Role of the ENPP1 K121Q Polymorphism and Susceptibility to Type 2 Diabetes in North Indian Punjabi Population

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Abstract
In many worldwide populations, the K121Q variant of ENPP1 gene are associated with increased risk of type 2 diabetes mellitus (T2DM). The objective of the present study was to investigate the association of K121Q variant of ENPP1 gene with T2DM in North Indian Punjabi populations. A total of 500 participants consisting 250 T2DM patients and 250 healthy unrelated subjects were recruited for this study. The PCR-RFLP method was used for genotyping of K121Q polymorphism. The minor allele Q was found to be significantly associated with increased risk of T2DM (OR: 1.44, 95% CI: 1.06-1.94, p=0.015). A significant difference in QQ genotype was observed between T2DM patients and control groups (OR: 2.04, 95% CI: 1.04-3.98, p=0.033). It is also observed that co-dominant model would be best fit to predict the susceptible gene effect (OR: 1.36, 95% CI: 1.04-1.80, p=0.026). This study confirmed the association of ENPP1 polymorphism with increased risk of T2DM.

Keywords: ENPP1; K121Q variant; T2DM; Punjabi population

Introduction
Type 2 Diabetes Mellitus (T2DM) has become a growing public health problem associated with socioeconomic lifestyle factors in both developed and developing countries like India. It is a complex heterogeneous metabolic disorders characterised by both environment and genetic factors [1-4]. It is estimated that the prevalence of T2DM worldwide is around 6% and it is likely to rise many folds over next decade due to economic transition and increasing the age of population [5,6]. In recent time several candidate genes have been suggested for defects in insulin signalling pathways through genome-wide association study [7,8]. However, one of such gene Ectoenzyme Endonucleotide Pyrophosphatase Phosphodiesterase 1 (ENPP1) has shown a strong association with increased risk of T2DM. It has suggested that this membrane glycoprotein (ENPP1) down regulates insulin signalling by inhibiting insulin receptor's tyrosine kinase activity. Therefore, it has also proposed that Q allele of K121Q in ENPP1 gene enhance the risk of developing insulin resistance [9,10]. The ENPP1 gene is located on 6q22-23, a locus has been found to be associated with T2DM and obesity in many ethnic populations [11-18]. In contrast, negative associations of Q allele with T2DM have also been demonstrated in many populations [19-24]. Therefore, in context of these ambiguous findings motivated us to investigate the association between ENPP1 K121Q variant and T2DM in an Indian Punjabi population. To our knowledge very scanty studies with this SNP have been available in the North Indian Punjabi populations.

Material and Methods
Subjects
The studied population consisted of 250 unrelated T2DM patients and 250 normoglycemic controls. The T2DM patients were recruited from the different clinical centres (Heart Station and Diabetic Clinic, A.P. Hospital, Heart Care Centre, Diabetic Clinic and Research Institute) in Amritsar district in Punjab, a North Indian state. Age and sex matched unrelated individuals without T2DM were recruited randomly from the same area to serve as control subjects. All participants provided written informed consent. T2DM patient diagnosis has been done based on the criteria of American Diabetes Association [25], with fasting plasma glucose ≥ 126 mg/dL, 2-hr plasma glucose ≥ 200 mg/dL during oral glucose tolerance test. The detailed protocol of the study was approved by the Ethical Committee of Guru Nanak Dev University.

Inclusion/Exclusion Criteria
At the time of data collection, the individuals over 40 years old and meeting above diagnostic criteria from north Indian Punjabi Population were included in the study. Only one member from one family without having family history of T2DM at least before two generations was taken. Individuals with type I diabetes, a family member with type I and type II diabetes, with any illness/ chronic disease interfering with patients and with any secondary diabetes were excluded from the study.

Measurements of clinical characteristics
Anthropometric measurements of height, weight, waist and hip circumference, skinfold thickness (biceps and triceps) were taken on each individual using standard anthropometric techniques and tools [26,27]. The Body Mass Index (BMI) was calculated as the weight in kg divided by square of height in meters. Waist to Hip Ratio (WHR) was calculated as waist circumference (cm) divided by hip circumference (cm). Height and weight were measured to the nearest 0.5 cm and 0.1 kg respectively. A Lange skinfold calliper was used to measure the skinfolds to the nearest 0.2 mm. Actual age and age on onset of the disease were recorded from the subject's health-card provided by the clinical centres.

Physiometric measurements
Left arm blood pressure (first phase systolic and fifth phase diastolic)

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Received August 26, 2014; Accepted September 18, 2014; Published September 24, 2014

Citation: Badaruddoza, Barna B, Matharoo K, Bhanwer AJS (2014) Role of the ENPP1 K121Q Polymorphism and Susceptibility to Type 2 Diabetes in North Indian Punjabi Population. J Diabetes Metab 5: 450 doi:10.4172/2155-6156.1000450

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were taken from each participant with mercury sphygmomanometer after a 5 minute rest. The average of the two subsequent measurements was used for the analysis. All efforts were made to minimize the factors which affect the blood pressure like anxiety, fear, stress, laughing and recent activities [28]. The radial artery at the wrist was used to count the pulse. It was counted over one minute. The difference of SBP and DBP was used as pulse pressure.

DNA extraction and genotyping

Genomic DNA was extracted from the peripheral blood using the phenol/Chloroform method.

The PCR mixture contained 20ng genomic DNA, 0.2 µM of each primer, 200 µM of each dNTP, 1.5 mM of tris-HCl buffer (pH 9.0), 1.5 mM MgCl₂, and 0.024 unit of Tag DNA polymerase in a final reaction volume of 15 µl. The amplification cycle was performed on Master cycler personal (Eppendorf AG 5332). The initial denaturing was set up at 94˚C for 5 minutes followed by 30 cycles of denaturation at 94˚C for 30 seconds, annealing at 55˚C for 40 seconds, extension at 72˚C for 40 seconds and the final extension was at 72˚C for 5 min. The PCR product was stored at 4˚C until further use. The amplified PCR product (208bp) was digested with 1 units of Ava II restriction enzyme (New England Biolabs, USA) at 37˚C for 15 hrs with 1x NEB buffer4 in a final volume of 15 µl. Reaction followed by heat inactivation for 20 min at 65˚C and analyzed on 2% agarose gel.

Statistical Analysis

All statistical analyses were done on SPSS (Statistical Package for Social Sciences, version 19.0, Chicago, IL, USA). A p value of <0.05 (two tailed) was considered to be significant. Genotypic and allele frequencies were compared using the Chi-square probability test. The t-test was applied to compare the means of quantitative traits between control groups. The criteria based on WHO (2004), it was observed maximum central obesity in T2DM patients group (BMI=27.72%) as compared to control groups (BMI=24.5). There was a significant (p<0.001) difference observed in fasting and random glucose level among diabetic group (181.23 ± 20.60 for fasting glucose and 218.25 ± 20.80 mg/dl for random glucose level respectively) compared to non-diabetic control (103.64 ± 18.58 mg/dl for fasting glucose and 118.05 ± 15.28 mg/dl for random glucose level respectively). The means of SBP, DBP, pulse rate and pulse pressure have also been significantly higher (p<0.001) in T2DM patient groups.

Table 2 summarizes the comparison of clinical characteristics between T2DM patients and non-diabetic control groups stratified by the genotypes of ENPP1 K121Q polymorphism. It was observed that among diabetic individuals carrying KK genotype, a significantly higher almost all clinical characteristics were observed (p<0.001). Similarly, among diabetic individuals carrying KQ+QQ genotypes have also shown significantly higher clinical characteristic values (p<0.001) as compared to non-diabetic controls with same genotypes.

In case-control association study, we genotyped (Table 3) ENPP1 K121Q variant in 250 unrelated T2DM patients and 250 non-diabetic control subjects. The genotype relative risk was calculated when compared with baseline genotypes.

The frequencies of KK, KQ and QQ genotypes in T2DM patients were 59.20, 30.00 and 10.80% respectively as compared to the non-diabetic controls (67.20, 26.80 and 6.00%). A significant difference (p<0.023) in the distribution of KK, KQ and QQ genotypes was observed. The minor allele Q of the ENPP1 K121Q variant was found to significantly increase T2DM risk, with allele Odds Ratio (OR) of 1.44 (95% CI: 1.06-1.94, p=0.05) in this study population. The Q allele frequency was 25.80% in T2DM patients and 19.40% in normoglycemic controls. The genotype relative risk was calculated when compared with baseline genotypes.

