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Andleeb Batool  
GC University, Lahore, Pakistan, bandleeb@yahoo.com

Riffat Iqbal  
GC University, Lahore, Pakistan

Maryam Mukhtar  
GC University, Lahore, Pakistan

Hong Xue  
Hong Kong University of Science and Technology, Clear Water Bay Kowloon, Hong Kong

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SUSCEPTIBILITY OF NPPA AND IL6 WITH TYPE 2 DIABETES AND HYPERTENSION IN PUNJAB PAKISTAN

Andleeb Batool1*, Riffat Iqbal1, Maryam Mukhtar1, Hong Xue2

1Department of Zoology, G.C. University, Lahore
2Applied Genomic Centre and Division of Life Sciences, Hong Kong University of Science and Technology, Clear Water Bay Kowloon, Hong Kong

Email: bandleeb@yahoo.com

ABSTRACT

Type 2 Diabetes (T2D) and Hypertension are the major health issues affecting quality of life of young ages throughout the world, especially the third world countries facing more complications with diabetes due to poor disease management. The present study was conducted to explore the association of genetic polymorphism with T2D and hypertension in the Punjabi population. The case control study was conducted comprising of 288 patients (118 male, 170 female) and 170 controls (104 male, 66 female). The selected genes along SNPs were NPPA (rs5064 G>A, rs5063 C>T) and IL6 (rs1800796 C>G). DNA was amplified by Nested PCR and sequencing was performed for genotyping. The rs5063 and rs5064 from NPPA was not associated with hypertension and not involved in the predisposition of diabetes (p < 0.05). Moreover, rs1800796 (IL6) showed an association (p < 0.001) with diabetes (OR = 0.394 (0.265, 0.584). SNPs analysis with demographic data confirmed that rs1800796- CC (p = 0.008) is significantly associated with positive family history of T2D. Risk of T2D development associated with IL6 was confirmed, whereas NPPA was not associated with hypertension.

Keywords: NPPA, IL6, T2D, SNPs

INTRODUCTION

Type 2 Diabetes (T2D) and hypertension have emerged as major health problems worldwide, and they are increasing rapidly in most Asian countries (Rees et al., 2011). Hypertension is a quantitative trait that varies among subjects according to environmental factors for a short duration and diurnal patterns for longer-term changes (Lewington et al., 2002). It has been reported that T2D is 4 to 6-fold more common in South Asians than in Europeans (Rees et al., 2011). According to the International Diabetes Federation, T2D prevalence is higher in Asian countries and the top five countries with a diabetic population were India (50.8 million), China (43.2 million), Pakistan (7.1 million), Japan (7.1 million) and Indonesia (7 million) (Yang et al., 2010).

Many studies suggested that hypertension is heritable, although environmental factors and obesity were sustainably contributing to the high incidence of hypertension (Luft, 2001; Haslam and James, 2005; Kearney et al., 2005, Tobin et al., 2005). Tobin et al estimated that the chance of hypertension heritability in a population based study is about 65% (Tobin et al., 2005). The incidences of Hypertension varied across the world. Its highest incidences were reported in Poland (68.9% in men and 72.5% in women) and lowest incidences were reported in rural areas of India (3.4% in men and 6.8% in women) from 1980 to 2003 (Kearney et al., 2004). Hypertension is more common in urban areas (22.7%) as compared to rural areas (18.1%) in Pakistan (Jafar et al., 2003). As the incidences of
hypertension and T2D increase worldwide, it is important to determine the genetic risk factors of hypertension (Cowley, A.W. 2006). T2D and hypertension are multifactorial diseases; undoubtedly both genetic as well as environmental factors contribute to the development of disease (Rees et al., 2011).

A Natriuretic Peptide Precursor A gene (NPPA; chromosome location 1p36.21) containing rs5064 was reported to be associated with hypertension, which is involved in the predisposition of diabetes. The Interleukin-6 (IL6; chromosome location 7p21) gene was reported to be involved in insulin resistance and increased the risk of T2D development (Conen et al., 2007; Meiner et al., 2008; Yin et al., 2013).

Pakistanis are more vulnerable to T2D and hypertension because of the rapid urbanization, susceptible lifestyle (more indoor activities) and cousin marriages. The population of Pakistan consists of vast heterogeneity due to diverse geographical area and ethnic groups. Diversity within our population depicted a different genetic makeup in relevance to Europeans and Americans (Shera et al., 2007). There is no study available on selected genes in our population. Family history is a major risk factor in diabetes development in the Punjabi population of Pakistan (Zafar et al., 2007; Ralph and Coop, 2013). Three single nucleotide polymorphisms (SNPs) are selected from NPPA and IL6. The aim of the current study was to report the genetic association of selected SNPs with T2D and Hypertension.

**MATERIAL AND METHODS**

**Ethical Approval**

The current study was approved by the Board of Advanced Research, G.C. University, Lahore, Pakistan and Diabetic center of Jinnah Hospital Lahore, Pakistan.

