CODEN [USA]: IAJPBB ISSN: 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES
http://doi.org/10.5281/zenodo.2642268

Available online at: http://www.iajps.com Research Article

Developing Amplification Refractory Mutation System –PCR system for rapid detection of CYP2C9*2 (rs1799853) C>T gene variation in T2D patients of Tabuk-Saudi Arabia

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Article Received: February 2019 **Accepted:** March 2019 **Published:** April 2019

Abstract:

Background: *The CYP2C9 is a drug-metabolizing enzyme and the second expressed CYP isoforms in hepatocytes. Single nucleotide polymorphisms (SNPs) influence the catalytic activity of* CYP2C9.*The* CYP2C9*2 *(rs1799853) is an important SNP in CYP2C9 gene results in replacement of Arginine144 to Cysteine. This SNP has been reported to reduce the activity of CYP2C9. Therefore the aim of this study was to establish a rapid and sensitive molecular based assay for the detection of CYP2C9*2 (rs1799853) C>T gene variation in T2D patients.*

Methodology: *This study was conducted on 25 clinically confirmed cases of T2D patients and 25 healthy controls. DNA extraction was performed with the Qiagen Kit. The amplification-refractory mutation system (ARMS)-PCR was optimized to detect CYP2C9*2 (rs1799853) *2 C>T gene variation.*

Results: *This study was conducted on 50 samples (25 were T2D patients and 25 were healthy controls). ARMS – PCR was optimized to detect CYP2C9*2 (rs1799853) *2 C>T gene variation in T2D patients and healthy controls.*

Conclusion: *ARMS-PCR system for detection of (rs1799853) gene variation was successfully optimized. The assay proved to be fast, accurate, simple and economical that does not require any special equipment other than a thermocycler. The ARMS-PCR system can be used as a potential molecular tool for the detection of CYP2C9 gene variations in T2Ds.*

Key Words

*Type 2 diabetes (T2D) - CYP2C9*2 (rs1799853) *2 C>T (Arg>Cys) - amplification-refractory mutation system (ARMS)-PCR*

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Please cite this article in press Imadeldin Elfaki et al., ***Developing Amplification Refractory Mutation System – PCR System for Rapid Detection of CYP2c9*2 (RS1799853) c>t Gene Variation in T2d Patients of Tabuk-Saudi Arabia.,*** *Indo Am. J. P. Sci, 2019; 06(04).*

INTRODUCTION:

The cytochromes P450s are heme-containing monooxygenases[1]. They constitute a large superfamily of enzymes widely distributed in animals, plants, fungi and prokaryotes [2]. The cytochromes P450s need electrons for their activity. The electrons donor is the NADPH catalyzed by a cytochrome P450 reductase [2]. They are expressed endoplasmic mitochondria and cell surface and present in hepatocytes, small intestine and kidney[1]. In humans, the cytochrome P450s have been arranged into 18 families and 43 subfamilies[1]. Cytochromep450s are metabolizing Xenobiotic and Endogenous Substrates[3]. Examples of the endogenous substrates include steroids and retinoic acid[3]. Exogenous substrate of Cytochrome P450s include carciongens and about 80% of all drugs[4]. Cytochrome P450 2C9 (CYP2C9) has very important roles in xenobiotic metabolism (15-20% of all drugs) and some endogenous compounds such as melatonin and arachidonic acid [5]. It is the second main CYP isoform present in the liver cells[5]. Adverse drug reactions have been associated with gene variations of CYP2C9[4]. For instance, the rs1799853 and rs1057910 are result in CYP2C9 with poor catalytic activity [6].

The prevalence of Diabetes Mellitus (DM) is increasing all over the world and become one of the major health concerns[7]. It is estimated that in the year 2013 there was 382 million people with DM and expected to increase to more than 590 million by the year 2035[7]. Saudi Arabia has been ranked by WHO as number two in Middle East, and number seven in world in term of the rate of DM[8]. Mainly, there are two types of Diabetes. Type 1 diabetes (T1D) which occurs in childhood, and by destruction of pancreatic Beta cells[9], and can be regarded as an autoimmune disease in addition to genetic, environmental and dietary risk factors[7, 10]. T2D constitutes majority (80-90%) of all cases of MD[11]. T2D is developed by the interaction between environmental and genetic factors[12]. T2D is characterized by insulin resistance and relative pancreatic beta cell dysfunction[7]. Insulin resistance is defined as a reduced cell or tissue response to the stimulation by insulin. It is initiated in the insulin sensitive tissues such as liver, muscles, and adipose tissues [13]. Risk factors for T2D include genetic, obesity, physical inactivity, and others[7]. Genome-wide association studies (GWASs) have revealed the association of certain genes with susceptibility to T2D and other diseases [1, 14-18]. Moreover, polymorphisms of insulin signaling genes and others have been associated of metabolic syndromes in certain populations [19, 20]. In the present study, we have optimized an the ARMS-PCR protocol for the detection of an important single nucleotide polymorphism SNP (rs1799853) of the drug-metabolizing CYP2C9 gene in Tabuk population.

