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The Frequency of *rs1799889* in Plasminogen Activator Inhibitor Type-1 Gene in Sudanese Type 2 Diabetic Patients, Gezira State, Sudan, 2020-2021

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Abstract

Background and Objectives: The cornerstone of the regulation fibrinolytic system is plasminogen activator inhibitor type-1. The 4G/5G polymorphism in the *PAI-1* gene is a key genetic predictor of increased plasma levels which is the most polymorphism associated with cardiovascular complications. The 4G carriers have six times higher *PAI-1* levels than 5G carriers leading to an increase in the level of plasma inhibitor by about 25% more than 5G allele (wide type). Type 2 diabetes presents symptoms of hypercoagulability and hypofibrinolytic system that lead to contribute in the atherothrombosis and then the myocardial infarction (MI). These findings supported the hypothesis that there is a link between diabetes patients and this SNP. There is no data about the prevalence of this allele in Sudanese diabetic patients with type 2 and the allele differs in prevalence according to ethnicity, for these reasons, the aim of this study was to determine the allele and genotype frequency of the *rs1799889* among Sudanese T2DM patients. **Methods:** A case-control study was conducted using 70 diagnosed diabetes type 2 patients and 50 healthy individuals as the control group. AS-PCR technique was used to genotype the *rs1799889*, and the allelic frequency was calculated according to Hardy-Weinberg equilibrium. Allelic frequencies were assessed using gene counting (SNP-STAT software V. Release 3.13), and genotypes were scored. **Results:** The result showed that 4G allele frequency was 28% among Sudanese diabetic patients without statistical difference when compared with con-

trol group (P-value = 0.998) but, high when compared with other studies in African population 13% and very low when compared with white and Indian populations studies. **Conclusion:** By this study, the allele frequency was higher in Sudanese diabetic patients with type 2, and also we need another study to evaluate the effect of this polymorphism in thrombophilic complications in Sudanese diabetic patients with type 2.

Keywords

4G/5G Polymorphism, Diabetic Mellitus Type 2 and *PAI-1* Gene

1. Introduction and Background

Type 2 diabetes is a progressive condition in which, the cells ability to secrete insulin deteriorates over time as a result of the patient's age and diabetes duration, eventually failing to fulfill body requirements [1]. The term diabetes describes a group of metabolic disorders characterized and identified by the presence of hyperglycemia in the absence of treatment. The heterogeneous aetio-pathology includes defects in insulin secretion, insulin action, or both, and disturbances of carbohydrate, fat and protein metabolism [2]. It is among the top 10 causes of death in adults and was estimated to have caused 4 million deaths globally in 2017 [3]. T2DM can lead to microvascular (neuropathy, nephropathy and retinopathy) and macrovascular complications (coronary artery disease, stroke and peripheral vascular disease) which are associated with an increased risk of atherosclerosis. Thrombosis is a major cause of death in 80% of patients with diabetes. Of these deaths, more than 75% are due to cardiovascular complications, while the remaining is due to cerebrovascular events and peripheral vascular complications [4] [5]. Chronic hyperglycemia is not the only cause of these complications; these ischemic events are also associated with hyperactivity of platelets, abnormal activation of coagulation proteins, abnormal endothelial function and hypo-fibrinolysis [6]. Also, patients with T2DM have presented hypercoagulability and hypofibrinolysis lead to imbalance between coagulation and fibrinolysis mainly contributes to excess fibrin deposition in the vascular and then results in the pathogenesis of atherothrombosis, the latter one which is a negligible cause of death and high healthcare costs. So, diabetes and its related complications will remain serious challenges for health authorities of Sudan.

Impaired fibrinolysis or hypofibrinolysis, caused by hereditary or environmental factors origin, may be linked to the development of thrombosis both venous and arterial, and has been associated with atherosclerosis, obesity, diabetes and hyperlipidemia. Researchers have looked at global assays for biomarkers of fibrinolysis capacity that would indicate reduced fibrinolysis, such as elevated plasminogen activator inhibitor-1 (*PAI-1*) which is important hypofibrinolytic marker, alpha-2-antiplasmin (Plasmin Inhibitor), and thrombin activated fibri-

nolysis inhibitor (TAFI) or changes in active t-PA levels. The incomplete dissolution of clots may also lead to smaller pieces of the clot breaking off and travelling down the vasculature, which can result in an embolism when a smaller blood vessel downstream is blocked. Therefore, the ability of the body to dissolve or break down clot plays an important role in CVD [7].