The sample size calculation showed that the minimum 210 samples of each cases and control groups were sufficient for 80% power (α=0.05) to detect an association with OR of 1.5. Allele frequencies were used from previous studies in this population.

Results

The anthropometric and physiometric characteristics of the study subjects are summarized in Table 1. Among the individuals with T2DM, the mean age of diagnosis was 48.12 ± 8.20 years. The duration of diabetes was 8.30 ± 6.20 years. The mean height, weight, BMI, waist and hip circumferences, WHR and thickness of skinfolds were significantly (p<0.001) higher in type 2 diabetic patients as compared to control subjects. The criteria based on WHO (2004), it was observed maximum central obesity in T2DM patients group (BMI=27.72%) as compared to control groups (BMI=24.5). There was a significant (p<0.001) difference observed in fasting and random glucose level among diabetic group (181.23 ± 20.60 for fasting glucose and 218.25 ± 20.80 mg/dl for random glucose level respectively) compared to non-diabetic control (103.64 ± 18.58 mg/dl for fasting glucose and 118.05 ± 15.28 mg/dl for random glucose level respectively). The means of SBP, DBP, pulse rate and pulse pressure have also been significantly higher (p<0.001) in T2DM patient groups.

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To find out which model would fit the effect of ENPP1 K121Q variant, dominant, co-dominant and recessive models were considered. In the dominant model (KQ/QQ vs. KK), the genotype frequencies were compared between the T2DM patients and control groups and no significant association was observed (OR: 1.41, 95% CI: 0.98-2.03, p=0.063). In the recessive model (QQ vs. KK/QQ), a significant association was observed (OR: 1.90, 95% CI: 0.98-3.66, p=0.05). However, the strong association was found in co-dominant model (OR: 1.36, 95% CI: 1.04-1.80, p=0.026). Based on 2% agarose gel electrophoresis, the polymorphism chain reaction-restriction-fragment length polymorphism (PCR-RFLP) product of K121Q polymorphism
of ENPP1 gene was determined. The Ava II restriction enzyme has been used to digest the PCR product to detect the homozygous wild (KK) fragment (208 bp), homozygous mutant (KQ) fragment (153 bp and 55 bp) and heterozygous fragment (208 bp, 153 bp and 55 bp) (Figure 1).

Discussion

The aim of the present study was to identify the association of ENPP1 K121Q polymorphism with T2DM in North Indian Punjabi population. The study observed that minor allele Q was significantly associated with the increased risk of T2DM in this population. Many studies have shown stronger association between ENPP1 K121Q gene with T2DM risk [6,9,29-31]. In these studies indicate that insulin resistance is the major cause of the pathogenesis of type 2 diabetes. The ENPP1 K121Q variants inhibit the insulin receptor's tyrosine kinase activity and confer insulin resistance. It has also observed the increased odds ratio between type 2 diabetes and ENPP1 K121Q variant in other ethnic populations such as Domican [12], South Asian, Caucasian [9], Finnish [32] and French population [33]. Furthermore, in meta-analysis for the association between ENPP1 K121Q polymorphism and type 2 diabetes showed significant association through odds ratio [34]. Many studies failed to show the significant association between ENPP1 K121Q variant and T2DM in many populations [19,22,35]. One available study from Punjabi Population [24] has not also been detected any significant association between ENPP1 K121Q variant, Body Mass Index (BMI), Waist to Hip Ratio (WHR), and level of cholesterol with type 2 diabetes. However, in the present study, the frequency of the QQ genotype presence of two minor allele Q (risk allele) was significantly higher in T2DM patients as compared to normoglycemic controls, therefore, suggesting a gene dosage effect. The frequency of KQ genotype was higher in T2DM patients as compared to controls and no significant difference was observed between T2DM. All important clinical characteristics such as BMI, WHR, skinfold thickness, SBP, DBP, pulse pressure and pulse rate were found to be significantly higher in T2DM in both K and Q allele homozygotes. The study has proved that K121Q variant of ENPP1 gene to be significantly associated in North Indian Punjabi population. Therefore, in the view of no apparent association was found in previous study [24], it is possible due to genetic heterogeneity of samples which was collected from different states in India. However, further similar study would be required to test the validity of this association between K121Q variant of ENPP1 gene with T2DM in this population. In conclusion, it is likely to be that the K121Q variant in ENPP1 gene has a significant effect on susceptibility to T2DM in Punjabi population in India.

Authors Contribution

All the authors have read and approved the final manuscript. The author Basanti

Table 2: Differences of clinical characteristics between type 2 diabetic and non-diabetic control subjects stratified by ENPP1 (K121Q) polymorphism.

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>KK genotype</th>
<th>KQ + QQ genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>T2DM</td>
</tr>
<tr>
<td>Age (years)</td>
<td>Mean (n=168)</td>
<td>Mean (n=148)</td>
</tr>
<tr>
<td>Age of Onset (years)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Duration (years)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>156.36</td>
<td>8.95</td>
</tr>
<tr>
<td>Weight (Kg)</td>
<td>65.19</td>
<td>10.47</td>
</tr>
<tr>
<td>Body Mass Index (Kg/m²)</td>
<td>24.49</td>
<td>4.55</td>
</tr>
<tr>
<td>Waist Circumference(cm)</td>
<td>91.56</td>
<td>10.73</td>
</tr>
<tr>
<td>Hip Circumference(cm)</td>
<td>96.84</td>
<td>9.45</td>
</tr>
<tr>
<td>Waist Hip Ratio</td>
<td>0.92</td>
<td>0.07</td>
</tr>
<tr>
<td>Bioeps Skinfold (mm)</td>
<td>10.19</td>
<td>4.98</td>
</tr>
<tr>
<td>Triceps Skinfold (mm)</td>
<td>16.75</td>
<td>6.63</td>
</tr>
</tbody>
</table>

Table 3: Genotypic, allelic frequencies and estimates of relative risk for ENPP1 K121Q polymorphisms in type 2 diabetic and non-diabetic control subjects.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Control N (%)</th>
<th>T2DM N (%)</th>
<th>P value</th>
<th>Test of Association Odds ratio (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>KK</td>
<td>168 (67.20)</td>
<td>148 (59.20)</td>
<td>&lt;0.023</td>
<td>1.27 (0.854 - 1.88)</td>
<td>0.236</td>
</tr>
<tr>
<td>KQ</td>
<td>67 (26.80)</td>
<td>75 (30.00)</td>
<td></td>
<td>2.04 (1.04 – 3.98)</td>
<td>&lt;0.033</td>
</tr>
<tr>
<td>QQ</td>
<td>15 (6.00)</td>
<td>27 (10.80)</td>
<td></td>
<td>1.44 (1.06 – 1.94)</td>
<td>&lt;0.015</td>
</tr>
<tr>
<td>K allele (%)</td>
<td>403 (80.60)</td>
<td>371 (74.20)</td>
<td>&lt;0.015</td>
<td>1.27 (0.854 - 1.88)</td>
<td>0.236</td>
</tr>
<tr>
<td>Q allele (%)</td>
<td>97 (19.40)</td>
<td>129 (25.80)</td>
<td></td>
<td>2.04 (1.04 – 3.98)</td>
<td>&lt;0.033</td>
</tr>
</tbody>
</table>

Figure 1: Restriction enzyme fragments of K121Q polymorphism using AvaII in 2% agarose gel electrophoresis. Lane M shows the 100bp genomic DNA Ladder; Lane 1 & 3 show the heterozygous fragments (208, 153 and 55 bp); Lane 5 shows a mutant fragment (155 and 53 bp) and Lane 2&4 show the wild type fragments (208 bp).
Barna has collected data and carried out the molecular genetic studies, the other authors (Badaruddoza, Kawaljit Matharoo and AJS Bhanwer) participated in the recruitment of the study subjects, study design, statistical analysis, interpretation and manuscript writing and editing.

Acknowledgement

The work was supported by University Grants Commission, New Delhi, [DRS I (UGC-SAP)] and also partially supported by UGC major project (study of genetic polymorphisms of short tandem repeat loci in Punjabi Population of North west Punjab) sancion to Dr. Badaruddoza. The authors would like to extend their gratitude to Dr Rohit Kapoor; Dr. A. P. Singh and Dr. Puneet Arora and to all the subjects who were involved in this study.

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