Pakistan J. Zool., pp 1-8, 2024. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20231226112338

A Cohort Study on Response of T2DM Patients to Oral Antidiabetics and their Association with *CYP2C9*3* Gene Polymorphism (rs1057910) in Khyber Pakhtunkhwa, Pakistan

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Article Information
Received 26 December 2023
Revised 04 January 2024
Accepted 11 January 2024
Available online 26 March 2024
(early access)

Authors' Contribution
IA conducted the study collected the data, performed the experimental work and wrote the manuscript. OM helped in data analysis. SS critically reviewed and facilitated in research article and designed the research project. MZ supervised the project

Key words
T2DM, Metformin, Glimepiride, *CYP2C9* gene rs1057910 and Responders

ABSTRACT

Type 2 diabetes mellitus (T2DM) is an important public health problem all over the world, affecting approximately 417 million adults which is expected to reach to 592 million by the year 2035 globally. Metformin, sulfonylureas (SFUs) are widely used oral anti-diabetic drugs metabolized by cytochrome P450 belonging to family 2 subfamily C member 9 (CYP2C9). CYP2C9 has shown better glycemic control and reduced the treatment failure rates compared to metformin and its combination therapy. To study the association of CY2PC9 gene variant rs1057910 in T2DM patients of Khyber Pakhtunkhwa (KP) origin, the cohort study was conducted for a period of 12 months including 150 patients receiving metformin, glimepiride and its combination therapy. Purposive sample technique was used for sample collection. Data collection was done by proper questionnaire. DNA was extracted from 200 µL of whole blood samples obtained from the patients by using the Wiz-Prep DNA extraction kit (Wiz-Prep no. W54100). Sanger sequencing was used for genetic analysis. Data analysis was done by using IBMS-KMU SPSS 23 version. The Hardy-Weinberg equilibrium for T2DM subjects was determined using an online HWE calculator. The rs1057910 polymorphism in CY2PC9 gene responsible for drug metabolism was analyzed which showed that our population has high frequency of major allele CC which confirmed that patients receiving metformin, glimepiride and its combination therapy were effective for glycemic control through-out in a 12 months cohort with a p value of <0.0001. The SNP rs1057910 in CYP2C9 was inconsistent with H.W equilibrium and shows disequilibrium in our population. The study found a modest but non-significant effect of the CYP2C9*3 rs1057910 polymorphism on susceptibility to type 2 diabetes in KP Pakistan, highlighting the need for further research on gene-environment and gene-gene interactions.

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INTRODUCTION

Globally, type 2 diabetes mellitus (T2DM) has become one of the most challenging chronic public health issue affecting 425 million people which accounts for at least 90% of all diabetes cases (Liu *et al*., 2010; Alwin *et al*., 2017; Ahluwalia *et al*., 2019). In Pakistan, the T_2DM prevalence was previously 11.7%, but it has now risen to 26.3% making it the third most diabetic country after India and China (Bowe *et al*., 2018; Iqbal *et al*., 2021). Sulfonylureas (SFUs) are the most extensively used among the oral anti-diabetic drugs which stimulate the beta cells to release insulin from beta cells of the pancreas which reduce the glucose level in the blood (Proks *et al*., 2002). However, the patients using these drugs are at risk of decreased glucose levels leading to hypoglycemia which has fatal consequences particularly renal impairment, stroke, heart attack (Brunetti and Kalabalik, 2012; van Dalem *et al*., 2016).

SFUs are metabolized by cytochrome P450 (CYP450) family enzymes which metabolize many other drugs. The cytochrome P450 enzyme CYP2C9 is primarily responsible for the oxidative metabolism of drugs with a narrow therapeutic index such as warfarin, tolbutamide and phenytoin, sulphonylureas, such as glimepiride, glibenclamide, and glipizide irbesartan and torasemide, as well as many anti-inflammatory (Zhou *et al*., 2010; Zanger and Schwab, 2013).

