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CLINICAL & BASIC RESEARCH

Expression Levels and Genetic Polymorphism of Scavenger Receptor Class B Type 1 as a Biomarker of Type 2 Diabetes Mellitus

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abstract: ***Objectives:*** This study aimed to determine whether the expression level and genetic polymorphism scavenger receptor class B type 1 (*SCARB1*) rs5888 may be used as biological markers in type 2 diabetes mellitus (T2DM). ***Methods:*** This case-control study was conducted at King George's Medical University, Lucknow, India, from September 2018 to December 2019. Blood samples were collected from each individual with T2DM and each healthy individual. Total proteins were determined using western blot analysis. Additionally, restriction fragment length polymorphism analysis was achieved to detect the incidence of genetic polymorphisms. ***Results:*** A total of 600 individuals, including 300 individuals with T2DM and 300 healthy individuals, were enrolled in the study. Western blot analysis results revealed that the protein expression of SRB1 was significantly decreased in T2DM of *SCARB1* CC variant when compared with controls (P = 0.007). The genotype distribution and the allelic frequencies for the SRB1 polymorphism were significantly different between T2DM and controls (P = 0.03). The CC genotype of the *SCARB1* polymorphism showed a potential association with the incidence of T2DM (odds ratio = 1.19, 95% confidence interval = 0.63–2.25; P = 0.577). ***Conclusion:*** The expression levels and genetic polymorphisms of the *SCARB1* CC variant may be potential biomarkers for the occurrence of T2DM.

Keywords: Genetic Polymorphism; Type 2 Diabetes Mellitus; Human SRB1 Protein; Biomarker; India.

Advances in Knowledge

- *This was the first study to identify the scavenger receptor class B type 1 (SCARB1) variant's association with type 2 diabetes mellitus (T2DM) at the protein level and revealed that the lower expression level of the SRB1 protein might be due to variation at the SRB1 rs5888 locus.*
- *SCARB1 gene polymorphism could be considered as an independent risk factor for the development of T2DM and may be used as a biomarker for early diagnosis.*

Application to Patient Care

- *Early identification of disease susceptibility could explain disease risk and prognostics.*

Type 2 diabetes mellitus (T2DM) persists as a major health problem worldwide; it is well accepted that T2DM is a metabolic disorder caused by hyperglycaemia, which arises from insufficient pancreatic insulin secretion to peripheral tissues.[1] However, long-term defects in beta-cells could also impair insulin levels and inhibit the production of insulin.[2,3] Insulin resistance is associated with increased serum insulin and dysfunctional beta-cells that could result in lipoprotein abnormalities and increased glucose levels.[4] Furthermore, genetic studies have shown that more than 45 genetic variants are associated with susceptibility to T2DM, including hereditary factors that also play a role in the development of diabetes.[5]

Scavenger receptor class B type 1 (SRB1) protein plays a major role in the uptake of cholesteryl ester from high-density lipoprotein (HDL) and it eliminates extra cholesterol from peripheral tissues as bile through the process of reverse cholesterol transport.[6,7] Lack of SRB1 protein can result in an imbalance of cholesterol and dyslipidaemia and eventually lead to insulin resistance.[8,9] McCarthy *et al.* reported that the rs5888 variants of *SCARB1* were associated with insulin resistance.[10] Similarly, Tetik Vardarli *et al.* found that *SCARB1* C1050T polymorphism is a risk factor for the development of diabetic dyslipidaemia.[11] Constantineau *et al.* studied the effects of C1051T polymorphism on SRB1 protein expression and function in an in vitro study.[12] Moreover, there are numerous specificity protein-1 (Sp1) binding positions in this DNA region, and previous results designated that the glucose inhibition of SRB1 expression is to some extent, intervened by the activation of the p38 mitogen-activated protein kinase (MAPK)-Sp1 pathway regulatory genes; this inhibits inflammation and other inflammatory signalling pathways such as nuclear factor-κB and arachidonate-signalling.[13] As a result, hyperglycaemia could activate the p38 MAPK pathway that might considerably add to the development of diabetes. This inhibitory effect of hyperglycaemia on *SCARB1* promoter activity is

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mediated by the p38 MAPK/Sp1 signalling cascade. These findings create the possibility of hyperglycaemia affecting reverse cholesterol transport by controlling SR-BI expression in diabetic patients.[13] The current study aimed to determine whether the expression level and genetic polymorphism of *SCARB1* rs5888 may be used as biological markers in T2DM.

