Association of rs865429 C/T polymorphism in SOST gene with Coronary Heart Disease in Iraqi Type 2 Diabetes Mellitus Patients

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Abstract

Sclerostin, encoded by the SOST gene, It has also been shown that sclerostin is expressed in aortic VSMC (vascular smooth muscle) and upregulation of SOST gene has inhibitory effects on the aortic aneurysm and atherosclerosis development. **Aims:** The current study aims to explore potential the association between SOST gene single nucleotide polymorphisms (**rs865429 C/T)** and coronary artery diseases (CHD) in type 2 diabetes mellitus (T2DM) patients, in addition to the effect of this SNP on the level of serum sclerostin and other glycemic parameters. **Material and methods:** From the Iraqi population, we enrolled 300 T2DM patients (150 T2DM with CHD and 150 T2DM without CHD). Serum blood glucose, serum insulin, HbA1C, and sclerostin were estimated. Genotyping for **rs865429 C/T** in SOST gene was achieved by RFLP (polymerase chain reaction-restriction fragment length polymorphism). **Results:** T2DM patients having CT + TT genotype included in the studied were at increased risk for CHD in T2DM (odd ratio: 0.4444 CI: 0.2800 to 0.7054) and related to a high serum sclerostin level in comparison with type 2 diabetic patients with CC genotype. **Conclusions:** Type 2 diabetic patients with T allele who have elevated plasma concentrations of sclerostin are at high risk for coronary artery diseases.

Key words: *single nucleotide polymorphism, polymerase chain reaction restriction fragment length polymorphism, type 2diabetese mellitus, coronary artery diseases.*

Introduction

Worldwide, the cardiometabolic risk is increased, and it is the leading cause of mortality and disability, such as impaired lipid and glucose metabolism, high blood pressure, obesity, and systemic inflammation [1]. Also, these metabolic features are present in many individuals with T2DM, which may contribute to the approximate doubling of coronary heart diseases (CHD) risk in persons with diabetes[2]. Sclerostin is a glycoprotein (190 amino acids) is the major antagonist of the Wnt- pathway. Sclerostin especially binding to the LRP 5/6 complex inhibits Wnt signalling and lowering β-catenin translocation into the nucleus, as well as a reduction in cholesterol uptake[3]. Osteocytes are a major source of sclerostin, liver, though chondrocytes, kidney, and avascular wall (aorta) may also secrete it. The relationship between Wnt inhibitors (DKK1 and sclerostin) and arterial wall calcification and cardiovascular events is unclear[4]. Higher serum level of sclerostin is observed in T2DM patients when compared with healthy subjects, and serum sclerostin level is correlated positively with the diabetes duration, and glycated haemoglobin (HbA1c) [5]. Sclerostin has been reported to have positive[6-8] negative[9] or no[10]correlation with arterial calcification. Even its connection with cardiovascular events is controversial. Sclerostin, encoded by the SOST gene, It has also been shown that sclerostin is expressed in aortic vascular smooth muscle and upregulation of SOST gene has inhibitory effects on aortic aneurysm and atherosclerosis development [11]. This study is intended to evaluate the association between **rs865429 C/T** in SOST gene and coronary artery diseases in T2DM patients.

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Experimental

Study Design

Three hundred patients (150Women, 150 Men) age 57.7 ±1.64 years, with a diagnosis of T2DM according to the American Diabetes Association criteria. Subjects, including 150 T2DM patients with CHD (T2DM-CHD), and 150 T2DM patients without CHD (T2DM). The coronary heart disease patients were selected from patients in the catheterization ward of Najaf Center for Cardic Surgery, at Al-Sadder Medical City, Najaf, Iraq (with more than 70% stenosis in each of the main coronary vessels). The protocol of the work was approved by the Ethics Committee of College of Science at Kufa University. Also, each subject signed an informed approval before participating in the study. From the patients, written informed consent was obtained. All patients were Iraqi, without congenital heart disease, cardiomyopathy, severe liver or kidney disease. Patients with vasculitis, connective tissue diseases and familial hypercholesterolemia were also excluded.

Biochemical analysis

At the same time of the day, blood samples were taken for all subjects and after 12-hour fasting. Blood samples collected in two tubes. The first was left to allow clot formation for 10 min, and then centrifuged within 20 min at 3000 Xg, and sera were separated. The reminder blood samples were collected in the tubes containing K_2EDTA for measurement percentage of glycated haemoglobin (HbA1c). The percentage of Hemoglobin A1c was measured by a colorimetric method by using the HbA1c kit (Stanbio, USA). Serum insulin and sclerostin levels were determined using a solid phase enzyme-linked immunosorbent assay kits (Elabscience, USA).