CYP450 enzymes are mostly released by liver cells although they are also present in the kidneys,

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0030-9923/2024/0001-0001 $ 9.00/0

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pancreas and gastrointestinal tract (Liu *et al*., 2023). *CYP2C9*2* (Arg144Cys) and *CYP2C9*3* (Ile359Leu) are two important genetic variants of the *CYP2C9* gene. Lower *CYP2C9* enzyme activity is due to substitutions in amino acids in the polypeptide (Yin *et al*., 2008). Patients who have same copy of CYP wild type (*CYP2C19*1/*1*) alleles are normal metabolizers, so the enzyme produced by it is having optimum activity. Heterozygous genotype (*CYP2C19* (**1/*2* and **1/*3*)), having one wild and the other mutant allele showing reduced function (**1/*2*) or one wild and one loss of function (*1/*3*) and homozygous genotypes having two copies of reduced-function alleles (**2/*2*) is an intermediate metabolizer. The individuals having two copies of loss of function alleles (homozygous (**3/*3*)) or with one copy of loss of function and the other with reduced function heterozygous genotype (**2/*3)* are poor metabolizers showing no or poor enzyme activity (Roosan and Sharma, 2021; Chamboko *et al*., 2023; Jan *et al*., 2023).

The inter-individual variation in response to the drugs is due to the genetic polymorphisms of drug metabolizing enzymes. The 13% of the clinically available drugs are metabolized by CPY2C 9 gene (Zanger and Schwab, 2013). According to Ahmed *et al*. (2020) approximately 20% of Pakistani population is having *CYP2C9* genotype that have at least one reduced function allele. So, the patients having this genotype may have poor response to the drugs metabolized by the CYP2C9 enzyme (Ahmed *et al*., 2020). Similarly, Jan *et al*. (2023) also reported that CYP2C9*2 allele was more frequent in T2DM patients from Pashtun population of Khyber-Pakhtunkhwa. Moreover, they also found that hypoglycemia was common in patients treated with SFUs. In Chinese population 12 variants of CYP2C9 that have been identified (Wang *et al*., 2014). In most ethnicities CYP2C9 1*, 2*and 3* alleles have been reported and individuals having 2* and 3* alleles of CYP2C9 have shown decreased function in metabolism (Kim *et al*., 2017). Because the various other alleles may also cause impaired metabolism which result in drug toxicity (Cario, 2016). Therefore, the dose drug may be advised according to the variant of CYPC29. Because very little information is available regarding pharmacogenomics of CYP 450 enzyme, the present study was conducted to evaluate the impact of the CYP2C9 polymorphism, rs1067910, on metformin, SFUs and its combination therapy response in a group of Pakistani patients from (KP) for the first time. Because the allele frequency is variable in different ethnicities, the following study is conducted with an aim to determine the mutant alleles and genotype frequencies of *CYPC29* gene in the population of KP, Pakistan.

MATERIALS AND METHODS

Subjects

The study technique was purposive sampling. A prospective cohort study was conducted for a period of 12 months from January 2022 to Jan 2023 in the Diabetes Hospital and Research Centre, Hayatabad, Peshawar, KP. Sample size was one hundred and fifty (with extra 10% as drop out), with open epi software for cohort study incidence rate of 95% CI, and with 5% margin of error.

Data was collected from the one hundred and fifty enrolled T2DM patients taking Sulfonylureas (Glimeperide) monotherapy, Bigunides (Metformin) monotherapy, and combination therapy. Patients with a clear clinical diagnosis of Type II Diabetes Mellitus T2DM, aged 30-70 years, receiving metformin and glimepiride for glycemic control with HbA1C levels greater 6.5% (48mmol/mol) were included in the study, whereas patients taking medications other than Sulfonylureas combination therapy of Sulfonylureas and Bigunides, and with other autoimmune disorders were excluded from the study. The patients were followed for 12 months.