Methods

This case-control study was conducted between September 2018 and December 2019 among the general population presenting at the Out-Patient Department of Medicine, King George's Medical University (KGMU), Lucknow, India. All participants had to follow a careful screening programme, which involved the determination of clinical and biochemical parameters. Following screening, subjects were selected based on the inclusion/exclusion criteria resulting in an equal number of T2DM subjects and control subjects. According to the American Diabetes Association, T2DM is defined as a fasting plasma glucose level with a fasting blood sugar (FBS) ≥126 mg/dL and glycated haemoglobin (HbA1c) ≥6.5%.[14] Subjects who were diagnosed with T2DM were included in this study. Those with congenital disorders, mental disorders, cardiovascular disease, heart failure or diabetes complications were excluded. For the control subjects, apparently healthy participants with no individual or familial history of T2DM or any other serious illness were included. Furthermore, those with identified systemic diseases, including T1DM, hypertension, heart disease, liver cirrhosis, renal disease, gastrointestinal disease, pulmonary disease or cancer were excluded. To gather the participant's demographic characteristics, a prepared questionnaire was administered by qualified staff.

The authors preferred two diabetes risk-associated polymorphisms *SCARB1* rs5888 C > T from 12q24.31 loci with minor allele frequency (MAF) > 5%. Previous literature reported that this locus is responsible for predisposition to diabetes.[15] For analysis, a total of 3 mL of venous blood was withdrawn from each participant under the supervision of a general practitioner. Out of this, 1 mL of blood was transferred into ethylenediaminetetraacetic acid (EDTA) vials, mixed up and down gently and then used for the isolation of genomic DNA and genetic analysis. The remaining 2 mL of whole blood was centrifuged at 1,500 rpm for 10 minutes and the isolated serum was used for the analysis of blood sugar and lipid parameters.

DNA was extracted from peripheral blood leukocytes (obtained from EDTA admixed whole blood) using the phenol-chloroform process.[16] NEB cutter, Version 2.0 (New England Biolabs, Massachusetts, USA), was used to select the restriction enzymes that cut at the sites of single nucleotide polymorphisms (SNPs).

Since the molecular weight of the DNA fragments digested with restriction enzymes were small (100–300 bp), they were checked by using 2% agarose gels. Gels were prepared in 1× tris-acetate-EDTA buffer (in diethyl pyrocarbonate water) and stained with 0.5 μg/mL ethidium bromide (EtBr). DNA samples (2 μL) were mixed with DNA loading buffer (xylene cyanole/bromophenol dye), loaded onto the gel and run at 50–70 volts until the dye migrated 3/4 of the way up gel. DNA was analysed in a gel documentation system (Bio-Rad Gel Doc TM EZ Imager, Bio-Rad Laboratories Inc, Hercules, California, USA).

SCARB1 gene polymorphism was detected by polymerase chain reaction (PCR; Applied Biosystems™ Veriti™ HID 96-Well Thermal Cycler, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) followed by restriction fragment length polymorphism. *SCARB1* gene was amplified by the following PCR conditions: 93˚C for 5 minutes, followed by 33 cycles of 91˚C for 35 seconds, 61˚C for 38 seconds, 69˚C for 39 seconds and final elongation at 71˚C for 5 minutes with precise *SCARB1 rs5888* gene forward primer 5'-CTTGTTTCTCCTCGACGC-3' and reverse primer 5'-CACCACCCCAGCCAGCAGC-3' (Eurofins Genomics, Ebersberg, Germany). Amplification was done with a 12 μL PCR mixture comprising 0.5 μL template DNA, 0.5 μL of both primers and 2× PCR master mixes (New England Biolabs). Enzyme digestion was conducted in a 15 μL final volume consisting of 1 unit of the Hin1I enzyme (Thermo Fisher Scientific Inc.). The reaction was conducted at 37˚C overnight and the digested products were separated on 2% agarose gel electrophoresis containing EtBr (0.5 μg/mL); agarose gel was observed by gel documentation system (Bio-Rad Laboratories Inc.). The genotypes recognised were labelled according to the presence or absence of the enzyme restriction sites. As a result, the TT genotype was a wild homozygote, the CT genotype was a heterozygote and the CC genotype was a variant homozygote.