Genotyping analysis

The genomic DNA extraction kit (Promega, USA) was used to extract genomic DNA from the peripheral blood, concerning manufactures guidelines. Genotyping for the studied SNP was accomplished using polymerase chain reaction-restriction fragment length polymorphism (PCR- RFLP). The primer sequences of rs865429 C/T SNP were present in table 1, and the restriction enzymes name and length of the restriction fragments were presented in table 2.

Table 1: Primer sequence for rs865429 C/T SNP in SOST gene[12]

SNP in SOST gene	Primer sequence (5 to 3)
Rs865429C/T	Forward primer: CAGGAGGTGAACCCCCAGCTCGAAGGGG
	Reverse primer: AGGCAAGGTTGGGACTGGGGTGGCTGCT

Table 2: Restriction enzyme name and length of the restriction fragments.

SNP in SOST gene	Restriction enzymes	Cut Temperatures.	Amplification length	Restriction fragments
Rs865429C/T	NcoI	37	365bp	365,102,263

Statistical Analysis

The present study data are expressed as mean ± standard deviation (Mean ± SD). The two groups parameters were compared used students *t*-test. All statistical analyses were performed using the statistics is a powerful statistical software platform (SPSS) software (V20;0 IBM Corporation, Armonk, NY. USA). Calculated probability (p-value) less than 0.05 was considered significant. Hardy-Weinberg equilibrium and Proportions of genotypes of alleles were tested using the standard $\chi 2$ test, in addition to odds ratios (ORs) and 95% confidence intervals (CI) were calculated.

Results and Discussion

The demographic data and clinical of all participants are existing in table 3. As point out in table 3, the levels of HbA1c and fasting insulin were significantly higher in the T2DM with CHD when compared with T2DM without CHD (P < 0.0001). HOMA-IR in T2DM-CHD patients was elevated significantly (11.111 ± 3.617) in comparison to the pathological control group (27.672 ± 9.519). Moreover, T2DM-CHD pointed out a significant increase in the level of sclerostin compared to the non-CHD diabetic pathological control group (181.357±10.959, and 154.285±12.377 Pg/ml, respectively) ($p \leq 0.0001$).

Table3: Hemodynamic characteristics and Clinical of participants and comparison of Sclerostin in the studied groups.

Variables	T2DM-CHD	T2DM	P-Value
Number	75 Female/75Male	75 Female/75Male	
Age years	56.617± 8.128	57.167± 7.764	0.549
T2DM duration	30.063± 5.216	29.969±1.996	0.829
FBG (mg/dl)	241.390± 79.586	253.048±92.963	0.243
HbA1C %	9.381± 1.445	7.373±1.779	0.0001
Serum insulin [µIU/ml]	36.539± 8.566	27.672± 9.519	0.0001
HOMA-IR	11.111 ±3.617	6.668 ±2.901	0.0001
Sclerostin (Pg/ml)	181.357±10.959	154.285±12.377	0.0001

Sclerostin gene was amplified for the detection of **rs865429C/T** in the patients involved in this study. The amplicon size of the amplification product was 365bp. Then it was digested by NcoI restriction enzyme. The digestion products were analyzed by 2% agarose gel electrophoresis. Patients with the wild type homozygote CC exhibited 365bp uncut fragment. While, patients with the of heterozyote CT type showed three bands with sizes of 365bp, 102bp, and 263bp. Those with the homozygote TT type yielded 2 fragments of 102bp, and 263bp fragments (Figure 1).