SNP selection and genotyping

SNPs associated with T2D mellitus were identified in both the HapMap database and the National Center for Biotechnology Information (NCBI). To create the SNPs, the NCBI website was used, while longer SNP sequences were obtained from http://genome.ucsc.edu/. Primers were generated using http://frodo.wi.mit.edu/cgi-bin/primer3/primer3www.cgi, and their specificity was confirmed using BLAST against the human genome in the NCBI database.

DNA extraction from blood collection

Blood samples were collected at time of enrollment from 150 T2DM patients (base cohort), then after 4 months at each follow-up and patients were followed for 12 months. Venous blood samples were collected in 5ml EDTA tubes from for DNA isolation (Molecular genetic studies). The samples were taken and kept in the pharmacogenomics lab at Khyber Medical University in Peshawar at 20 °C.

DNA was extracted from blood samples obtained from the T2DM patients using the Wiz-Prep DNA extraction kit (Wiz-Prep no. W54100) according to the guidelines provided in the kit. The DNA quantification was done by using Nano drop in which the final concentration of DNA was adjusted to 5 ng/µL.

PCR amplification and Sanger sequencing

From Purified DNA, CYP2C9 region containing the selected variant (rs rs1057910) was targeted for

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sequencing. The final product was purified and send to Tsingke Biotecnology China for Sanger sequencing.

The Staeden pack-age and Finch TV vl.4 (Geospiza, Inc, Seattle, WA, united states) were used to find the accuracy of DNA sequences.

Statistical analysis

The collected information was entered into MS excel sheet and then exported to SPSS Version 23 for analysis. Chi-square test was applied and p values were determined for responders and non-responders. Online Hardy-Weinberg (H-W) equilibrium was applied CYP2C9 gene rs1057910 analysis.

RESULTS

In the 12 months prospective cohort study oral glycemic drugs metformin, glimepiride and combination therapy were used in T2DM patients. The rs1057910 polymorphism in CYP2C9 gene responsible for drug metabolism was analyzed which showed that our population has high frequency of major allele CC which confirmed that patients receiving metformin, glimepiride and its combination therapy were effective for glycemic control through-out in a 12 months cohort ($P < 0.0001$).

Table I. Distribution of genotypes among responders and non-responders to the drugs.

Cyp2c9 (rs1057910)	Genotypes (%)	Respond-ers (%)	Non-Re-sponders (%)	P value
CC	80 (53.3%)	51(68.91%)	29 (38.1%)	<0.001
CT	6 (4%)	3 (4%)	3 (3.94%)	
TT	64 (42.6)	20 (27%)	44 (57.9%)	
Total	150 (100%)	74(49.33%)	76 (50.6%)	

In the present study a total number of 150 samples of T2DM subjects were included. Chi-squared test was applied for genotypes frequency and p value in which drug response with Cyp2c9 rs1057910, major allele for Cyp2c9 rs1057910 was CC, minor allele was TT and mutant was CT. A total number of major allele for CC was 80 (53.3%) in which were 51(68.91%) responders and 29(38.1%) were non-responders (Table I). Mutant allele CT was 6 (4%) recorded in which 3(4%) were responders and were 3(3.94%) non-responders. While minor allele TT was 64 (42.6%) recorded in which 20(27%) were responders and 44(57.9%) were non-responders.

Hardy-weinberg equilibrium

The Hardy-Weinberg equilibrium for T2DM subjects were determined by online HWE calculator. CYP2C9 rs1057910 was not in consistent with Hardy-Weinberg equilibrium and disequilibrium as shown in Table II.

Table II. Hardy-Weinberg equilibrium for CYP2C9 rs1057910.

SNPs	Genotypes	Ob-served #	Expect-ed #	X^2 test value	P value
Cyp2c9 rs1057910	Homozygote reference	80	45.9	126.7	<0.0001
	Heterozygote	06	74.1		
	Homozygote variant	64	29.9		

Table III. Genotype and Allelic frequency T2DM patients for CYP2C9 rs1057910 with chi-square test.