Western blot assay was carried out to assess the protein expression of SRB1 in a subset of T2DM cases and controls. The determined protein was then transferred to the polyvinylidene fluoride membrane through a western blot procedure at 30 volts for 120 minutes using a wet electrotransfer apparatus (Bio-Rad Laboratories Inc.). The membrane was blocked for 60 minutes in a tris-buffered saline (TBS) buffer containing 5% skimmed milk powder. The primary polyclonal antibody was added at a 1:1000 dilution in

TBS buffer and kept overnight at 4°C. The membrane was washed three times for 5 minutes each in TBS+polysorbate buffer; a secondary antibody (goat anti-mouse immunoglobulin G-alkaline phosphatase [AP]) was added at a 1:1000 dilution in TBS buffer and incubated for 60 minutes at room temperature. Detection of AP-conjugated antibody was done by Supersignal West Pico Chemiluminescent Substrate (Elabscience Biotechnology Inc., Houston, Texas,USA; Catalogue number: SC-358798). Membrane images were examined by a gel documentation system. ImageJ software was used for the investigation of serum protein bands.

The differences between all parametric variables were analysed using the student's t-test. Chi-square test was used to determine the significance of differences in allele and genotype frequency. Analysis of variance was used to test for variance in parameters between genotypes. Differences were considered statistically significant when *P* was <0.05. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) software, Version 16.0 (IBM Corp., Chicago, Illinois, USA).

Institutional ethics committee's at KGMU approved this study (Code: 98th ECMIIA/P4). All subjects signed the approved consent form.

Results

A total of 663 subjects were recruited for this study but 63 subjects were excluded because they refused to participate or were not willing to provide consent. Of the remaining participants, 300 individuals with T2DM and 300 healthy controls were included.

No significant differences were observed between the two groups in terms of age and alcohol consumption; however, there were significant differences in smoking

Table 1: Characteristics and biochemical parameters of participants with type 2 diabetes mellitus participants and healthy controls (N = 600)

Characteristic	Mean ± SD		OR (95% CI)	*P* value
	Controls n = 300	T2DM cases n = 300		
Age in years	47.5 ± 7.3	48.6 ± 9.7	-	0.11
BMI in kg/m^2	23.4 ± 4.90	26.5 ± 5.10	-	0.02
Smoking			1.5 (1.09–2.09)	0.01
Yes	169	138		
No	131	162		
Alcohol consumption			1.12 (0.81–1.55)	0.51
Yes	165	156		
No	135	144		
Biochemical parameter				
HDL in mg/dL	42.3 ± 8.5	46.5 ± 7.9	-	0.001
LDL in mg/dL	97.8 ± 12.7	139.2 ± 22.5	-	0.001
VLDL in mg/dL	44.5 ± 11.3	42.6 ± 8.5	-	0.001
TG in mg/dL	122.6 ± 11.7	206.2 ± 34.5	-	0.001
TC in mg/dL	184.4 ± 21.5	215.2±33.4	-	0.001
FBS in mg/dL	78.4 ± 13.7	133.2 ± 17.4	-	0.001
HbA1c in %	5.6 ± 0.7	7.94 ± 0.99	-	0.001

SD = standard deviation; OR = odds ratio; CI = confidence interval; T2DM = type 2 diabetes mellitus; BMI = body mass index; HDL = high-density lipoprotein; LDL = low-density lipoprotein; VLDL =very low-density lipoprotein; TG =triglycerides; TC = total cholesterol; FBS =fasting blood sugar; HbA1c =glycated haemoglobin.

