Association of RETN C-420G single nucleotide polymorphism with type 2 diabetes mellitus in Pakistani Punjabi Rajput population

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Abstract

Objectives: To determine the association of human resistin gene RETN C-420G single nucleotide polymorphism with type 2 diabetes mellitus in a specific ethnic population.

Methods: The controlled study was conducted from June 2012 to January 2015 at Military Hospital, Rawalpindi, Army Medical College, Rawalpindi, and the Institute of Biomedical and Genetic Engineering, Islamabad, Pakistan. Patients with type 2 diabetes and healthy controls belonging to Pakistani Punjabi Rajput ethnic group were genotyped for human resistin gene RETNC-420G single nucleotide polymorphism. Serum resistin, serum insulin, fasting blood sugar, lipid profile, body mass index and insulin resistance was determined and correlated with genotypes. SPSS 18 was used for data analysis.

Results: Of the 789 subjects, 539(68%) were diabetics and 250(32%) were controls. Serum resistin levels were significantly higher in diabetics than controls (p<0.05). The frequency of GG, GC and CC was 15(2.8%), 322(59.75%) and 202(37.5%) in diabtics. This single nucleotide polymorphism was associated with diabetes (p<0.02). Human resistin gene RETN C-420G single nucleotide polymorphism was not associated with serum resistin, insulin, body mass index, insulin resistance and dyslipidaemia in both groups (p<0.05 each).

Conclusions: Human resistin gene RETN C-420G single nucleotide polymorphism was found to be a risk factor for type 2 diabetes in Pakistani Punjabi Rajput population.

Keywords: Diabetes mellitus, Resistin, Single nucleotide polymorphism, RETN C-420G, Body mass index. (JPMA 68:1584; 2018)
RETNC-420G SNP is associated with T2DM, augmented serum resistin levels, IR, BMI and dyslipidaemia. A few studies have reported contrary results as well. Various studies regarding frequencies of genotypes, association with risk of T2DM and parameters of metabolic syndrome show inter-ethnic variations.

No study has been done yet to determine genotypes of RETN gene in Pakistani population. The current study was planned to determine the frequency of RETN C-420G SNP and its association with T2DM in Pakistani Punjabi Rajput ethnic group.

Materials and Methods
The controlled study was conducted from June 2012 to January 2015 at Military Hospital, Rawalpindi, Army Medical College, Rawalpindi, and the Institute of Biomedical and Genetic Engineering, Islamabad, Pakistan. The study was approved by the ethical committees of the institutions and written informed consent was obtained from the participating subjects. A total of 789 subjects were selected by non-probability convenient sampling technique out of which, 539 were patients of T2DM and 250 were healthy controls belonging to Pakistani Punjabi Rajput ethnic group. Diagnosed cases were enrolled on the basis of WHO criteria of T2DM. Patients were selected with less than 5 years duration of the disease and without clinical evidence of acute infections. In control group, healthy subjects with normal blood glucose levels and without clinical evidence of acute infections were included. Smokers and pregnant females were excluded from the study.

Blood glucose fasting levels were determined to exclude undiagnosed T2DM cases. Blood samples were collected under aseptic measures. Plasma glucose was measured by glucose oxidation method, serum TC, HDL and TG were estimated by enzymatic method (Vitalab Selectra E) while LDL was calculated by using Friedewald formula. Plasma fasting insulin levels were measured by enzyme immunoassay (EIA) method (Life Technologies, Frederick, USA). IR was calculated by homeostatic model of assessment of insulin resistance (HOMA-IR) by the formula FBG (mmol/L) x fasting plasma insulin (picomol/L)/22.5. Quantitative measurement of serum resistin was done using Enzyme-linked immunosorbent assay (ELISA) (Life Technologies, Invitrogen Corporation, Frederick, USA). The intra-assay and inter-assay coefficient of variation for resistin was 2.86% and 7.2% respectively and the minimum detectable concentration was 100 pg/ml. Standard laboratory procedures were followed for all biochemical tests.