CYP2C9 rs1057910 genotype and Allelic	No. (%)	P-value
CC	80 (53.3%)	0.001
CT	6 (4%)	
TT	64 (42.7%)	
C	166 (55.3%)	
T	134 (44.7%)	

Table IV. Drug response of metformin, glimepiride and its combination therapy in T2DM subjects with CYP2C9 rs1057910 with genotypes and allele frequency in responders and non-responders.

	Cyp2c9 rs1057910				
	Genotypes no.			Respond-ers no.	Non-respond-ers no.
Metformin	50	CC	29(58%)	17(70.8%)	12(46.2%)
		CT	2(4%)	1(4.2%)	1(3.8%)
		TT	19(38%)	6(25%)	13(50%)
		C	60(60%)	35(35%)	25(25%)
		T	40(40%)	13(13%)	27(27)
Glimepiride	50	CC	43(86%)	33(86.8%)	10(83.3)
		CT	2(4%)	1(2.6%)	1(8.3%)
		TT	5(10%)	4(10.5%)	1(8.3%)
		C	88(88%)	67(67%)	21(21%)
		T	12(12%)	9(9%)	3(3%)
Combined therapy	50	CC	8(16%)	1(8.3%)	7(18.4%)
		CT	2(4%)	1(8.3%)	1(2.6%)
		TT	40(80%)	10(83.3%)	30(78.9%)
		C	18(18%)	3(3%)	15(15%)
		T	82(82%)	21(21%)	61(61%)
Total No: 150					

Sanger sequencing was used for the analysis of genotype and allelic frequency of T2DM patients for CYP2C9 rs1057910 in KP. Major allele CC was 80(53.3%), mutant allele was 6(4%) and minor allele was

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64(42.7%). While allele frequency was for Major allele was 166(55.3%) and for minor allele were 134(44.7%) with a p value 0.001 (Table III).

In Table IV, the subjects receiving metformin had major genotype CC with (70.8%) in responders. While lowest genotypic percentage was recorded for mutant genotype CT with (4.2%) in responders.In subjects receiving glimepiride had higher percentage of major genotype CC with (86.8%) in responders. while lowest genotype percentage was that of mutant genotype CT with (2.6%). The subject receiving combined therapy had shown highest percentage of minor genotype TT with (78.9%) in non-responders,while lowest in mutant genotype with (2.6%) in non-responders.

Genotypic analysis of Cyp2c9 rs1057910

A total of 150 samples of T2DM subjects were sent to China for sequencing. The results were determined by finch TV. The homozygous (TT) gives one peak, also homozygous (CC) gives one peak while heterozygous (CT) gives two peaks (Fig. 1).