MATERIALS AND METHODS:

Study Population: In order optimize rs1799853 gene variation in T2D patients; we selected 50 specimens among which 25 were clinically confirmed cases of T2D and 25 healthy controls.

Inclusion criteria: The study included 25 clinically confirmed cases of T2D patients and 25 healthy controls. All participants were of Saudi origin.

Exclusion Criteria: Patients with any other disease. Non-Saudi Arabian were excluded.

Sample collection: **A**fter obtaining a written informed consent form as well as assessing all Laboratory findings, a 3ml sample of peripheral blood was collected by venipuncture in EDTA tubes from T2D patients and healthy controls in EDTA tube.

Genomic DNA extraction:

Genomic DNA was extracted from the peripheral blood of clinically confirmed cases of T2D patients and healthy controls using QIAGEN DNeasy Blood & Tissue Kit (Cat No-69504) as per the manufactures instructions. The DNA quality and yield was assessed with Nanodrop (optical density) and 1% agarose gel electrophoresis. The DNA was isolated from T2D patients and controls was dissolved in nuclease-free water, and stored at 4°C until use.

Genotyping for rs1799853 with ARMS-PCR

ARMS-PCR primers were designed using Primer3 software as shown table 1. The ARMS-PCR primers for rs1799853 gene were performed in a reaction volume of 25µL containing template DNA (50ng). The cocktail was prepared for five samples as shown in Table 2. F1-0.25µL, R -0.25µL, F2-0.25µL, R -0.25µL of 25pmol of each primers and 5µL from 2X DreamTaq green Master Mix (Thermo Scientific, USA). The final volume of 25 µL was brought with nuclease free ddH_2O. Finally, 2-3µl of DNA was added from each T2D patient.

Table 1: ARMS primers for rs1799853

Direction	Primer sequence	PCR product size	AT
Outer primer FO	5- CTGTCTTGGGGATGGGGAGG -3	544bp	60
Outer primer RO	5-CCGCAAAGTTCAGGAGAACATG-3		
Inner primer FI G	5-GGAAGAGGAGCATTGAGGACC-3	323bp	
Inner primer RI A	5-CGGGCTTCCTCTTGAACACA-3	259bp	

Table 2: Preparation of PCR cocktail for rs1799853

Reagent	1x	5 x
PCR master mix	5ul	25 ul
Forward FO	0.25 ul	1.25 ul
Reverse RO	0.25 ul	1.25 ul
Forward FI	0.25 ul	1.25 ul
Reverse RI	0.25 ul	1.25 ul
Nuclease free water	17.00 ul	85ul
Total volume	**23ul**	**115ul**
Add sample		2ul
Total reaction volume		**25ul**

Thermocycling conditions:

The amplification conditions used were at 94 ºC for 10 minutes followed by 40 cycles of 94ºC for 35sec, 59 ºC for 40 sec, 72 ºC for 43 sec followed by the final extension at 72 ºC for 10 minutes.

Gel electrophoresis:

The amplification products were separated by electrophoresis through 2% agarose gel stained with 0.5µg/mL ethidium bromide and visualized on a UV transilluminator.

Statistical analysis:

All statistical analyses were performed using Graph Pad Prism 6.0 or SPSS 16.0.

Results

Study Population:

All demographic features of the subjects are shown in (Table 3). This population-based case–control study was done on 25 T2D patients and 25 healthy controls with no history of any type of disease.

Table3: Baseline Characteristics of T2D Patients

	T2D patients		Controls	
Number of subjects	N=25	%	N=25	%
Males	15	60%	17	68
Females	10	40%	8	32
Age >30	19	76%	14	56
Age <30	06	24%	11	44

Figure1: Nanodrop analysis of genomic DNA extracted from Healthy control

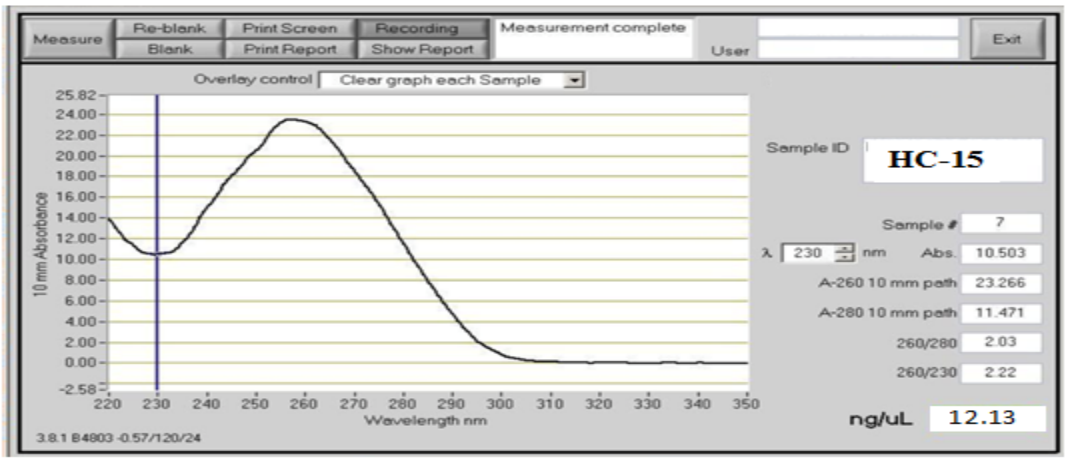
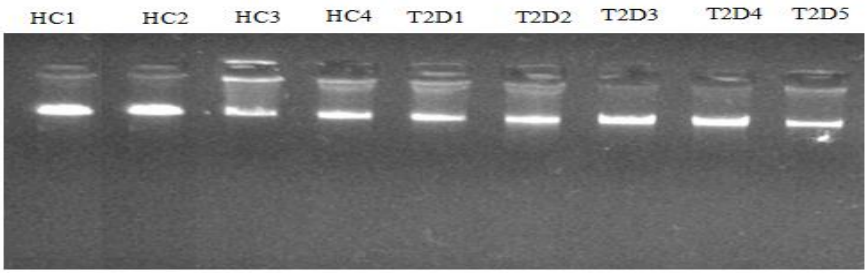


Figure1:Analysis of genomic DNA extracted from T2D cases and healthy controls: The Quality and integrity of DNA were checked at 260 nm by NanoDrop™ (Thermo Scientific, USA)

Figure 2: 1% gel electrophoresis of genomic DNA extracted from T2D patients and healthy controls



The extracted DNA was assessed by running in 1% gel electrophoresis.

Optimization of ARMS primers for rs1799853 gene variation: Primers FO and RO flank the exon 3 of the CYP2C9 gene, resulting in a band of 544 bp to act as a control for DNA quality and quantity. Primers Fwt and RO amplify a wild-type allele (C allele), generating a band of 323 bp, and primers FO and Rmt generate a band of 259 bp from the mutant allele, T allele (Figure 3).

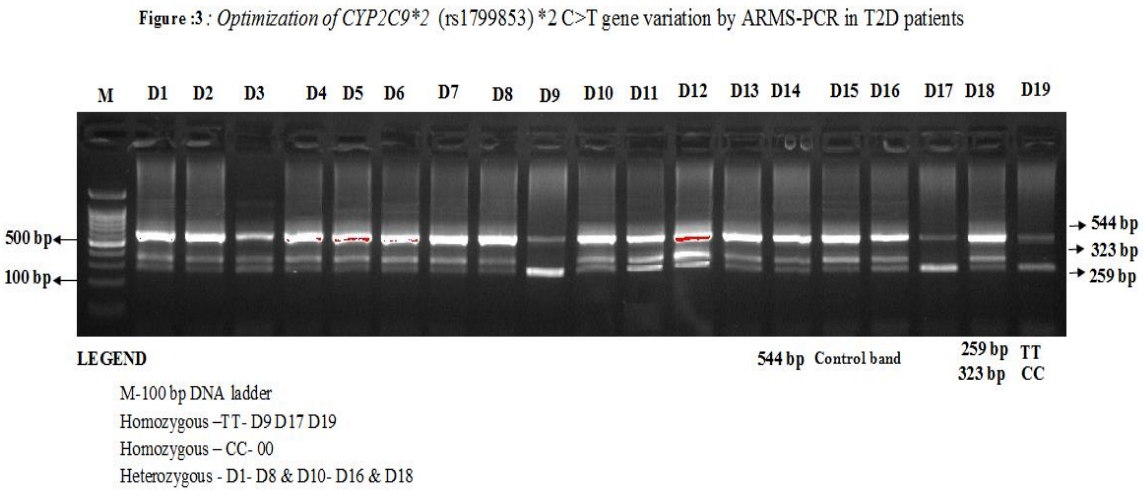


Figure 3: The ARMS-PCR amplification products were separated by electrophoresis through 2% agarose gel stained with 0.5µg/mL ethidium bromide and visualized on a UV transilluminator.