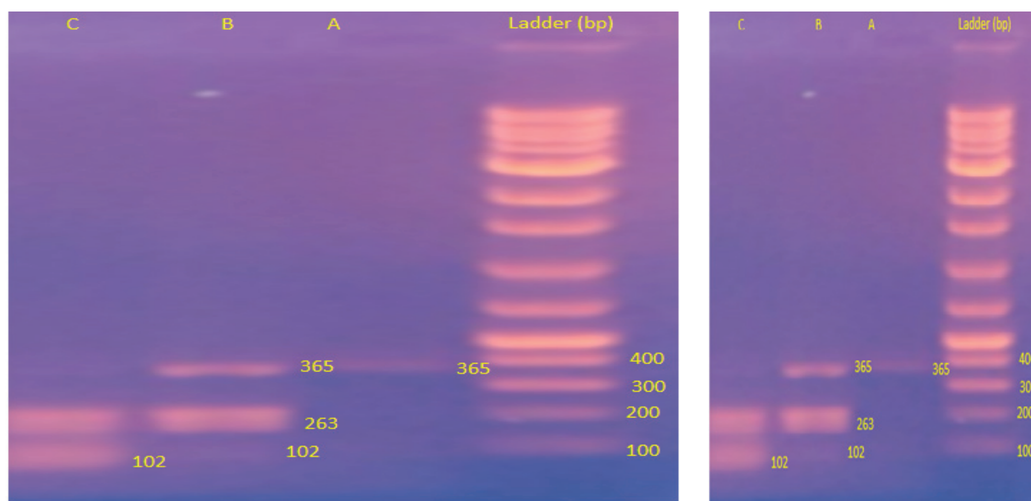

Figure 1: The PCR product of rs865429C/T SNP in SOST gene analyzed by agarose gel electrophoresis. Lanes A: for patients with the wild type homozygote CC genotype that revealed one fragment with the size of 365bp. Lane B: for patients have the heterozygote GT genotype exhibited three fragments with sizes of 365bp, 263bp, and 102bp. Lanes C: for individuals has the mutant homozygote TT of 253bp, 102bp.

The genotyping of the studied polymorphism in T2DM patients are seen in table 4. Data indicated no significant difference between the observed and expected genotypes. The genotyping results of the studied SNP were found to be consistent with Hardy-Weinberg equilibrium.

Table 4: Hardy-Weinberg equilibrium analysis of rs865429C/T SNP in SOST gene.

Results	Genotyping number			P-value
	CC	CT	TT	
Observed	29	71	150	0.526
Expected	27	126	148	

To evaluate the gene-disease associations for the investigated SNP, different inheritance models were considered. The selection of the most appropriate model was achieved via the estimation of the odd ratio. The statistical analysis of the genotype distribution of rs865429C/T SNP in the SOST gene under various inheritance models significant differences under co-dominant and recessive models**,** when the data of T2DM-CHD and T2DM groups were compared. The results were found to be associated with the recessive and dominant models for the rs865429C/T SNP in the SOST gene. Allele association of rs865429 SNP was analyzed for the relevance with the risk of development of coronary heart diseases in diabetic patients. The Results indicated a frequency of the C allele of 36.33% and 23.33% and the T allele of 63,67%, 76.67% in T2DM-CHD and T2DM groups respectively with an insignificant difference (Table 5).

Table 5: Analysis of rs865429C/T Genotype Association with Coronary Heart Diseases under Different Inheritance Models.

Model	Genotype	Frequency %		Odd ratio (CI 95%)	P-value
		T2DM	T2DM-CHD		
Co-dominant	CC	19	10		0.0001
	CT	71	50	1.3380 0.5737 - 3.1209	
	TT	60	90	2.8500 1.2396 -6.5523	
Dominant	CC	19	10	0.4925 0.2209-1.0982	0.0834
	CT +TT	131	140		
Recessive	CC +CT	90	60	0.4444 0.2800 to 0.7054	0.0006
	TT	60	90		
Allele	C	0.3633	0.2333		0.073
	T	0.6367	0.7667		

The biochemical characteristics T2DM-CHD patients were analyzed in relevance to the genotype of the selected SNP under the recessive model. For the rs865429C/T SNP in the SOST gene, the recessive model included patients with genotypes CC+CT vs TT. The analysis was carried out with the use of the student's t-test. The analyses of the data of the rs865429C/T SNP in SOST gene indicated significant elevations (P=0.0001) of levels of fasting blood glucose, fasting blood insulin, HbA1c, and HOMA-IR, in patients have CT+TT genotypes when compared with those of CC genotype (Table 6). Data of sclerostin concentration revealed a significant elevation (P=0.0001) in patients who have TT genotypes in comparison with those of CC+CT genotype.

Table 6. Differences of biochemical characteristics in relevance to the genotypes of rs865429C/T SNP in the SOST gene of T2DM-CHD patients under the recessive model.