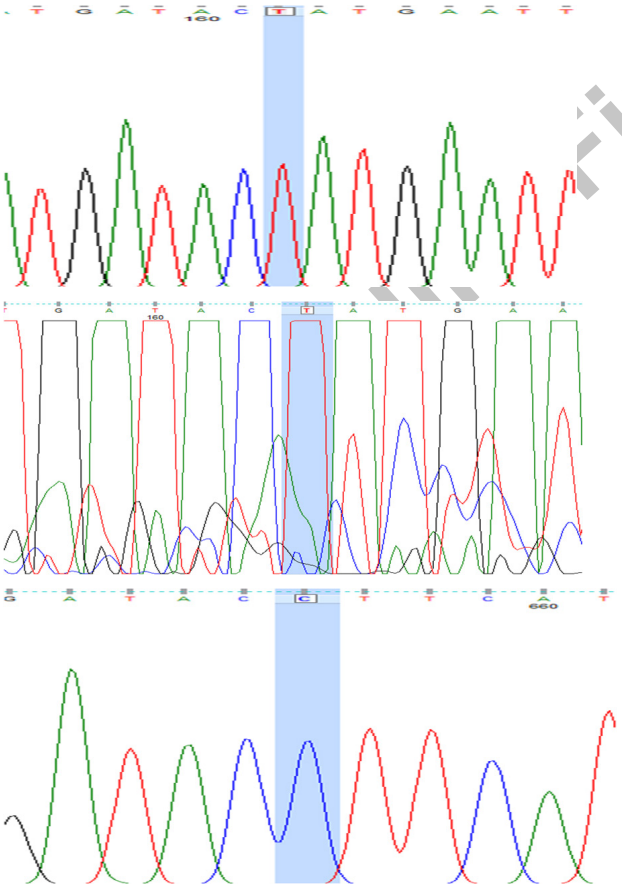

Fig. 1. Highlighted area of image shows homozygosity and heterozygosity of Cyp2c9 rs1057910.

DISCUSSION

In the present prospective cohort study CYP2C9 genetic polymorphisms were found to be associated with good glycemic control in patients treated with metformin monotherapy, glimepiride and their combination therapy. Metformin only has a glucoregulatory effect which reduce the glucose synthesis and decreases the insulin resistance by 20–30% (Powers and D'Alessio, 2011).

Combination therapy including SUs and metformin is mostly prescribed for treating type 2 diabetes mellitus. It is often recommended for treating various genotypes which have shown variation in the efficacy of SUs. Using the ADA-recommended cutoff of 130 mg/dl for managed fasting plasma glucose, a significant association between the genotype and diabetes management status was discovered (Goldman-Levine, 2011; Castelán-Martínez *et al*., 2018).

CYP2C9 genetic polymorphisms may also have an impact on glimepiride metabolism. In order to determine whether type 2 diabetic patients with various CYP2C9 genotypes would respond to glimepiride, metformin and its combination therapy this study examined the efficacy, safety, and pharmacokinetics of glimepiride, metformin, and their combination therapy.

The rs1057910 polymorphism in CYP2C9 gene responsible for drug metabolism was analyzed which showed that our population has high frequency of major allele CC which confirmed that patients receiving metformin, glimepiride and its combination therapy were effective for glycemic control through-out in a 12 months cohort with a p value of <0.0001. The Hardy-Weinberg equilibrium for T2DM subjects were determined by online HWE calculator. CYP2C9 rs1057910 was not in consistency with Hardy-Weinberg equilibrium and disequilibrium showed disequilibrium with a P = <0.0001 in Pakistani KP population.

Similarly, Suzuki *et al*. (2006) reported that among the polymorphisms of CYP2C9 gene in Japanese population showed CYP2C9*1/*3 improved HbA1c to greater extent in type 2 diabetes patients treated with glimepiride but one of the female subject reported increases in body weight when treated over long periods. The stronger effect of this drug might be due to less hydroxylation activity of the glimepiride that caused an increased plasma level in the blood of the patients with CYP2C9*1/*3 polymorphisms. These findings align with previously reported pharmacokinetic data that demonstrated a significant impact of CYP2C9 genetic polymorphisms on the pharmacokinetics of glimepiride in healthy individuals (Kirchheiner *et al*., 2005).

Subjects carrying the CYP2C9*3 enzyme may

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experience a stronger pharmacological effect from high concentrations of glimepiride. Response to glimepiride may also be affected by the duration of diabetes and prior treatment with oral antidiabetic agents (Soldin *et al*., 2011). According to (Salam *et al*., 2014) only 9% of patients had the double CYP2C9 genotype (CYP2C9*2/*3), while the wild type nucleotide sequence variations (CYP2C9*1/*1), the CYP2C9*2 allele, and the CYP2C9*3 allele were all present in 53% of patient populations as recessive homozygotes.