The *PAI-1* gene spans 12.3 kb and 50-kDa glycoprotein. It is located on human chromosome 7q21.3-22, with eight introns and nine exons [8]. It is the primary physiological inhibitor of tissue-type plasminogen activator (tPA) in the fibrinolytic system, which generates active plasmin from plasminogen and subsequently cleaves fibrin and then produced fibrin degradation product (FBPs) and other products like D-dimer. It belongs to the serine protease inhibitor (serpin) family. Increased level of plasma *PAI-1* expression causes impaired function of fibrinolytic system in patients with thrombotic illness [9]. There are numerous polymorphisms in *PAI-1* gene, that cause the rise in *PAI-1* level documented earlier by various researchers study.

The most commonly described are the following: *PAI-1* gene (*rs1799889*)-675 4G/5G insertion/deletion polymorphism [10], is considered an independent factor of cardiovascular risk in type 2 diabetes mellitus [4]; G-A substitution at position-844 (*rs2227631*) [11] [12]; c.43G < A (p.A15T, *rs6092*) [13], and p.I17V *rs 6090*; two (CA)n repeat polymorphisms, one in the promoter and one in intron 4 and Hind III RFLP13. In MI patients and control subjects, higher plasma *PAI-1* levels were significantly associated with the smaller alleles of the intron 4 (CA)n repeat. Studies of the Hind III, which is in strong linkage disequilibrium with the (CA)n repeat, demonstrated that plasma *PAI-1* levels were lower in both control subjects and MI patients with an additional Hind III site. Studies of the 5G/4G polymorphism have shown a higher plasma *PAI-1* level and activity in subjects with the 4G than with the 5G allele in MI patients and type 2 diabetic patients when compared with healthy control subjects.

The insertion/deletion of one guanidine nucleutide at position 675 in the promoter region known as 4G/5G polymorphism (*rs1799889*) is the most well-known [14].This polymorphism results in two alleles with four or five guanidines consecutive (4G allele and 5G allele), which regulate the *PAI-1* concentrations differently [15].

Plasma concentrations of *PAI-1* are approximately 25% greater in subjects who are homozygous for the 4G allele than in subjects who are homozygous for the 5G allele because; both 4G and 5G alleles bind with transcription-regulation proteins, the 5G allele bind repressor proteins in addition to transcription proteins but, the 4G allele just binds with transcription proteins without repressor proteins that lead to more and more transcribe of *PAI-1*. The 4G allele transcribe of *PAI-1* six times more than 5G allele [16]. Specific genotypes such as 4G/5G polymorphism of *PAI-1* gene interact with metabolic syndrome factors (plasma triglycerides, high-density lipoprotein, plasma insulin, and visceral fat; circumference width, and finally body mass index) to raise *PAI-1* plasma concentrations, potentially increasing the risk of intravascular thrombosis and re-

current myocardial infarction (MI) in young diabetics [16] [17].

Adipocytes, hepatocytes, endothelial cells, vascular and non-vascular smooth muscle cells all produce *PAI-1*. Also produced following platelet activation, may also be synthesized and stored by platelets. Platelet alpha granules contain about 90% of circulating *PAI-1* level, which leads to slows clot lysis by forming complexes with t-PA and u-PA, preventing these plasminogen activators from binding to plasminogen and generating plasmin [18]. When platelets become active, *PAI-1* is released into the bloodstream, increasing *PAI-1* circulation [19]. *PAI-1* exists in three molecular states in the blood: latent (inactive), active, and complexed to t- or uPA. *PAI-1* binds to t- or uPA quickly, producing a stable 1:1 complex that is removed from circulation by the liver. The majority of t-PA is linked to *PAI-1*, which has a negative relationship with fibrinolysis [20]. Increased *PAI-1* levels may predispose individuals to the development of prone-to-rupture atherothrombotic plaques with a high lipid-to-vascular smooth muscle cell ratio [11]. *PAI-1*'s genetic expression and polymorphisms are still poorly known [16], and there is no data on the prevalence of the 4G/5G polymorphism in Sudanese type 2 diabetic patients especially in Gezira state, central of Sudan.