Table 2: Association between lipid parameters and scavenger receptor class B type 1 rs5888 polymorphism in participants with type 2 diabetes mellitus compared to healthy controls

Lipid parameter	SRB1 rs5888 polymorphism, Mean ± SD			*P* value
	TT	CT	CC	
Controls				
Number of cases	80	147	73	
HDL in mg/dL	43.1 ± 11.5	39.5 ± 9.7	39.7 ± 10.2	0.03
LDL in mg/dL	97.2 ± 27.6	95.2 ± 30.1	104.1 ± 29.4	0.10
VLDL in mg/dL	41.4 ± 9.4	40.3 ± 9.1	43.1 ± 10.6	0.12
TG in mg/dL	144.2 ± 23.5	142.6 ± 33.1	147.5 ± 26.1	0.841
TC in mg/dL	172.1 ± 39.7	177.2 ± 29.6	176.8 ± 33.8	0.544
T2DM cases				
Number of cases	63	129	108	
HDL in mg/dL	35.1 ± 7.5	33.1 ± 12.5	30.3 ± 7.2	0.03
LDL in mg/dL	116.5 ± 22.4	123.7 ± 17.2	130.3 ± 36.4	0.02
VLDL in mg/dL	41.4 ± 10.3	43.5 ± 10.2	44.5 ± 12.5	0.31
TG in mg/dL	179.7 ± 28.2	182.5 ± 29.4	176.3 ± 32.3	0.835
TC in mg/dL	222 ± 28.1	228.3 ± 21.1	223.5 ± 26.1	0.16

SRB1 = scavenger receptor class B type 1; HDL = high-density lipoprotein; LDL = low-density lipoprotein; VLDL = very low-density lipoprotein; TG = triglycerides; TC = total cholesterol; T2DM = type 2 diabetes mellitus.

Table 3: Comparison of genotype and allele frequencies of scavenger receptor class B type 1 rs5888 in participants with type 2 diabetes mellitus compared to healthy controls (N = 600)

SRB1 (rs5888) polymorphism	n (%)		OR (95% CI)	*P* value
Genotype	**Controls n = 300**	**T2DM cases n = 300**		
TT	80 (27)	63 (21)	Reference (1.00)	
CT	147 (49)	129 (43)	1.11 (0.74–1.67)	0.67
CC	73 (24)	108 (36)	1.87 (1.20–2.92)	0.007 χ^2=7.19
Allele				
T	307 (51)	255 (43)		
C	293 (49)	345 (58)	1.41 (1.12–1.78)	0.003 χ^2=8.70

SRB1 = scavenger receptor class B type 1; OR =odds ratio; CI =confidence interval.

behaviour (*P* = 0.01) and body mass index (BMI; *P* = 0.02). Significantly higher levels of total cholesterol (TC), triglyceride, low-density lipoprotein (LDL), FBS and HbA1c and decreased HDL levels were observed in the individuals with T2DM compared to controls (*P* = 0.001 each) [Table 1]. Furthermore, the findings also revealed that the *SCARB1* TT genotype had significantly increased HDL levels when compared to the CC genotype (*P* = 0.03) [Table 2].

In *SCARB1* rs5888, the frequencies of the CC, TT and CT genotypes in the T2DM group were 36%, 21% and 43%, respectively, while in the control group, these were 24%, 27% and 49%, respectively. The distribution of all genotypes and alleles were in Hardy Weinberg equilibrium. It was observed that in *SCARB1* rs5888 polymorphism, the CC genotype was significantly increased in the T2DM group compared to control group (odds ratio [OR] = 1.87, χ^2 = 7.19, 95% confidence interval [CI]: 1.20–2.92; *P* = 0.007). The C allele frequency was associated with the T2DM group when compared to the control group (OR = 1.41, χ^2 = 8.70, 95% CI: 1.12–1.78; *P* = 0.003) [Table 3].

A total of 100 serum samples (n = 50 for each group) were analysed for possible consequences of the *SCARB1* genetic polymorphism on protein expression levels by western blot analysis. Beta-actin was used as an endogenous control. Results showed that the protein expression of SRB1 was significantly higher in the *SCARB1* TT variant of healthy controls compared to participants in the T2DM group and the variant might affect SRB1 protein expression levels [Figure 1].

