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: The present study aimed to determine whether the expression level and genetic polymorphism scavenger receptor class B type 1 (SRB1) rs5888 may be used as biological markers in type 2 diabetes mellitus (T2DM). Methods: A total of 600 individuals, including 300 T2DM and 300 healthy individuals, were enrolled in the study from April 2016 to April 2017. Blood samples were collected from each T2DM and healthy individual. Total proteins were determined using western blot analysis. Also, restriction fragment length polymorphism (RFLP) analysis was achieved to detect the incidence of genetic polymorphisms. Results: Western blot analysis results revealed that the protein expression of SRB1 was significantly decreased in T2DM of SRB1 CC variant as compared with controls. The genotype distribution and the allelic frequencies for the SRB1 polymorphism were significantly different than T2DM and controls. CC genotype of the SRB1 polymorphism showed a potential association with the incidence of T2DM (OR =1.19, 95% CI 0.63 - 2.25, P=0.577). Conclusions: The expression levels and genetic polymorphisms of SRB1 CC variant may be potential biomarkers for the occurrence of T2DM.
Keywords: Polymorphism; T2DM; SRB1, Protein expression; Biomarker
Advances in Knowledge
- This is the first study to identify SRB1 variant association with type 2 diabetes mellitus at the protein level, and revealed that the lower expression level of the SRB1 protein might be due to variation at SRB1 rs5888 locus.
- SRB1 gene polymorphism could be considered as an independent risk factor for the development of type 2 diabetes mellitus and possibly be used as a biomarker for early diagnosis.

Application to Patient Care
- Early identification of disease susceptibility could possibly explains disease risk and prognostics.

Introduction
Type 2 diabetes mellits (T2DM) persists to be a major health problem worldwide and it is well accepted that T2DM is a metabolic disorder caused by hyperglycemia, which arises from insufficient pancreatic insulin secretion to peripheral tissues\(^1\). Although, 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 cell that could result in lipoprotein abnormalities and increased glucose level\(^4\). Further, genetic studies shows more than 45 genetic variants are associated with susceptibility to T2DM including hereditary factors that also play role in developing diabetes\(^5\).

Moreover, scavenger receptor class B type 1 (SRB1) protein plays a major role in uptake of cholesteryl ester from high density lipoprotein (HDL) and eliminates extra cholesterol from peripheral tissues as a bile by the process of reverse cholesterol transport (RCT)\(^6\) \(^7\). Lack of SRB1 protein can result in an imbalance of cholesterol, dyslipidemia and eventually lead to insulin resistance\(^8\) \(^9\). Earlier, McCarthy et al.\(^10\) reported that the rs5888 variants of SRB1 were associated with insulin resistance. Similarly, Tetik Vardarli et al.\(^11\) too found that SRB1 C1050T polymorphism to be a risk factor for the development of diabetic dyslipidemia. Further, Constantineau et al.\(^12\) studied the effects of the C1051T polymorphism on SRB1 protein expression and function by an in vitro study. Moreover, there were numerous specificity protein-1 (Sp1) binding positions in this DNA region and previous results designate that the glucose inhibition of SRB1 expression is to some extent intervene by the
activation of the p38 MAPK-Sp1 pathway regulatory genes that inhibits inflammation and other inflammatory signalling pathways such as nuclear factor-κB and arachidonate-signaling. As a result, hyperglycemia could activate the p38 MAPK pathway that might considerably add to the development of diabetes. Previous findings also showed that hyperglycemia inhibits hepatic endogenous SR-BI expression. This inhibitory effect of hyperglycemia on SR-BI promoter activity is mediated by the p38 MAPK/Sp1 signaling cascade. These findings raise the possibility that hyperglycemia may affect reverse cholesterol transport by controlling SR-BI expression in diabetic patients. The present study aimed to determine whether the expression level and genetic polymorphism of SRB1 rs5888 may be used as biological markers in type 2 diabetes mellitus (T2DM).

Methods

Recruitment of participants

This is a case-control study. All participants had to follow a careful screening program, which involved determination of clinical and biochemical parameters. A total of 663 subjects were recruited between April 2016 to April 2017 from OPD Medicine, King George’s Medical University, Lucknow, India. 63 subjects were excluded from the study either they refused to participate or not willing to give consent. After screening, 600 subjects were selected based on the inclusion/exclusion conditions. Overall 300 T2DM subjects and 300 control subjects were recruited. The subjects were recruited from Lucknow, Uttar Pradesh, India from the general population. T2DM is defined as a fasting plasma glucose level FBS ≥ 126 mg/dL and HbA1C (≥ 6.5 %) level. This was defined according to the American diabetes association.

The control contestant had no individual or familial history of T2DM, or any other serious illness. Contestants with identified systemic diseases, including T1DM, hypertension, Heart disease, liver cirrhosis, renal disease gastrointestinal disease, pulmonary disease, or cancer were disqualified. Contestants were interrogated using a prepared questionnaire by qualified staff to gather demographic characteristics. Inclusion criteria for the cases are subjects with diagnosed type 2 diabetes mellitus and willing to contribute to the study. Whereas, exclusion criteria for the cases are subjects with congenital disorders, mental disorders, CVD, heart failure, diabetes complications and not keen to participate in the study. Moreover, inclusion criteria for the controls are healthy volunteers not having type 2 diabetes or any other specific
illness and willing to give written informed consent. Institutional ethics committee’s KGMU, Lucknow approved ethical consent. All subjects signed the approved informed consent.

**Polymorphisms selection**

We preferred two diabetes risk-associated polymorphisms SRB1 rs5888 C > T, from 12q24.31 loci with minor allele frequency (MAF) > 5%. Previous literature reported this locus is responsible for predisposition to diabetes. 

**Blood sample collection**

A total of 3mL of venous blood was withdrawn from each participant under the supervision of a general practitioner. Out of which 1mL of blood was transferred into Ethylenediaminetetraacetic acid (EDTA) vials and mingle up and down softly and then utilized for isolation of genomic DNA and genetic analysis. Further, 2mL of whole blood was centrifuged at 1,500 rpm for 10 min and the isolated serum was used for the analysis of blood sugar and lipid parameters.

**DNA isolation**

DNA was extracted from peripheral blood leucocytes (obtained from EDTA admixed whole blood) with phenol: chloroform process.

**Selection of Restriction Enzymes**

NEB cutter (v 2.0) was used to select the restriction enzymes cutting at the sites of SNPs.

**Electrophoresis**

Since the molecular weight (MW) of DNA fragments digested with RE were small (100-300bp), and were checked on 2% agarose gels. Gels were prepared in 1X TAE buffer (in DEPC water), stained with 0.5 µg/mL ethidium bromide (EtBr). DNA samples (2µL) were mixed with DNA loading buffer (Xylene cyanole/Bromophenol dye) and loaded onto the gel and run at 50-70 volts until the dye migrated 3/4 of the gel. DNA was analyzed in a gel documentation system (Bio-Rad Gel Doc TM EZ Imager, USA).
**Analysis of SNPs**

SRB1 gene polymorphism was detected by PCR (Applied Biosystems™ Veriti™ HID 96-Well Thermal Cycler) followed by RFLP. SRB1 gene was amplified by the subsequent PCR conditions: 93°C for 5 min; followed by 33 cycles of 91°C for 35s, 61°C for 38s, 69°C for 39s and final elongation at 71°C for 5 min with precise SRB1 rs5888 gene forward primer 5’-CTTGTTCCTCCTCGACGC-3’ and reverse primer 5’-CACCACCCAGCCAGCAGCAGC-3’ (Eurofins Genomics, Ebersberg, Germany). Amplification was done with a 12µL PCR mixture comprising 0.5µL template DNA, 0.5µL of both primers, and 2X PCR master mixes (New England Biolabs, USA). Enzyme digestion was conducted in a 15µL final volume consists of 1U of the HinII enzyme (Thermo Scientific Inc. USA). 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 Gel Doc TM EZ Imager, USA). The genotypes recognized were labelled according to the presence or absence of the enzyme restriction sites. As a result, TT genotype is wild homozygote, CT genotype is a heterozygote and CC genotype is variant homozygote.

**Western blotting**

We analyzed a total of 100 serum samples (each n=50) from healthy controls and T2DM subjects for the analysis of protein expression. Western blot assay was carried out to assess the protein expression of SRB1 in cases and controls. The determined protein is then transferred to the PVDF membrane through a western blot procedure at 30 volts for 120 minutes using a Bio-Rad wet electrotransfer apparatus. The membrane was blocked for 60 minutes in 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-each 5 minutes in TBST buffer and then secondary antibody (Goat Anti-Mouse IgG-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 Super Signal West Pico Chemiluminescent Substrate (Elabscience Biotechnology Inc. USA, Catalogue number: SC-358798). Membrane images were examined by a gel documentation system. Image J was used for the investigation of serum protein bands.
Statistical analysis
The differences between all parametric variables were analyzed by using the student’s t-test. Chi-square ($\chi^2$) test was applied to determine the significance of differences in allele and genotype frequency. Analysis of variance (ANOVA) was used to test for variance in parameters between genotypes. Differences were considered significant when $p$ was $<0.05$. All statistical analyses were achieved by performing SPSS software version 16.0 (SPSS, Chicago, IL).

Results
Clinical parameter between T2DM and healthy controls
This study involved 300 individuals with T2DM and 300 healthy controls. No significant differences were observed in age, alcoholic subjects, but there were significant differences observed in smoking ($p=0.01$), body mass index (BMI) ($p=0.02$) between cases and controls. Significantly higher levels of total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL), fasting blood sugar (FBS), Glycated hemoglobin (HbA1c), and decreased HDL levels were confirmed in T2DM compared to controls [Table 1]. Furthermore, our findings also revealed that the SRB1 TT genotype had significantly increased HDL when compared to CC genotype [Table 2].

Genetic variants association with T2DM susceptibility
In SRB1 rs5888, the frequencies of CC, TT and CT genotypes in the case group were 36, 21 and 43% respectively; in the control group, the corresponding frequencies were 24, 27 and 49% respectively. Distribution of all genotypes and alleles were in Hardy Weinberg Equilibrium. In SRB1 rs5888 polymorphism, we observed CC genotype with an odds ratio of 1.87 ($\chi^2=7.19$, 95% CI=1.20-2.92, $P=0.07$) was significantly increased in T2DM as compared to controls. C allele frequency was associated with T2DM compared to control with OR 1.41($\chi^2=8.70$, 95% CI=1.12-1.78) [Table 3].

SRB1 protein expression in the healthy control and T2DM
The possible consequences of SRB1 genetic polymorphism on protein expression levels were evaluated by western blot analysis, beta-actin was used as an endogenous control. Results showed that the protein expression of SRB1 was significantly higher in SRB1 TT variant of
healthy controls when compared to T2DM, and the variant might affect SRB1 protein expression levels. Descriptive western blots from healthy controls and T2DM are shown in [Figure 1].

Discussion
The results of numerous previous association studies between the SRB1 rs5888 SNP and diabetes are not constant in unlike ethnic groups. Therefore, the genetic confirmation for an association between the SRB1 SNP and diabetes in humans still prerequisites to be explained. Additionally, selecting control and case group is vital for genetic study as any systematic error among both groups can result in false-positive, false-negative results. There are several risk factors for type 2 diabetes that primarily includes increased age, gender, dyslipidemia, smoking, increase serum TC and LDL. To mitigate the effect of confounders caused by the mismatch between the two groups, type 2 diabetes and control subjects were matched in age, gender, BMI.

Furthermore, in the present study, we observed that SRB1 CC genotype was significantly associated with an increased T2DM risk, which is in concordance with previous findings that also reported a significant association between the SRB1 polymorphism and T2DM risk. In agreement with this, a previous study from the American Caucasians population also showed that SRB1 was significantly associated with an increased risk of T2DM. Although the precise role of SRB1 rs5888 concerning T2DM risk is still under investigation, evidences suggested that SRB1 gene variants may affect the disease susceptibility by altering the levels of SRB1 that might contribute to the risk of T2DM. More to the point, in the present study, we noticed that the SRB1 CC variant had decreased SRB1 protein expression which might be due to variation in the regulatory region of the SRB1 gene that could involve in differential expression of SRB1 as the variant was located in the promoter region of the gene and could hinder the binding of transcriptional factor. Additionally, previous reports also found that the rs5888 SNP affected SRBI RNA secondary structure, which changed its ability to undergo productive protein translation leading ultimately to significantly lower SRB1 protein expression. Our findings indicated that the lower expression level of the SRB1 protein might be due to variation at 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 and thus affecting the level of protein expression \(^{20}\).

Further, the involvement of numerous variants in the SRB1 gene and serum lipid profiles has been described in distinct populations worldwide. In the present study, control subjects carrying the SRB1 TT genotype had higher HDL levels when compared with the CC genotype. The result was in concordance with a previous study, which also revealed that the rs5888 T allele is significantly associated with higher HDL levels among the Tunisian population \(^{11}\). Also, type 2 diabetes subjects with CC genotype had a significantly low level of HDL. As mentioned above, the result indicated that patients with T2DM have significantly higher levels of SRB1 and significantly higher frequency of homozygous CC SNPs (rs5888). These findings suggests that SRB1 may be potential biomarker for incidence of T2DM.

**Conclusion**

Present study indicated an increased number of CC SNPs in T2DM with reduced SRB1 protein expression that might be associated with the occurrence of T2DM mellitus and could possibly be used as a biomarker for early diagnosis. Furthermore, after completion of research, the present study has certain limitations because it was performed on with a small sample size and single centric. Additionally, we suggest that the study be conducted on a large sample size with multicentric considering different ethnic groups to identify the potential relationship of these loci. Knowing the genes involves will help us to understand the mechanism of disease better and make improved treatment. Future efforts should use a genetic approach to increase the functional efficiency of SRB1 which could result in the decreased occurrence of diabetes mellitus. However, maintaining a healthy diet, physical activity and dedication to lifestyle changes in individuals with increased risk could delay or prevent diabetes.

**Acknowledgment**

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**Conflict of Interest**

The authors declare no conflicts of interest.
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References
10. McCarthy, J. J. *et al.* Polymorphisms of the scavenger receptor class B member 1 are associated with insulin resistance with evidence of gene by sex interaction. *Journal of*


20. Introduction, I. Monogenic Diabetes: What It Teaches Us on the Common Forms of Type 1 and Type 2 Diabetes. 37, 190–222 (2016).
Table 1: Clinical and biochemical parameters of cases and controls

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Controls</th>
<th>T2DM</th>
<th>OR (95% CI) P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>47.5±7.3</td>
<td>48.6±9.7</td>
<td>0.11</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.4 ± 4.9</td>
<td>26.5±5.10</td>
<td>0.02*</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>169</td>
<td>138</td>
<td>1.5 (1.09- 2.09) 0.01*</td>
</tr>
<tr>
<td>No</td>
<td>131</td>
<td>162</td>
<td></td>
</tr>
<tr>
<td>Alcoholic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>165</td>
<td>156</td>
<td>1.12 (0.81-1.55) 0.51</td>
</tr>
<tr>
<td>No</td>
<td>135</td>
<td>144</td>
<td></td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>42.3 ± 8.5</td>
<td>46.5 ± 7.9</td>
<td>0.001*</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>97.8 ± 12.7</td>
<td>139.2 ± 22.5</td>
<td>0.001*</td>
</tr>
<tr>
<td>VLDL (mg/dL)</td>
<td>44.5 ± 11.3</td>
<td>42.6 ± 8.5</td>
<td>0.001*</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>122.6 ± 11.7</td>
<td>206.2 ± 34.5</td>
<td>0.001*</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>184.4±21.5</td>
<td>215.2±33.4</td>
<td>0.001*</td>
</tr>
<tr>
<td>FBS (mg/dL)</td>
<td>78.4 ± 13.7</td>
<td>133.2 ± 17.4</td>
<td>0.001*</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.6 ± 0.7</td>
<td>7.94 ± 0.99</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

Values are mean ±SD, *=P ≤ 0.05, HDL- high density lipoprotein, LDL- low density lipoprotein, VLDL- very low density lipoprotein, TG- triglycerides, FBS- fasting blood sugar, HbA1c- glycated hemoglobin

Table 2: Association between lipid parameters and SRB1 rs5888 polymorphism in T2DM and comparison with healthy control

<table>
<thead>
<tr>
<th>Lipid</th>
<th>TT N=80</th>
<th>CT N=147</th>
<th>CC N=73</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>97.2±27.6</td>
<td>95.2±30.1</td>
<td>104±29.4</td>
<td>0.10</td>
</tr>
<tr>
<td>VLDL (mg/dL)</td>
<td>41±9.4</td>
<td>40.3±9.1</td>
<td>43.1±10.6</td>
<td>0.12</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>172.1±39.7</td>
<td>177±29.6</td>
<td>176.8±33.8</td>
<td>0.544</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>144.2±23.5</td>
<td>142.6±33.1</td>
<td>147.5±26.1</td>
<td>0.841</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>43.1±11.5</td>
<td>39.5±9.7</td>
<td>39.7±10.2</td>
<td><strong>0.03</strong></td>
</tr>
<tr>
<td>Cases</td>
<td>N=63</td>
<td>N=129</td>
<td>N=108</td>
<td></td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>116.5±22.4</td>
<td>123.7±17.2</td>
<td>130.3±36.4</td>
<td><strong>0.02</strong></td>
</tr>
<tr>
<td>VLDL (mg/dL)</td>
<td>41.4±10</td>
<td>43.5±10.2</td>
<td>44±12.5</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>Controls (n=300)</td>
<td>T2DM (n=300)</td>
<td>OR (95% CI)</td>
<td>P-Value</td>
</tr>
<tr>
<td>---------------</td>
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<td>---------</td>
</tr>
<tr>
<td><strong>SRB1 (rs5888)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>80 (27%)</td>
<td>63 (21%)</td>
<td>Reference (1.00)</td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>147 (49%)</td>
<td>129 (43%)</td>
<td>1.11 (0.74-1.67)</td>
<td>0.67</td>
</tr>
<tr>
<td>CC</td>
<td>73 (24%)</td>
<td>108 (36%)</td>
<td>1.87 (1.20-2.92)</td>
<td><strong>0.007</strong>*</td>
</tr>
<tr>
<td><strong>T</strong></td>
<td>307 (51%)</td>
<td>255 (43%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>C</strong></td>
<td>293 (49%)</td>
<td>345 (57%)</td>
<td>1.41 (1.12-1.78)</td>
<td><strong>0.003</strong>*</td>
</tr>
</tbody>
</table>

OR= Odds Ratio, CI- Confidence Interval, Chi square test (χ²)
*bold represents P ≤ 0.05 was considered statistically significant
Figure 1: (A) Representative western blot, using β Actin as the loading control and protein expression levels of SRB1 in the healthy control and T2DM. Total proteins were isolated and separated by SDS PAGE and subjected to immunoblot analyses. (B) Protein expression levels of SRB1 in healthy control and T2DM. P < 0.05; SRB1, Scavenger receptor class b type 1; T2DM, Type 2 diabetes mellitus.