Parameters	CC+CT (60)	TT(90)	P-value
FBG (mg/dl)	216.193± 98.104	283.720 ± 76.810	0.0001
HbA1C (%)	7.984± 2.029	10.002 ± 2.779	0.0001
Insulin (µU/ml)	26.930 ± 8.566	37.982 ± 6.910	0.0001
HOMA-IR	9.987 ±4.819	14.944 ±3.012	0.0001
Sclerostin (pg/ml)	131.402 ±19.878	194.006±28.298	0.0001

The current study assesses the relationship between rs865429C/T single nucleotide polymorphism in the SOST gene with CHD risk in T2DM. Interestingly, in our population, the selected SNP data show similarity with Hardy-Weinberg equilibrium. Statistically, a significant association was observed between the TT genotype with the risk of CHD in T2DM. Iraqi carriers of the TT genotype seem to be more susceptible to the disease. The pooled odd ratios of multiple comparisons of genotyping models, co-dominant, dominant, and recessive were 2.963 (0.906-4.8366, P=10-4), 0.4925 (1.2396- 6.5523, P=0.0834), and 0.444(0.2800- 0.7054, P= 0.0006), respectively. To realize the impact of SOST gene polymorphism (rs865429 C/T) in the development of CHD in type 2 diabetics, we have to focus on mechanisms by which Sclerostin is engaged.

The GWA has proved that SOST gene is one of the hub genes that play a role in osteoporosis pathology [13]. While, Deveci *et al,;* reported that SOST gene rs865429C/T influences weight and body mass index of Turkish women [12]. And, Piters *et al.* ; evaluated SOST rs10534024 polymorphism on 783 young and 600 elder Danish men. They reported an interaction between body shape and SOST rs10534024 polymorphism[14]. We also observed an association between CHD in T2DM and rs865429C/T polymorphisms in the SOST gene. T2DM is a major cardiovascular disease risk factor. There are numerous certificates that both genetic and environmental factors contribute to this risk. Gene risk factors identified for cardiovascular diseases is crucial to understand the aetiology of the diseases. Although, mechanisms of atherogenesis in large part stay to be defined. Observational researchers have explained that the progression of the disease involves crisscross between immune cells with both endothelial cells and VSMCs [15]. The fundamental cells inside the media layer of arteries are VSMCs and are important to maintaining the arterial wall integrity of 16. They take part in the remodeling of the arterial wall and have serious roles in atherosclerosis through all disease stages. The VSMCs display marked plasticity in response to lipoprotein accumulation, vascular injury, and inflammation during progression of disease through re-programming gene expression, i.e., phenotype switching, a shift to a proliferative, promigratory, and activated phenotype[17]. There is rising evidence on the extraskeletal functions of sclerostin, highlighting to its role in many vascular disorders. Recent studies have pointed that under calcifying conditions, the VSMCs are able of producing a phenotypic transition to osteoblast-like cells, under calcifying conditions, which are capable of expressing the typical bone markers, like sclerostin [18].

Various researchers notified the link between sclerostin level and vascular tissue calcification [7,19]. Besides the contribution of sclerostin in some disorders associated with processes of vascular calcification [20,21]. It is controversial in the mechanism by which sclerostin can affect the calcification process[9,22,23] , some of them propose that sclerostin has a protective role[24], while others propose the opposite [25]. In aortic tissue, sclerostin is expressed in VMSCs [11]. While, in atherosclerotic plaques which obtained from carotid endarterectomy, sclerostin is detectable by immunohistochemical staining in VMSCs as well as in macrophages[26].

It is reasonable to suggest here that this SNP is an important genetic marker that predisposes the occurrence of CHD in type 2 diabetics. Hence, more prospective research should be performed to provide the use of sclerostin and rs865429 SNP in the SOST gene as a good biomarker to identify an increased predisposition to coronary heart diseases in type 2diabetic Iraqi patients.

Conclusion

The present work describes the **rs865429C/T SNP** in the SOST gene encoding sclerostin in a T2DM patient with CHD. The mutation has a devastating effect on the biological function of sclerostin by increasing extracellular sclerostin levels, thereby, its antagonistic activity on canonical Wnt signalling contributes to the disease phenotype. Also, The association between CHD and rs 865429C>T polymorphism in the SOST gene may open new insights on pharmacogenetics studies of CHD in T2DM.

Limitation:

Our study has a limited number of T2DM patients; events might have restricted the power of our study to detect statistically significant associations.

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Conflicts of Interest: Declared none.

Ethics Statement: This experiment was approved by the Central Committee for Bioethics in college of Sciences/ Kufa, Iraq.

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