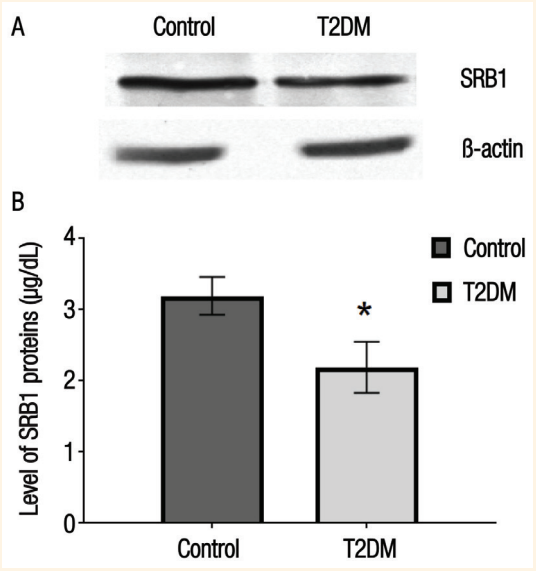

Figure 1: A: Representative western blot using beta-actin as the loading control and protein expression levels of scavenger receptor class b type 1 (SRB1) in a healthy control versus a participant with type 2 diabetes mellitus (T2DM). **B:** Protein expression levels of SRB1 in a healthy control and a participant with T2DM.
**P <0.05.*

Discussion

The results of numerous previous association studies between the *SCARB1* rs5888 SNP and diabetes were not constant between different ethnic groups. Therefore, genetic confirmation for an association between the *SCARB1* SNP and diabetes in humans needs to be explored. In the present study, it was observed that the *SCARB1* CC genotype was significantly associated with an increased T2DM risk, which was in concordance with previous findings that also reported a significant association between the *SCARB1* polymorphism and T2DM risk.[10] In agreement with this, a previous study from the Han and Guangxi population also showed that *SCARB1* is significantly associated with an increased risk of T2DM.[17] Although the precise role of *SCARB1* rs5888 in regard to T2DM risk is still under investigation, evidence has suggested that the *SCARB1* gene variants may affect susceptibility to the disease by altering the levels of SRB1 that might contribute to the risk of T2DM.[13] In the present study, it was noticed that the *SCARB1* CC variant had decreased SRB1 protein expression, which might have been due to the variation in the regulatory region of the *SCARB1* gene; this could have contributed to the differential expression of SRB1, as the variant was located in the promoter region of the gene and could hinder the binding of the transcriptional factor.[15] Previous reports

also found that the rs5888 SNP affected the SCARBI RNA secondary structure, which changed its ability to undergo productive protein translation, ultimately leading to significantly lower SRB1 protein expression.[18] Findings indicated that the lower expression level of the SRB1 protein might be due to the variation at the rs5888 (exonic C1050T) locus, which affects the functionality and stability of the protein. Interestingly, SNPs located in the exonic region of the gene might manipulate mRNA translation, thereby affecting the level of protein expression.[19]

The involvement of numerous variants in the *SCARB1* gene and serum lipid profiles has been described in distinct populations worldwide. In the present study, control subjects carrying the *SCARB1* TT genotype had higher HDL levels compared with the CC genotype. This result was in concordance with a previous study that also revealed that the rs5888 T allele is significantly associated with higher HDL levels among the Turkish population.[11] Additionally, T2DM subjects with the CC genotype had a significantly low level of HDL. This result indicated that patients with T2DM have significantly higher levels of *SCARB1* and a significantly higher frequency of homozygous CC SNPs (rs5888). These findings suggest that SRB1 may be a potential biomarker for the incidence of T2DM.

This study selected control and case groups which is vital for a genetic study, as any systematic error in either groups can result in false-positive and false-negative results.[20] There are several risk factors for T2DM that primarily include increased age, gender, dyslipidaemia, smoking, increased serum TC and LDL. To mitigate the effect of confounders caused by the mismatch between the two included groups, T2DM and control subjects were matched in age, gender and BMI. However, the present study was subject to certain limitations. This study was conducted using a small sample size and was single centric. A study with a large sample size that is multicentric and considers different ethnic groups should be conducted to identify the potential relationship of these loci.

Conclusion

The present study showed an increased number of CC genotype in T2DM participants with reduced SRB1 protein expression; this might be associated with the occurrence of T2DM and could be used as a biomarker for early diagnosis. Identification of the genes involved will help in better understanding the mechanism of the disease and lead to improved treatment. Future efforts should use a genetic approach to increase the functional efficiency of SRB1, which could result in a decreased occurrence of T2DM. However, a healthy diet, physical activity and dedication to lifestyle changes could delay or prevent T2DM in individuals with increased risk.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHORS' CONTRIBUTION

MW, WA and DH designed and performed the study. MW and WA analysed the data. MW drafted the manuscript. All authors approved the final version of the manuscript.

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