DNA was extracted from blood cell by standard Phenol and Chloroform method. Genotyping of samples was done for RETN C-420G (rs 1862513) SNP by PCR-restriction fragment length polymorphism (PCR-RFLP) method and was confirmed on deoxyribonucleic acid (DNA) sequencing. Two replicates of positive and one replicate of negative controls were included in each PCR. For PCR-RFLP, allele-specific primers for PCR-RFLP for RETN C-420G polymorphism was designed at link (primer3.ut.ee). A 484-bp fragment of RETN gene was amplified. The forward and reverse primer sequences were RETN- 420 F: 5’- CTGTGCTGGTTGCCCAGACT- 3’ and RETN- 420 R: 5’ - TGGGCTCAGCTAACCAAATC- 3’ respectively. The reaction was done in a final volume of 20 µl containing 2 µl of 10 X PCR buffer without magnesium chloride(MgCl2) (Fermentas, Lithuania), 1 µl of 25 mM MgCl2 (Fermentas, Lithuan4a), 1 µl 2mM deoxyribonucleotide triphosphate (dNTPs) (Fermentas, Lithuania), 0.1 µl of 5U Taq DNA polymerase enzyme (Fermentas, Lithuan4a), 1 µl of 20 µM of forward and reverse primers, 2 µl of 40 ng/µl human genomic DNA and volume of the reaction was made up to 20µl with autoclaved deionised water. DNA was denatured for 5 min at 95°C and then subjected to 35 amplification cycles. Each PCR cycle comprised denaturation at 95°C for 45 seconds, annealing at 61°C for 45 seconds and extension at 72°C for 10 minutes. Products were held at 4°C.

In the next step, 10 µl of 484 bp PCR products, one µl of restriction enzyme Bst6I (Fermentas), 3 µl of 10 X buffer and 16 µl of deionised water was mixed to make a total volume of 30 µl reaction mixtures. The reaction mix was incubated at 37°C for 16 hours on a shaking water bath and was then analysed using 2.5% agarose gel. GG homozygotes were resolved as 275 and 206 bp fragments; CC minor alleles were resolved as 484 bp fragments and GC heterozygotes were revealed as 484, 275 and 206 bp fragments (Figure-1). Validation of genotypes by PCR-RFLP were confirmed by DNA sequencing (Applied Biosystems® 3130 Genetic Analyser by Life Technologies; USA) done on samples selected randomly (Figure-2). Statistical analysis was done using SPSS 18. Distribution of data was estimated by applying Kolmogorov-Smirnov test. Continuous variables with Gaussian distribution of
both study and control groups were compared using Independent student’s t-test which included age, BMI, lipid profile and fasting blood glucose (FBG). Variables with non-Gaussian distribution included duration of disease, HOMA-IR and serum resistin levels which were determined using Mann-Whitney test and their median and inter-quartile range (IQR) were calculated. Genotypes and allele frequencies were estimated and odd ratios (OR) were calculated and compared by regression analysis for risk estimate between study and control groups. Difference between RETN gene polymorphism with serum resistin level, BMI, lipid profile, FBG, serum insulin levels and IR were assessed by analysis of variance (ANOVA) and Post-hoc Tukey’s test. Bivariate and stepwise multivariable logistic regression analysis was performed to estimate the correlations between various variables. Logistic regression analysis was applied to determine the odds ratio (OR) between independent variables including age, BMI, duration of the disease, dyslipidaemia, FBG, serum insulin, HOMA-IR and serum resistin levels, and the presence of risk allele as dependent variable. The distribution of genotypic frequencies of RETN C-420G within the Hardy-Weinberg equilibrium was determined using Fischer exact test. The results were graphically illustrated by drawing triangular ternary plot through DeFinetti diagram. Values were considered statistically significant when p<0.05.

Results

Of the 789 subjects, 539(68%) were diabetics and 250(32%) were controls. Demographic features and baseline biochemical parameters were recorded for all subjects (Table-1). BMI was significantly raised in patients compared to controls (p=0.0001). In lipid profile, only serum TC was significantly higher in patients compared to controls (p=0.0001). FBG, serum insulin and IR were significantly higher in patients compared to controls (p=0.0001 each). Serum resistin levels were also significantly higher in the
patients compared to controls (p=0.001).

In multivariate regression analysis, serum resistin was a predictor of IR along with raised serum insulin in obese but not in lean T2DM patients (p=0.0001). There was no significant difference in serum resistin levels in patients for the three genotypic profiles (p=0.74). Non-significant difference was found for HOMA-IR (p=0.58), BMI (p=0.43) serum insulin (p=0.61) and dyslipidaemia (p=0.42). On sub-grouping the two groups based on genotypic profiling, CC, GC and GG genotypes did not differ from each other in relation to serum resistin levels, IR, BMI, serum insulin levels and lipid profile (p>0.05 each).

The allele frequency of RETN C-420G SNP for patients was GG15 (2.8 %), GC 322 (59.75 %) and CC 202 (37.5 %) compared to 4 (1.6 %), 32 (52.8 %) and 114 (45.6 %) in controls. Regression analysis showed RETN C-420GSNP was significantly associated with T2DM (OR = 1.41; 95% confidence interval CI= 1.03-1.89, p< 0.02) by. No association was found between any of the demographic and biochemical parameters with presence of risk allele (Table-2).