DISCUSSION:

DM is a growing health problem all over the world in become a global epidemic. It is expected that high prevalence rate of DM in Middle East. DM is one of the big health problems facing Saudi Arabia [21]. T2D is a metabolic disease characterized by hyperglycemia resulting from pancreatic dysfunction of the beta cells and impaired insulin action [22]. T2D representing the majority of DM cases accounting for about 90%. This percentage may be increase in the year 2030[23]. T2D is developed by the combination of genetic and environmental risk factors [24]. It is well-established that modifying the risk factors such as heathy diet, regular exercise, reducing body wait prevents or delay the onset of T2D[25]. Sulfonylureas are an important component in the treatment of T2D [26]. It is classified as an insulin secretagogs [26]. They are usually prescribed when the normal blood glucose level is not reached with metformin only [26]. The CytochromeP450s are large superfamily of enzymes involved in metabolism of endogenous substrates and xenobiotics including about 80% of drugs[4]. CYP2C9 is the expressed in the liver and has the second highest expression level after CYP3A4 in the liver[6]. It metabolizes about 20% of the drugs. Examples of the drugs that are metabolized by the CYP2C9 are Phenytoin (Antiepileptic), losartan (antihypertensive), Tamoxifen (Anti-estrogen), Fluvastatin (inhibits 3-hydroxy-3-methylglutaryl coenzyme A reductase), Diclofenac, Ibuprofen, Warfarin (Anticoagulant), the Sulfonylurea drugs (hypoglycemic) such as the Glibenclamide, Glipizide and Tolbutamide[6].

The CYP2C9 gene is very polymorphic, and these polymorphisms affect the enzyme catalytic activity and hence the metabolism of the drugs that are substrates of CYP2C9. Individuals with the CYP2C9 are at risk of adverse drug events in particular with the substrates exhibit a narrow therapeutic index such as the anticoagulant drug, warfarin, and the hypoglycemic agent's glipizide, and tolbutamide[5, 27]. Two important SNPs have been reported in

CYP2C9 gene and lower its catalytic activity, the rs1799853 that result in substitution of the Arginine 144 to Cysteine, and rs1057910 that result in the substitution of the Isoleucine 359 to Leucine [5, 27]. Individuals with these two SNPs have CYP2C9 with reduced catalytic activity, and therefore when treated with anti-coagulant warfarin may develop bleeding complications. They also at the risk of hypoglycemia when treated with the glucose lowering drugs such as glipizide and tolbutamide [5]. Furthermore, gene polymorphisms of Cytochromep450 have been associated with T2D and other diseases [5, 16, 28].

In the present study, we successfully optimized tetra ARMS-PCR for the detection of SNP rs1799853. The PCR has been used successfully in diagnosis of different infectious diseases [29, 30]. Genotyping of SNPs has been applied in various genetic contexts [16-18]. Tetra-primer amplification refractory mutation system (T-ARMS) polymerase chain reaction (PCR) is reported as a prominent assay for SNP genotyping [31]. It is a rapid and inexpensive, accurate tool for determining SNPs in which only the PCR amplification followed by the agarose gel electrophoresis are required. In the T-ARMS, four primers are used, the external forward (OF), external reverse (OR), internal forward (IF) and internal reverse (IR) primers [32]. The mixture of OF/OR primers produce the outer band of the SNP gene and used as a control for the PCR, whereas the mixtures of the IF/OR and OF/IR primers generates allele-specific bands of the SNP under the investigation [32]. The internal primers are annealed in different positions from the external primers producing bands with different sizes for each allele. These different sized bands are easily separable in gel electrophoresis and the genotyping is according performed [32].

CONCLUSION:

This study successfully developed the ARMS-PCR technique for the detection of rs1799853 gene polymorphism in T2D population of Taluk. The assay proved to be fast, accurate, simple and inexpensive and required simple equipment such thermocycler. The ARMS-PCR system could be used as a potential molecular tool for the detection of CytochromeP450 single nucleotide polymorphism.

Competing interests:

The authors declare that they have no competing interests.

Authors' contributions:

IE and RM designed the study. IE performed the experiments; IE and RM wrote and approved the manuscript.

ACKNOWLEDGMENTS:

- We are most thankful to the subjects for their contributions in this study.
- This project is funded by the DEANSHIP OF SCIENTIFIC RESEARCH, UNIVERSITY OF TABUK, grant number (**No.0121-1439-S**) for IE.

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