The similar case-control study conducted by Jan *et al*. (2023) to find the association of the sulphonylurea-Induced hypoglycaemia (SIH) with CYP2C9 gene in T2DM patients in KP revealed that the patients with SIH showed higher frequency of low-activity allele CYP2C9*2 (CYP2C9*1/*2 CYP2C9*2/*2) compared to the control group. These results are in alignment with our study which also showed the SIH in T2DM patients with similar alleles.

Another case control study conducted by Muhammad *et al*. (2020) to find the variants of CYP2C9 gene in diabetic patients treated with SFUs found that CYP2C9*1/*2, CYP2C9*1/*3 were found in control while CYP2C9*3/*3, genotype was found in the cases which support our result that the mutant alleles are linked to the hypoglycemia as well as poor blood sugar control. Moreover, they also reported that CYP2C9*61 in the heterozygous state was more frequent in the diabetic patients.

According to another study the rs1799853 variant in *CYP2C9* gene in T2DM patients showed that the 90.9% of the patients treated with SFUs were having wild type (CC) genotype while mutant heterozygous (CT) genotype were present only in 9.1%. But the wild type genotype showed uncontrolled HbA1C and fasting blood glucose (Perwitasari *et al*., 2021). Similarly in our study it was found that major allele for CC for rs1057910 was 80 (53.3%) of the patients in which 51 (68.91%) responded to the metformin, glimepiride and combination therapy of both of these drugs while 29 (38.1%) did not responded effectively. The difference in results may be attributed to the different SNPs present in the patients which responded differently to the drugs. Hence it supports the idea that the variation in response to the drugs is due to variation in genotypes.

Holstein *et al*. (2012) reported slightly higher rates of the wild genotype CYP2C9*1/*1 (65%, 66.5%, and 65%) in their three age groups. Our study also supports their discovery that all patients experienced hypoglycemia. According to Salam *et al*. (2014) the frequency of genotypes found in the previous study was similar to ours, with the CYP2C9*1/*2 genotype occurring at a frequency very close to ours (20%, 18.4%, and 19.6%), a lower frequency of the CYP2C9*1/*3 genotype (5%, 11.6%, and 11.5%), a lower frequency of the CYP2C9*2/*3 genotype (5%, 1.5%, and 1.7%), and a frequency very close to ours for the CYP2C9*2/*2 genotype (0%, 1.5%, and 1.8%) and CYP2C9*3/*3 genotype (5%, 0.6%, and 0.4%). Our findings are also same with the findings of Salam *et al*. (2014) showing study most of the subjects were ranged in the age group of 14 (9%) in 30-40 years, 63 (42 %) were in the range of 41-50 years, 49(33%) were in the range of 51-60 years and 24 (16%) were in the range of 61-70 years in both genders. They found that *CYP2C9*2/*3* genotype in carriers is responsible for good glycemic control and consequently prolonged half-life of the glimepiride.

Weise *et al*. (2010), evaluated the prevalence of CYP450 gene variants in 101 individuals with type 2 diabetes, the following genotypes were identified: CYP2C9**2 (20%), CYP2C9**3 (22%), as well as CYP2C9**2 (24%) and CYP2C9**3 (21%) in 102 non-diabetics. This analysis demonstrated significant resemblances in genotype frequencies to those observed in our study.

Holstein *et al*. (2012) found that those using sulfonylureas with the CYP2C9*3/*3 or CYP2C9*2/*3 genotype were 5.2 times more likely to experience severe hypoglycemia events than those with the other CYP2C9 genotype groups. They concluded that CYP2C9 genotypes suggestive of poor enzyme activity (CYP2C9*3/*3 and CYP2C9*2/*3) should be considered as one but not the main risk factor for severe hypoglycemia resulting from therapy with sulfonylurea oral hypoglycemic medications). Holstein *et al*. (2012) have also reported similar type of results.

According to Kirchheiner *et al*. (2005) CYP2C9 single nucleotide polymorphism considerably effect glimepiride pharmacokinetics in strong healthy individuals. The result is support by previous pharmacokinetic data. Soldin *et al*. (2011) glimepiride drug response is effected by diabetes period and other anti-diabetic drugs. Patients having strong CYP2C9*3 enzyme having raised levels of glimepiride drug. According to Salam *et al*. (2014), The occurrence of the CYP2C9 genotype revealed that only 9% of patient populations were double heterozygous (CYP2C9*2/*3), 20% were mutant alleles for the CYP2C9*2 gene mutation, 18% were mutant alleles for the CYP2C9*3 allele, and 53% of patient populations were homozygous for the wild type allele (CYP2C9*1/*1). Our findings are also in consistency with the findings of Salam *et al*. (2014).

In contrast to the present research, according to Holstein *et al*. (2012), the frequency of major allele CC) of CYP2C9*1/* was greater among three different age groups in the three different ages (64, 65, and 66 years). Our findings are also in favor with the findings of

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Holstein *et al*. (2012) who has recorded hypoglycemia in all patients Salam *et al*. (2014) found that the frequency of the CYP2C9*1/*2 and CYP2C9*2/*2 genotype was very similar to ours while that of the CYP2C9*1/*3 genotype was lower and that of the CYP2C9*2/*3 and CYP2C9*3/*3 genotype was higher. In the present study most of the subjects were ranged in the age group of 14 (9%) in 30-40 years, 63 (42 %) were in the range of 41-50 years, 49(33%) were in the range of 51-60 years and 24 (16%) were in the range of 61-70 years in both genders.

Weise *et al*. (2010) with the genotypes CYP2C9**3* (21%), CYP2C9**3* (22%), and *CYP2C9**2 (24%) respectively while in healthy individuals *CYP2C9**2 were 20%. Notably, these frequency findings bear a striking resemblance to our own study.

Similar to our results, those diabetic patients taking sulfonylureas having genotypes CYP2C9*3/*3 or CYP2C9*2/*3 in CYP2C9 gene experienced severe hypoglycemia. The reason explained was that polymorphisms of CYP2C9*3/*3 and CYP2C9*2/*3 was a strong drug metabolizers causing hypoglycemia while in minor form of CYP2C9 gene (TT) allele prevalence low enzymatic action of CYP2C9 gene was observed (Holstein *et al*., 2012). Results determined by finch TV. Thus, knowledge of a patient's *CYP2C9* genotype may aid in reducing the likelihood of side effects during the initial phase of glimepiride therapy, in conjunction with clinical data regarding the duration of diabetes and previous SU therapies. The importance of genes encoding members of the cytochrome P450 CYP2C9 rs1057910 subfamily in Pakhtuns' susceptibility to T2DM has not yet been investigated in any studies.

CONCLUSION

Our results suggest a modest but statistically non-significant effect of the CYP2C9*3 rs1057910 in susceptibility to T2DM, particularly in population of KP, Pakistan. More work will be required for future association studies, especially those which are properly powered, effectively control for confounding factors, Moreover, gene–environment and gene–gene interactions should also be taking into consideration for future studies. The goal of this study was to determine the effects of the CYP2C9*3 rs1057910 polymorphism on the efficacy, safety, and pharmacokinetics of metformin, glimepiride and its combination therapy in type 2 diabetes patients in KP, Pakistan.

ACKNOWLEDGMENT

Authors express their gratitude to the leadership teams at Peshawar's Diabetes Hospital and Research Centre and the Institute of Pharmaceutical Sciences at Khyber Medical University, Peshawar.

Funding

The study received no external funds.

IRB approval

Institutional BioEthical Committee of Islamia College Peshawar, Pakistan approved the project.

Ethical approval

Before taking the consent from the enrolled patients, the ethical approval of the study was taken from the ethical review committee of Islamia College Peshawar and Khyber medical university.

Statement of conflict of interest

The authors have declared no conflict of interes.

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