2. Material and Methods

This was analytical case control study for determination of the 4G allele frequency of 4G/5G polymorphism in the *PAI-1* gene (*rs1799889*) in diabetic patients with type 2. A total of 70 patients known diagnosed with diabetes by Glucose Tolerance Test (GTT) and HbA1c attending the Diabetic Clinic of the Aldaraja health center during period from June 2020 to January 2021, in Wad Medani, Gezira state, central of Sudan. Both males and females aged between 28 - 86 years of controlled and uncontrolled type 2 diabetic patients were selected by simple random sampling technique using comprehensive questionnaire including personal and clinical information and laboratory results applied in case and control groups.

Patients selection:

Age less than 18 years and other types of diabetes patients were excluded also; pregnancy and women using contraceptive pills; neuropathy and nephropathy; smoking or patients who were taking standard anticoagulant treatment with either coumarin derivatives or heparins at the time of admission and patients with liver disease and finally patients had hypertensive before diabetes mellitus.

Samples collection:

6 mL of venous blood was collected from each subject using aseptic procedure after a 12 hours fasting. 3 mL of blood was drown in to 0.75 mL of EDTA tube to estimate HbA1c used fine care rapid quantitative test by principle fluorescence immunoassay technology and the reminder of sample store at −20˚C for DNA extraction. The reminder of blood sample was drown in to lithium heparin tube for estimate FBG used SPINREACT reagent using spectrophotometric method after centrifugation of the sample at 2000 rpm for 5 min [21].

Genetic analysis:

DNA Extraction: Genomic DNA was isolated from whole blood leucocytes using **G-DEX™ IIb** Genomic DNA Extraction Kit [for Blood] **Intron** company. DNA quantification was performed using GenQant photometer by diluted the DNA (10 µL DNA to the 90 µL nuclease free water), vortex for 15 second and kept at room temperature for 10 min to make the DNA homogeneous, the diluted DNA was read at 260 nm, protein at 280 nm and the DNA yield was obtained automatically, the mean ratio was 1.71 obtained for samples.

Genotyping of 4G/5G polymorphism of *PAI-1* gene: Using Allele Specific Primer PCR, this technique was used to screen 4G/5G polymorphisms (*rs179989* insertion/deletion) in the -675 promoter rejoin in study subjects and controls. Three primers are designed for 4G/5G polymorphisms (*rs179989* insertion/deletion) ASP-PCR genotyping method and two primers for internal control. Primers sequence 5'CAACTTCATCCACGTTCACC'3 and 5'GAAGAGCCAAGGACAGGTAC'3 for HB1 and HB2 respectively as internal control. For 4G/5G polymorphism genotyping used primers downstream 5'TGCAGCCAGCCACGTGATTGTCTAG'3, 4G specific primer 5'GTCTGGACACGTGGGGA'3 and 5G specific primer 5'GTCTGGACACGTGGGGG'3.

Principle: Two PCR reactions were run per sample (1 for each allele). A 264-bp control band resulted from the upstream (HB1) and downstream (HB2) primers. Each allele-specific primer and downstream primer amplified 138-bp bands. 4G/4G homozygotes yielded 268- and 138-bp bands for the 4G allele-specific reactions and a 268-bp band for the 5G allele-specific reaction. 4G/5G heterozygotes demonstrated 268- and 138-bp bands for both reactions, whereas 5G/5G homozygotes demonstrated a 268-bp band for the 4G allele-specific reaction and 268- and 138-bp bands for the 5G allele-specific [22].

Master Mix Composition: Master mix that was used Add Taq PCR Master Mix. (2× conc.) 1.0 mL APSTP1100A, Taq DNA Polymerase 5 U/µL, Tris-HCl (pH8.8) PCR 20 mM, KCl 100 mM, Triton® X-100 as 0.2%, $MgCl_2$ 4 mM, dNTP 0.5 mM and Protein stabilizer sediment loading dye 1×.

