
Mean HOMA2-IR	2.12	1.89	0.455	
Neuropathy	72.22%	58.67%	0.166	
Nephropathy	61.11%	58.67%	0.806	
Retinopathy	44.44%	44%	0.965	
Severe hypoglycemia	19.44%	12.28%		2.045
Mild hypoglycemia	27.6%	72.4%		0.8

* means S: significant, OR: odd ratio

12.6, 12.22, 9.02, and 14.1 for the genotypes 1*/*1, *1/*2, *2/*2, and *1/*3 respectively. The mean HOMA2-IR were found to be 1.89, 2.14, 1.47, and 2.35 for the carriers of genotypes 1*/*1, *1/*2, *2/*2, and *1/*3, respectively.

The association between the mutant and wild alleles groups and DMT2 were demonstrated in Table 3, complication and hypoglycemic events. Statistically significant differences were found in mean HbA1c between the mutant and wild alleles group (P = 0.044). The individuals with CYP2C9*2 and *3 alleles (n = 38) have mean HbA1c (8.4750) whereas the mean HbA1c was (9.3177) for the individuals with CYP2C9*1 (n = 75) allele. Although no statistically significant difference was not found in the mean FBS, the carriers of *CYP2C9*2* and *3 alleles had a lower mean FBS than the wild allele carriers, 10.511 mmol/L against 11.040 mmol/L. Mean FI was higher in *CYP2C9*2* and *3 alleles group 13.553 uIU/ml, against 11.863 uIU/ml in the wild allele group. The last observation can be explained by the fact that plasma level of glibenclamide increased in the patients

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with the *CYP2C9*3* allele followed by CYP2C9*2[12] and glibenclamide is secretagogues that stimulate the beta cell to secrete insulin. The current study revealed that the odd of having *CYP2C9*2* and *3 alleles is associated with severe hypoglycemia two time higher than *CYP2C9*1* or the wild allele (odd ratio [OR] = 2.045). The magnitude of the OR suggests a strong association between *CYP2C9*2* and *3 alleles and hypoglycemic events.

Discussion

There are three sources of diversity in a population: mutation, recombination, and gene immigration. Relocation into a population from other groups with varying genetic alleles is another cause of variability. The allele frequency in the resulting mixed population will be somewhere in the mid of its original value and the donor group's frequency.[12] Regarding the frequency distribution, the results of this study are related to Arici and Özhan[13] study. They reported percentages of 79.36%, 19.38%, and 13% for CYP2C9*1, *2 and *3 respectively among Turkish population. Another study found that the percentages of CYP2C9*1, *2, and *3 were 76.10, 16.10, and 7.80, respectively in Iran.[14] Considering the Saudi population, the percentages of CYP2C9*1, *2, and *3 were 79,2%, 11.7%, and 9.1% respectively.[15] Yousef *et al.* (2012) reported a percentages of (79.7 %) for CYP2C9*1, (13.5 %) for *CYP2C9*2* and (6.8 %) for *CYP2C9*3* in the Jordanian population.[16]

The influence of CYP2C9 on DMT2 was investigated and the results revealed that the carriers of the genotypes *1/*3 were the most beneficial patients for the combination of metformin/SU therapy as all the participants of the current study.[17] The results reflect these findings and the carriers of *1/*3 genotype (n=9) mean HbA1c concentration was 8.91% against those with the wild genotype (HbA1c= 9.23). The studies of glibenclamide pharmacokinetic showed that the median total area under the plasma concentration–time curve of glibenclamide in patients with the *1/*3 genotype was 267 percent of what it was in those with the CYP2C9*1/*1 genotype. The pharmacokinetics of glibenclamide were dramatically impacted by CYP2C9 genotypes, and glibenclamide oral clearance was reduced by 75% in CYP2C9*1/*3 carriers compared to *1/*1 carriers.[18] Suzuki *et al*., 2006 conducted compatible study with the current on Japanese DMT2 patients and found out that the decrease in the HbA1c was significantly higher ($P < 0.05$) among CYP2C9*1/*3 against the CYP2C9*1/*1 genotype carriers.[19] Koren & Koren, (2015) declared that the carriers of the wild and mutated alleles had similar HbA1C levels. The results showed there is no significant differences in mean FBS and FI between carriers of CYP2C9 variations.[20]

Saberi and his colleagues[21] observed a significant increase in retinopathy and nephropathy *CYP2C9*2/*2* andCYP2C9*2/*3* genotypes in DMT2 patients. This increase was due to the marked increase in micro-albuminuria and decrease in the glomerular filtration rate in 66.7% of the patient with *CYP2C9*2/*2* and *CYP2C9*2/*3* genotype. In contrast, the presence of wild type alleles (*1/*1, *1/*2 and *1/*3) protects the normal function of kidney in DMT2 patients. Although this study did not find significant differences, the incidence of neuropathy was higher in the carriers of the *CYP2C9*2* and *3 alleles than the wild allele.

Another study concluded that the neuropathy in wild allele carriers has lower frequency than CYP2C9 *1/*2 and that the carriers of heterozygous genotype for CYP2C9*2 have the highest neuropathy frequency.[22] In contrast, study presented by Koren and his colleagues[20] did not reveal any significant differences between the wild and *CYP2C9*2* and*3 alleles for *CYP2C9* gene, but the results of their study indicated that the frequency of DMT2 complications was more abundant in the wild allele carriers and consistent with current regarding nephropathy and retinopathy. Finally, the odd ratio calculation revealed that the neuropathy is more likely to occur in carriers of CYP2C9*2 and *3 DMT2 patients (OD= 1.832).

The association between the *CYP2C9* genotypes and enzyme activity was established in study conducted on 20 diabetic patients with severe hypoglycemia, after treatment with sulphonylurea they compared to a control group of 337 individuals with type 2 diabetes but with no history of severe hypoglycemia in a case-control study. The results revealed that *CYP2C9* genotypes *3/*3 and *2/*3 are prognostic of low enzyme activity and were more common in the hypoglycemic group than in the contrast groups (10% vs < 2%, respectively: OR= 5.2).[23] It is well documented that hypoglycemia is associated with Sulphonylureas in DMT2 patients. SU-induced hypoglycemia was detected in a large proportion of patients on a sulfonylureametformin combination.[24]In general and regardless any genetic variability influence. In addition, GB has a long action time and metabolites with hypoglycemic activity, leading to an increased risk for chronic hypoglycemia. Hypoglycemia caused by GB is more common in the elderly, those with irregular eating routines, and those with renal impairment.[25]

Conclusion

1. There is a strong association between hypoglycemia and CYP2C9 genotype. CYP2C9*2 and *3 enzyme variants are at increased risk of having hypoglycemic episodes.
2. Glibenclamide should be taken with caution In the Iraqi population. All patients on GB and others SUs should be taught on the signs and symptoms of hypoglycemia.

3. Patients with certain traits, such as renal failure and advanced age, may be predisposed to drug-induced hypoglycemia.
4. Patient evaluation is essential for identifying who are most at risk and avoiding problems.
5. Patients with T2DM who had variant CYP2C9 genotypes in this study responded better to GB medication than those who had the wild genotype.
6. The T2DM patients with variants CYP2C9*2 and *3 are at increases risk of having neuropathy and hypertension.

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Conflicts of interest

There are no conflicts of interest.

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