A written consent was taken from patients and controls before sampling.

**Subjects**

A total of 458 Punjabi subjects (case = 288; controls = 170) were included in the study. The cases were diagnosed with T2D by a physician according to WHO criteria. All control subjects were healthy and had a negative family history of T2D. Demographic characteristics such as age, age of diagnosis, Body Mass Index (BMI), hypertension and complications of the subjects were studied.

**DNA Isolation**

The blood samples (3 ml) were collected in EDTA coated tubes and stored at 4°C for DNA extraction. DNA extraction was performed by Fermentas DNA purification kit (#K0512). DNA samples were stored at -20°C for further genetic analysis.

**Nested PCR**

Primers for Polymerase Chain Reaction (PCR) along annealing temperatures are listed in Table 1. DNA fragments of interest were amplified by using nested PCR. DNA amplification was carried out in a 20 μl reaction mixture consisting of 50-60ng of genomic DNA, 2.5 mM magnesium chloride (MgCl2, Sigma: M2670), 200 µM of each deoxyribonucleotide triphosphate (dNTPs: promega USA U1515), 125 nM of each primer and 1U TaqDNA polymerase (Amersham Bioscience, Uppsala, Sweden). PCR cycle conditions were as follows: 5 min for denaturation at 95°C, 30 cycles of the three steps of 95°C for 30 sec, 30 sec at 56°C, 2 min at 72°C, and then final extension at 72°C for 8 min. PCR products were purified by manual method (ethanol) which is as follows: PCR products were precipitated with chilled 100% ethanol and
then washed with 70% ethanol. After washing, pellets were dried and dissolved in

Table 1: The primer sequences used for PCR along with optimized conditions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP ID</th>
<th>1st PCR</th>
<th>Nested PCR</th>
<th>PCR Product (bp)</th>
<th>Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPPA</td>
<td>rs5063</td>
<td>F: CCCTCGTCTTCCATTGGCGGT</td>
<td>F: AFIGTGGGGGCACGACCTCAT</td>
<td>568</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>rs5064</td>
<td>R: TGTGACCTTGTTGGCTCGGT</td>
<td>R: CTGTGACAAAGCCTGCGGATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL6</td>
<td>rs1800796</td>
<td>F: ACAATAGCGCGCGGGAATCTCT</td>
<td>F: CACAGTTGCTGAGGAGGCTGTC</td>
<td>674</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TGCGAGGTGCTTTGGAGCA</td>
<td>R: AGCCAGACATTCAACAAGAGCACC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

bp = Base Pairs; * = Annealing Temperature; F = Forward Primer; R = Reverse Primer.

DEPC water. It was stored at 4°C for genotyping.

**Direct DNA sequencing**

Direct sequencing of the nested PCR product was executed for SNP genotyping (Lo et al., 2007). Sequencing reaction contained 0.75 µl of Big Dye (ABI, 1201096), 14.5 µl Milli Q H₂O, sequencing buffer 3.00 µl (ABI 1203142), 240 nM primer and 1.5 µl of PCR product. The sequencing reaction was initially denatured at 96°C for 1 min followed by 25 cycles, and each cycle was of 10 sec at 96°C, 5 sec at 50°C, and 4 minutes at 60°C. After the cleanup of the post-sequencing mixture with ethanol, the air-dried sequencing sample was dissolved by adding 10µl of Hi-Deionized formamide (Applied Biosystems Inc.). It was denatured at 95°C for 5 min and chilled to 4°C prior to sequencing (Model 3130 Genetic Analyzer, Applied Biosystems).

**Statistical Analysis**

The distributions of demographic characteristics among studied subjects were presented in numbers and percentages (%). First, the selected SNPs were analyzed by the Hardy-Weinberg Equilibrium (HWE) using the Genepop software on the web. All allelic and genotypic frequencies in case-control analysis were compared and calculated by using the Chi square test (χ²) as well as by SHEs: http://analysis.bio-x.cn/myAnalysis.php. Associations between age of diagnosis, gender, hypertension and genotypes by logistic regression (SPSS version 18) P values less than 0.05 were considered to be significant. Changes in amino acid sequences were determined by aligning sequences on Mega 6 software.

**RESULTS**

A total of 458 subjects were included in the study with 20 and 22 years minimum age of diabetes diagnosis for males and females respectively (Table 2). Certain SNPs were associated with T2D. The selected SNPs did not deviate from the Hardy-Weinberg equilibrium (p > 0.05), but rs1800796 did (p > 0.01). From the three studied SNPs, only rs1800796 [p - 0.0001, OR- 0.394 (95% CI 0.265-0.584)] was associated with T2D in single site analysis (Table 3).