PCR temperature profile for 4G allele: Initial Denaturation at 94˚C/5 min for 1 cycle, Denaturation 94˚C/1 min, Annealing 58˚C/45 sec, Extension 72˚C/45 sec for 35 cycles Final Extension 72˚C/5 min for 1 cycle and Hold at 4˚C∞ for detection 138 bp.

PCR temperature profile for 5G allele: Initial Denaturation at 94˚C/5 min for 1 cycle, Denaturation 94˚C/1 min, Annealing 58˚C/45 sec, Extension 72˚C/45 sec for 40 cycles Final Extension 72˚C/5 min for 1 cycle and Hold at 4˚C∞.

Agarose gel electrophoresis:

2.5% of agarose gel was dissolved in 100 mL 1× Tris-borate EDTA buffer and stain with ethedium bromide 1.5 µL/ 10 mL of agarose gel. 15 µL PCR product and 5 µL of 100 bp DNA ladder (contain bromophenol blue dye which served as visual aid to monitor the progress of migration during agarose gel electrophoresis) was loaded into gel wells the gel was run at 120 V for 25 min. Then visuali-

zation by transilluminator under UV light and the product was captured by the soft wear program and stored as picture.

Ethics approval and consent to participate:

Written consent was obtained from all participants. The study was approved by the RCE research ethical committee of Gezira University and ministry of health, Gezira state. Before collection sample approval was taken from the local Research Medical Ethics Committee (REC) in university of Gezira and ministry of health of Gezira state, the permission was taken from Diabetic Clinic of the Aldaraja health center also the consent was taken from diabetic patients and control subjects.

Statistical analysis:

It was carried out by Statistical Package for Social Sciences (SPSS) Version 16. The significances of the differences of alleles and genotypes between diabetic patient and control groups were tested using the Chi-squire analysis. The allelic frequencies were estimated by gene counting used (SNP-STAT program) and genotypes were scored. The observed numbers of each *PAI-1* genotype were compared with that expected for a population in Hardy-Weinberg equilibrium $(p + q)^2 = p^2 + 2pq + q^2 = 1$, p = allele 4G frequency, q = allele 5G frequency.

3. Results

120 samples were taken, 70 for diabetic patient type 2 from different ages (Their ages ranging from 28 - 86 years old with an average age of 56.2 ± 11.9 years) and different gender (41.4% were males and 58.6% were females) matched with 50 healthy individuals as control group as shown in Table 1.

The frequency of 4G allele in all study population was 28% (n = 67). The *PAI-1* alleles of the presented patient group were represented according to Hardy-Weinberg equilibrium (Table 2).

The genotype distribution of 4G/5G polymorphism of *PAI-1* gene in the diabetic patients type 2 group and control subjects were 4G/4G, 6% (n = 4 and 3), 4G/5G, 44% (n = 31 and 22), and 5G/5G, 50% (n = 35 and 25) respectively (Table 3).

There were no statistical differences in genotyping distribution when compared the diabetic patients with control group for 4G/5G polymorphism of *PAI-1* gene (P value = 0.998) as shown in Table 4 and Figure 1.

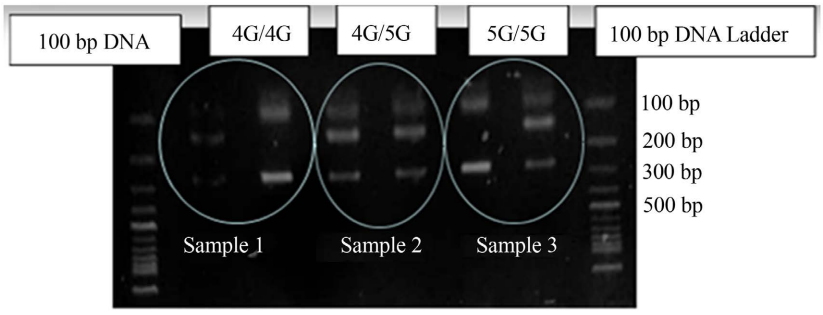

Figure 1. Result of gel electrophoreses.

Table 1. Demographical data in case and control subjects.

Parameters	Case	Control	P-Value
Demographic data:			
Subject	70	50	-
Age/years	56.2	41.8	-
Gender: M/F	29/41	16/34	-
Duration of diabetes/years	10.4	-	-
Family history of diabetes: yes/No	58/12	39/11	-
BMI, Kg/m^2	28.1	26.3	NS
Nutritional: Health/Unhealth	22/48	24/26	-
Mobility: good/bad/some	42/24/3	43/3/4	-

Table 2. The alleles frequency of *PAI-1* gene in study population.

Genotype allele frequencies (n = 120)						
	All subjects		Control		Diabetic patients	
Allele	Count	Proportion	Count	Proportion	Count	Proportion
5G	173	0.72 = 72%	72	0.72	101	0.72
4G	67	0.28 = 28%	28	0.28	39	0.28

Table 3. Distributions genotyping of 4G/5G polymorphism of *PAI-1* gene among study population.

Genotype frequencies (n = 120)						
	All subjects		Control		Diabetic patients	
Genotype	Count	Proportion	Count	Proportion	Count	Proportion
4G/4G	7	0.06	3	0.06	4	0.06
5G/4G	53	0.44	22	0.44	31	0.44
5G/5G	60	0.5	25	0.5	35	0.5

Table 4. Crosstabulation for genotyping 4G/5G polymorphism of *PAI-1* gene in case and control.

Model	Genotype	Control	Diabetic patients	P-value
Co-dominant	5G/5G	25 (50%)	35 (50%)	0.998
	4G/5G	22 (44%)	31 (44.3%)	
	4G/4G	3 (6%)	4 (5.7%)	

4. Discussion

Genetic factors may help develop new strategies to reduce cardiovascular morbidity and mortality in patients with diabetes mellitus, particularly type 2, so a

better understanding of the mechanisms that lead to vascular thrombosis, rather than traditional factors was genetic factors. The *PAI-1* gene, also known as serpin E1, spans 12.3 kb and has nine exons. It is found on human chromosome 7q21.3 - q22. *PAI-1* is a fibrinolytic inhibitor that works quickly. Increased levels of *PAI-1* in the blood have been linked to an increased risk of thrombophilia. The guanine deletion polymorphism (4G allele) at position 675 of the promoter rejoin relative to the transcription start site is the most widely studied functional variation of the *PAI-1* gene, and the 4G allele exhibits greater transcriptional activity than the 5G allele [16]. There was no information on the prevalence of the 4G allele of the *PAI-1* gene's 4G/5G polymorphism in Sudanese diabetes patients. In this study the risk allele 4G frequency was 28% among Sudanese diabetic patients type 2 as shown in **Table 2** without statistical difference when compare with control group (P value = 0.998) as shown in **Table 4**, and this prevalence high when compared with study of Aburto-Mejía, 2017 in Mexico population with diabetes his study contain three different African ethnic groups the frequency of 4G allele was lower in African 13% when compare with other population Indian and white individuals (54% and 58%) respectively [17]. In a research conducted by Zelda de Lange *et al.* in South Africa in 2013, the prevalence of the 4G allele was determined by 15% [18]. In study of Fatima Khalaf's 2019 on Egyptian diabetes patients revealed that the prevalence of the 4G allele was 54.7% [16]. In compared the studies of FESTA 2003 and NARAN 2008 and our study, the most common genotypes of the 4G/5G polymorphism of *PAI-1* gene in Sudanese diabetes patients and controls were 5G/5G 50%, 4G/5G 44%, and lastly 4G/4G 6% as shown in **Table 4**, the latter genotype was greater than the African population 2.6% [23] [24]. According to these researches, the prevalence of the 4G allele and homozygous 4G genotype was lower in African diabetes groups, and higher in Sudanese diabetic populations, but not than Egyptian diabetic populations, this referees to ethnic differences.

5. Conclusion

The frequency of the 4G allele of the 4G/5G polymorphism of *PAI-1* gene in Sudanese diabetic patients was 28% as they in control subjects, which is higher than other African populations. The percentage of this polymorphism differs due to differences in ethnicity. A further study is needed to compare this polymorphism with clinical presentation of the patients especially with cardiovascular thrombosis.

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Authors' Contributions

All authors had participated actively in the research concept, study design, data collection, results interpretation and draft of the manuscript.

Conflicts of Interest

The authors have no conflicts of interest to disclose.

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