On multivariable logistic regression analysis, the presence of risk allele was not correlated to duration of the disease (p= 0.14), BMI (p= 0.99), serum TC (p= 0.27), serum TG (p= 0.22), serum LDL (p= 0.27), serum HDL (p= 0.13), FBG (p= 0.58), serum insulin (p= 0.83), HOMA-IR (p= 0.78) and serum resistin (p= 0.32).

### Table-1: Demographic features and biochemical parameters of type 2 diabetes mellitus and healthy controls in Pakistani Rajput ethnic group.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Study group</th>
<th>Control Group</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Age (years)</td>
<td>43.34±4.99</td>
<td>42.88±4.35</td>
<td>0.2</td>
</tr>
<tr>
<td>Gender F/M</td>
<td>265/274</td>
<td>126/124</td>
<td>0.69</td>
</tr>
<tr>
<td>Duration of disease† (years)</td>
<td>4 (2-6)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BMI (in kg/m2)</td>
<td>27.78±4.62</td>
<td>24.13±4.35</td>
<td>*0.0001</td>
</tr>
<tr>
<td>S. TC (mmol/L)</td>
<td>4.57±1.06</td>
<td>4.3±0.92</td>
<td>*0.0001</td>
</tr>
<tr>
<td>S. TG (mmol/L)</td>
<td>2.09±1.09</td>
<td>2.02±1.01</td>
<td>0.28</td>
</tr>
<tr>
<td>S. LDL (mmol/L)</td>
<td>2.55±0.85</td>
<td>2.45±0.82</td>
<td>0.14</td>
</tr>
<tr>
<td>S. HDL (mmol/L)</td>
<td>1.09±0.27</td>
<td>1.1±0.33</td>
<td>0.54</td>
</tr>
<tr>
<td>FBG (mg/mL)</td>
<td>8.38±2.2</td>
<td>5.2±0.58</td>
<td>*0.0001</td>
</tr>
<tr>
<td>Fasting Serum insulin† (µIU/L)</td>
<td>12.03(8.59-14.95)</td>
<td>8.53(6.04-10.58)</td>
<td>*0.0001</td>
</tr>
<tr>
<td>HOMA-IR†</td>
<td>4.37(3.08-5.92)</td>
<td>1.94(1.32-2.43)</td>
<td>*0.0001</td>
</tr>
<tr>
<td>S. resistin†</td>
<td>10.64(7.31-13.41)</td>
<td>9.58(6.53-11.89)</td>
<td>*0.001</td>
</tr>
</tbody>
</table>

†Median (IQR), *Significant p value, IQR: Inter-Quartile Range. BMI: Body Mass Index.

### Table-2: Multivariate and logistic regression analysis among metabolic parameters.

**Multivariate regression analysis**

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Independent Variable</th>
<th>R value</th>
<th>R² value</th>
<th>β value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOMA-IR</td>
<td>S. resistin</td>
<td>0.767</td>
<td>0.588</td>
<td>0.34</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>S. insulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Logistic regression analysis**

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Independent Variable</th>
<th>β coefficient</th>
<th>Sig.</th>
<th>Expo(β)</th>
<th>95% CI of Expo(β)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Risk allele G</td>
<td>Duration</td>
<td>-0.047</td>
<td>0.14</td>
<td>0.95</td>
<td>0.895</td>
</tr>
<tr>
<td></td>
<td>BMI</td>
<td>0.0001</td>
<td>0.99</td>
<td>1.01</td>
<td>0.962</td>
</tr>
<tr>
<td></td>
<td>TC</td>
<td>0.093</td>
<td>0.27</td>
<td>0.91</td>
<td>0.769</td>
</tr>
<tr>
<td></td>
<td>TG</td>
<td>0.099</td>
<td>0.22</td>
<td>1.1</td>
<td>0.940</td>
</tr>
<tr>
<td></td>
<td>LDL</td>
<td>0.119</td>
<td>0.27</td>
<td>0.88</td>
<td>0.719</td>
</tr>
<tr>
<td></td>
<td>HDL</td>
<td>0.067</td>
<td>0.13</td>
<td>0.54</td>
<td>0.247</td>
</tr>
<tr>
<td></td>
<td>FBG</td>
<td>0.025</td>
<td>0.58</td>
<td>1.02</td>
<td>0.936</td>
</tr>
<tr>
<td></td>
<td>S. insulin</td>
<td>0.006</td>
<td>0.83</td>
<td>1.006</td>
<td>0.952</td>
</tr>
<tr>
<td></td>
<td>HOMA-IR</td>
<td>0.021</td>
<td>0.78</td>
<td>1.02</td>
<td>0.879</td>
</tr>
<tr>
<td></td>
<td>S. resistin</td>
<td>0.02</td>
<td>0.32</td>
<td>1.02</td>
<td>0.98</td>
</tr>
</tbody>
</table>

HOMA-IR: Homeostatic Model for Assessment of Insulin Resistance.