**Table 2: Demographic characteristics of samples used in the study.**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Case (n = 288)</th>
<th>Control (n = 170)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Number</td>
<td>118</td>
<td>170</td>
</tr>
<tr>
<td>Age of diagnosis (y)</td>
<td>42.86±1.123</td>
<td>41.18±0.824</td>
</tr>
<tr>
<td>Minimum age of diagnosis (y)</td>
<td>20</td>
<td>22</td>
</tr>
<tr>
<td>Family history</td>
<td>74 (62.71%)</td>
<td>110(64.70%)</td>
</tr>
</tbody>
</table>
In analysis of genotype frequency shown in Table 3, Genotype CC of rs1800796 was increasing the risk of diabetes development in comparison to CG and GG genotypes, and rs1800796 is significantly associated with diabetes onset ($p < 0.001$). However rs5063 ($p = 0.408$) and rs5064 ($p = 0.552$) did not depict association with hypertension at the genotype level.

Haplotypes analysis of rs5063 and rs5064 indicated that CA, CG, TA and TG were not significantly associated with hypertension and T2D onset ($p > 0.05$), whereas the rest of the haplotypes were not associated with T2D development.

Protein alignment analysis on Mega 6 software showed a change in the nucleotide at the rs5063 polymorphic site, causing the change of alanine into threonine shown in Figure 1, (a). Mutation at rs5064 leads to the change of glutamine into arginine (Figure 1: (b). Similarly, in the case of the 1800796 polymorphic site, the change in the single nucleotide leads to the change of proline into arginine (Figure 1: (c).

The genotype CC of rs1800796 ($IL6$) was associated with a family history of
diabetes \( (p = 0.008) \) and was also gender specific in patients \( (p = 0.002) \).

![Figure 1: (a) Amino acid alignment of rs5063; (b) Amino acid alignment of rs5064; (c) Amino acid alignment of rs1800775.](image)

**DISCUSSION**

Diabetes is a multifactorial disease; genetic as well as environmental factors play a crucial role in the onset of diabetes. The minimum age of diagnosis was 20 for males and 22 for females in the investigated population. The survey found that a majority of the diabetic men and women had a family history of diabetes, with 62.7% and 64.7% respectively. Positive family history was reported as a strong risk factor for the onset of diabetes and an indicator for the risk of disease development \((Zafar \ et \ al., \ 2007; \ Ralph \ and \ Coop, \ 2013)\).

Out of the three studied genetic variants, one exhibited a significant association with the disease \((Table \ 3)\). To the best of our knowledge, this is the first study in any of Pakistani populations to report an association between the disease and the SNPs in the selected genes.

Family history is a strong predictor for the onset of T2D. It is a complex disease. A number of genes interact with each other and multiple genetic variants are involved in the development of diabetes. Province Punjab of Pakistan is a multicultural and multiethnic region with specific traditions from generation to generation. The caste system is strong and within caste or cousin marriages are very common. This tradition narrows down the gene pool with little genetic recombination. Even this mechanism sometimes disturbs the allele and genotype frequency in the population that ultimately leads to the violation of Hardy-Wienberg Equilibrium. The genome-wide associations throughout the world identified a number of loci associated with T2D.

Chromosome 1 was previously reported as a susceptible loci linked with T2D in different populations. Our genetic data provided no association between rs5063 and rs5064 in the NPPA gene with hypertension in diabetics \( [p > 0.01] \). The rs5064 form NPPA was reported to be associated with hypertension in a follow-up study of Americans \((Tun-Sen \ et \ al., \ 2013)\). Although rs5063 was not significantly associated with diabetes and hypertension, it is first time reported in Pakistani population and not reported in any ethnicity throughout the world.

Only rs1800796 in IL6 remained significantly associated with T2D after a number of tests performed. SNP rs1800796 in IL6 was found to be significantly associated with the development of T2D \([p = 0.001, \ OR = 0.394 \ (0.265, \ 0.584)] \) in south Asians \(especially \ Pakistanis\). The rs1800796-G effective risk allele contributed to the onset of diabetes in the Punjabi population, but its C allele was protective against diabetes with 0.719 frequencies in controls. Elevated IL6 level in plasma was found to be considerably associated with hypertension in females \((Cheung \ et \ al., \ 2011)\). It is controversial in various ethnic groups worldwide that rs1800796-C/G genotype has been the risk for the onset of
In line to our study, Yin et al. (2013) evidently found the significant association between the risk of disease and rs1800796 [OR = 1.29 (1.09-1.52), \( p = 0.002 \)] in the Chinese population. It is suggested that -572 G allele increased the risk of T2D in the corresponding population (Yin et al., 2013).

**CONCLUSION**

Conclusively, two SNPs in *NPPA* and one in *IL6* genes were studied in a Pakistani population by performing a case control analysis. We confirmed that the SNPs in *IL6* were associated with the risk of T2D. Consequently, these finding may be helpful in advanced clinical practices and public health genomics. Further studies are required in the Pakistani population with the prospective of genetics and environmental factors at large scale to reduce the risk of diabetes.

**Acknowledgment**

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