### Discussion

The RETN genotypes and allele frequency distribution in Punjabi Rajput ethnic group is reported for the first time in Pakistani population in our study. The present data shows 62.5% frequency of RETN C-420G risk allele in T2DM patients and 54.4% in healthy Pakistani population. The presence of G allele variant in RETN gene at -420 positions in promoter regions rendered the risk of T2DM 1.41 times more in our population. Many ethnic groups have shown
similar results. Homozygous RETN GG genotype is associated with T2DM in Egyptian, Japanese, Chinese and South Asian countries but a series of genome-wide association study (GWAS) researches conducted on Indian Asians revealed the positive association of RETN gene promoter variant with T2DM. Although sufficient data indicate the positive results, this association with T2DM has been challenged by many studies. Some studies reported even contrary results in which minor allele G was found to be associated with lower risk of T2DM. Our study is the first one reporting RETN C-420G SNP allele frequencies and association with T2DM in Pakistani population.

In our study, serum levels of resistin were significantly higher in T2DM compared to healthy persons, as was also found in other studies. Resistin suppresses the differentiation of adipocytes by 80% leading to fatty acid overload and lipotoxicity by increasing the production of LDL and degradation of LDL receptors in liver and muscles leading to metabolic syndrome. Resistin induces IR by suppressing insulin-induced suppression of gluconeogenesis in liver and hyperglycaemia by impairing glucose transport in skeletal muscles. In our study, serum levels became comparable when T2DM and healthy persons were sub-grouped into GG, GC and CC genotypes, indicating lack of association of RETN C-420G SNP with serum resistin levels. On multivariate regression analysis, serum resistin was a predictor of IR along with raised serum insulin in obese but not in lean T2DM patients. On sub-grouping, the two groups based on genotypic profiling of CC, GC and GG genotypes did not differ from each other in relation to IR, BMI and serum insulin levels. The findings are supported by studies in diabetic Americans, Egyptian obese subjects and healthy Italians. Contrary to our findings, RETN C-420G SNP was correlated with higher serum levels of resistin and IR in many studies. In this study, there was no association found between RETN C-420G allele and higher BMI or dyslipidaemia, shown in previous studies, although significant association was found in some other studies. Inter-ethnic variations cannot be explained easily. Although varying results are reported regarding association of RETN C-420G SNP with T2DM, serum levels, BMI, IR and dyslipidaemia, the findings do not exclude the association of risk allele G with these parameters. The difference in biochemical and genetic phase of the current study can be attributed to unequal division of subjects in genetically characterised sub-groups. Only 2.8% in T2DM and 1.6% in healthy group was of GG genotype in our study. This explains the significant difference in serum resistin levels between the groups but insignificant difference in the sub-groups based on genotypes. Larger sample size studies with significant number of individuals in each genotype are recommended to reach a definitive conclusion. It depicts that these associations are modified by various factors in different ethnic groups. Gene-gene, gene-environment and gene-nutrient interactions modulate these associations. For example, in Japanese healthy persons, presence of Pro/Pro genotype of peroxisome proliferator-activated receptor gamma (PPAR-γ) had synergistic effect with RETNC-420G allele in augmenting serum resistin levels. SNPs in regions outside the coding region may influence transcription or mRNA stability and thus affect the expression of the gene differently among various ethnic groups.

Some nuclear proteins specifically recognise one base difference at SNP -358 in RETN gene and A at RETN -358 is not associated with increased serum resistin levels, BMI, IR and dyslipidaemia in genetically susceptible individuals. In Caucasians, the correlation between SNP RETN C-420G and plasma resistin is not strong, and A at RETN-358 may not exist, suggesting that SNP RETN-358 could explain this ethnic difference. It is possible that Pakistani Punjabi Rajput ethnic group also lacks A at RETN -358 which can be verified by further studies.

Future studies with larger sample size of all ethnic groups of Pakistani population are required to substantiate the findings of the current study. Co-existent multiple SNPs in promoter region of RETN gene can influence the impact of this SNP at transcription and translation, and, thus, the serum levels. The study of all the haplotypes of RETN gene was beyond the scope of the current study.

Conclusion
Functionally significant sequence variation of RETN C-420G genes was found to be widely distributed in Pakistani population in Punjabi Rajput ethnic group at varying frequencies in T2DM and healthy persons. This risk allele is not associated with increased serum resistin levels, BMI, IR and dyslipidaemia in genetically susceptible individuals.
Reference:


