Calpain-10 Gene Polymorphism in Type 2 Diabetes Mellitus Patients in the Gaza Strip

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Key Words
Calpain-10 \cdot Cholesterol \cdot Gaza Strip \cdot Triglycerides \cdot Type 2 diabetes mellitus

Abstract
Objective: To examine the role of calpain-10 SNP-44, -43, -63 and del/ins-19 in genetic susceptibility to type 2 diabetes mellitus (T2DM) and associations with triglycerides and total cholesterol in a group of subjects residing in the Gaza Strip.

Subjects and Methods: Ninety-six individuals were examined: 48 T2DM patients and 48 controls. The groups were genotyped for calpain-10 SNP-44, -43, -63, and del/ins-19. Mutagenically separated polymerase chain reaction was used to examine SNP-44; del/ins-19 was examined by electrophoresis of the PCR product on agarose gel, while the restriction fragment length polymorphism method was used for SNP-43 and -63.

Results: There was evidence that the C allele at SNP-44 played a possible role in susceptibility to T2DM (p = 0.01). T2DM patients with G/A genotype were found to have higher levels of total cholesterol in comparison to those homozygous for allele 1 (G/G) in SNP-43. Total cholesterol levels increased in T2DM patients who are homozygous for del/ins-19 allele 2, in T2DM patients with the 121/221 haplotype combination, and in control subjects with the haplotype combination 111/121.

Conclusion: SNP-44 polymorphism of the calpain-10 gene has a significant association with T2DM patients in the Gaza strip. Certain polymorphisms of calpain-10 also have associations with the levels of total cholesterol in both T2DM patients and controls.

Introduction

Diabetes mellitus (DM) is a progressive and chronic endocrine disorder which primarily results in a hyperglycemic condition. The number of adults with diabetes in the world is expected to rise from 135 million in 1995 to 300 million in the year 2025 [1]. DM affects the body’s ability to metabolize fat, carbohydrates and proteins. The primary hormone that maintains homeostasis of the body’s glucose levels, insulin, is either insufficient or ineffective in individuals with DM [2].

Type 2 DM (T2DM) is preceded by a long period of impaired glucose tolerance, a potentially reversible metabolic state. Many patients with T2DM are asymptomatic, and their disease is undiagnosed for many years because the hyperglycemia is often not severe enough to provoke noticeable symptoms of diabetes. Nevertheless, such patients are at increased risk of developing macrovascular and microvascular complications. Studies suggest that
the typical patient with new-onset T2DM has had diabetes for at least 4–7 years before it is diagnosed [3]. Among patients with T2DM, 25% are believed to have retinopathy, 9% neuropathy and 8% nephropathy at the time of diagnosis [3].

In Palestine, DM seems to be a serious health problem among the population – especially the refugees – because the prevalence rate of DM in Palestine was about 9% in 2002, while the rate in other populations in the same year was about 5.2% (in the age group 20–79 years) [4]. T2DM is a heterogeneous disorder that may result from defects in one or more diverse molecular pathways [5]. It is a classic example of a multifactorial disorder, its etiology combining both genetic and environmental factors [6]. Identification of the genetic components of T2DM is the most important area of diabetes research because elucidation of the diabetes genes (alleles) will influence all efforts toward a mechanistic understanding of the disease, its complications, treatment, cure and prevention [5].

Recently the calpain-10 gene has been identified as a diabetes susceptibility gene. Variation in calpain-10 has been associated with a threefold increased risk of T2DM in Mexican-Americans and an increased risk of diabetes in Northern European populations [7]. Variation in the calpain-10 gene has also been associated with increased levels of total cholesterol and triglycerides [8, 9].

The calpains are a family of calcium-dependent non-lysosomal cysteine proteases [10]. The presence of calpains in mammalian cells was first reported over 30 years ago. Since then, at least 14 members of the calpain family have been identified and their chemistry and biology extensively studied. Although their physiological function is still not fully understood, they are implicated in a variety of calcium-regulated cellular processes, such as signal transduction, cell proliferation, cell cycle progression, differentiation, apoptosis, membrane fusion and platelet activation [11].

Calpain-10 is located on chromosome 2q37 and consists of 15 exons spanning 31 kb. Analysis of human cDNA clones revealed a complex pattern of alternative splicing, generating proteins of 672, 544, 517, 513, 444, 274, 139 and 138 amino acids. There is a complex relationship between susceptibility to T2DM and polymorphisms in calpain-10. Susceptibility is attributable not to a single polymorphism or allele, but rather to multiple polymorphisms (e.g. SNP-44, -43, -63 and del/ins-19), whose collective effects are not easily predicted without having the full genotype/haplotype information at all contributing sites [7]. The haplotypes in CAPN10 were defined from 4 polymorphisms spread across the gene: SNP-43 (G/A within intron 3: allele 1 = G and allele 2 = A), SNP-19 (two repeats of 32-bp sequence or 3 repeats of 32-bp sequence within intron 6; allele 1 = 2 repeats and allele 2 = 3 repeats), SNP-63 (C/T within intron 13: allele 1 = C and allele 2 = T), and SNP-44 (T/C within intron 3: allele 1 = T and allele 2 = C) [12, 13]. Genetic variation in calpain-10 seems to affect susceptibility to T2DM in both Mexican-Americans and Europeans. The 112/121 haplotype combination is associated with a similarly increased risk (threefold) of T2DM in both groups [7]. The knowledge of T2DM genetic background becomes very important due to its scientific, prognostic, prophylactic and also therapeutic significance [14]. Therefore, the aim of this study was to examine the role of calpain-10 SNP-44, -43, -63 and del/ins-19 in genetic susceptibility to T2DM and to the levels of triglycerides and total cholesterol in subjects in the Gaza Strip.

Subjects and Methods

Ninety-six individuals were included in this study: 48 T2DM patients (males/females: 10/38; age: 56 ± 9 years; duration of disease: 14 ± 10 years) and another 48 non-diabetic controls (males/females: 12/36; age: 41 ± 9 years). The control group contained only individuals with a normal fasting glucose level and negative family history among first-degree relatives. The study was performed according to the ethical guidelines in the Declaration of Helsinki after approval from the institution’s ethics committee. Written informed consent was obtained from all participants.

Blood samples were collected from patients and controls in the morning after fasting; 3 ml of venous blood were withdrawn from the antecubital vein into EDTA Vacutainer tubes.

Genotyping

DNA was extracted from peripheral blood lymphocytes by using the Wizard Genomic DNA Purification kit (Promega Corporation, Madison, Wisc., USA). We first genotyped the 4 polymorphisms indicating the highest risk for T2DM in the Mexican-Americans, SNP-44, -43, -63 and del/ins-19. Allele distribution of calpain-10 SNP-44, -43, -63 and del/ins-19 was examined in the T2DM patients and control subjects. We then examined the distribution of haplotype and haplotype combinations comprising these alleles.

SNP-44

The mutagenically separated PCR (MS-PCR) method was used to genotype for SNP. A common reverse primer and 2 allele-specific forward primers of different lengths were used: common reverse primer, 5’-CTCATCCTCACCAAGTCAAGGC-3’; allele 1 (T) primer, 5’-CAGGGGCTACACCTGCTAT-3’; allele 2 (C) primer, 5’-GTGCGCAGGGAGCTGTGGCGCAGCGATTGT-3’. PCR products were separated on 3% NuSieve agarose gel and were visualized by ethidium bromide staining. Allele 1 (T) should give a band of 71 bp and allele 2 (C) 86 bp [13].
SNP-43

The PCR restriction fragment length polymorphism (PCR-RFLP) method was used for SNP-43. It was amplified with primers: forward primer, 5’-GCTGGCTGTGACATCAGTG-3’ and reverse primer, 5’-ACCAACGGTCTAGCCTCCTAC-3’. PCR products were digested with 10 U NdeI for 16 h at 37°C. The digested products were separated on 4.0% agarose gel and were visualized by ethidium bromide staining. Allele 1 (G) was detected as a 254-bp band, and allele 2 (A) was detected as 223- and 31-bp bands [8].

SNP-19

This deletion/insertion polymorphism was amplified by PCR, primers used: forward primer, 5’-GTGTTGTTCTCTCCACG-GG-3’ and reverse primer, 5’-CATGAACCCCTGCGGAG-3’. The PCR products were separated on 3.0% agarose gel and were visualized by ethidium bromide staining. Allele 1 (2 repeats of 32-bp sequence) was detected as a 155-bp band, and allele 2 (3 repeats of 32-bp sequence) was detected as a 187-bp band [13].

Biochemical Tests

All samples for clinical chemistry analysis were collected after 12–14 h fasting. Fasting blood glucose, triglycerides and total cholesterol were assessed by enzymatic colorimetric methods (DiaSys kit, DiaSys Diagnostic Systems, Holzheim, Germany). Triglyceride levels were measured by colorimetric enzymatic test using glycero-3-phosphate-oxidase, total cholesterol levels by the ‘CHOD-PAP’ enzymatic photometric test and glucose levels by the ‘GOD-PAP’ enzymatic photometric test.

Statistical Analysis

Statistical analysis was carried out using the SPSS v.13 for Windows. For normally distributed data, means and SD were calculated. Odds ratios and 95% CIs were reported where appropriate. Triglyceride concentrations were log transformed before analysis to achieve a normal distribution. Statistical significance was at the 5% level or less.

Results

The allele distribution of calpain-10 to SNPs and del/ins-19, haplotype distributions, and haplotype combinations comprising these alleles are given in tables 1–3, respectively, for both patients and controls. According to the results of the current study, we did not find any association between T2DM and allele frequencies of calpain-10 to SNPs and del/ins-19 haplotypes in T2DM patients and controls.
SNP-43, del/ins-19 and SNP-63 individually; p values were 0.40, 0.66 and 1.0, respectively. We did, however, find a significant difference in the allele frequency of SNP-44 (p = 0.01), where the presence of allele 2 (C) was associated with a 2.7-fold increased risk of diabetes. Haplotype frequencies were 42.5% for 121, 38.0% for 111, 12.5% for 221, 3.0% for both 112 and 122, and 2.0% for 222.

We evaluated the frequency of the combinations comprising these alleles in the present study, and we found that neither the 112/121 haplotype combination nor the other haplotype combinations had significant impact on increasing the risk of T2DM in the Gaza Strip.

**Table 4.** Triglycerides and total cholesterol levels according to haplotype combination in patients

<table>
<thead>
<tr>
<th>Haplotype combination</th>
<th>Frequency</th>
<th>log(triglycerides), mg/dl</th>
<th>p value</th>
<th>Total cholesterol, mg/dl</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>111/111</td>
<td>9</td>
<td>2.35 ± 0.15</td>
<td>0.65</td>
<td>177 ± 32</td>
<td>0.051</td>
</tr>
<tr>
<td>111/112</td>
<td>3</td>
<td>2.03 ± 0.40</td>
<td>0.06</td>
<td>182 ± 32</td>
<td>0.43</td>
</tr>
<tr>
<td>111/121</td>
<td>8</td>
<td>2.25 ± 0.12</td>
<td>0.50</td>
<td>181 ± 27</td>
<td>0.15</td>
</tr>
<tr>
<td>111/122</td>
<td>2</td>
<td>2.46 ± 0.18</td>
<td>0.44</td>
<td>212 ± 29</td>
<td>0.64</td>
</tr>
<tr>
<td>111/221</td>
<td>5</td>
<td>2.28 ± 0.33</td>
<td>0.81</td>
<td>193 ± 43</td>
<td>0.73</td>
</tr>
<tr>
<td>121/121</td>
<td>11</td>
<td>2.25 ± 0.19</td>
<td>0.43</td>
<td>203 ± 34</td>
<td>0.69</td>
</tr>
<tr>
<td>121/122</td>
<td>8</td>
<td>2.46 ± 0.43</td>
<td>0.28</td>
<td>232 ± 28</td>
<td>0.005**</td>
</tr>
<tr>
<td>111/222</td>
<td>2</td>
<td>2.43 ± 0.03</td>
<td>0.53</td>
<td>240 ± 80</td>
<td>0.12</td>
</tr>
</tbody>
</table>

**Table 5.** Triglycerides and total cholesterol levels according to haplotype combination in controls

<table>
<thead>
<tr>
<th>Haplotype combination</th>
<th>Frequency</th>
<th>log(triglycerides), mg/dl</th>
<th>p value</th>
<th>Total cholesterol, mg/dl</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>111/111</td>
<td>8</td>
<td>2.05 ± 0.08</td>
<td>0.69</td>
<td>174 ± 29</td>
<td>0.41</td>
</tr>
<tr>
<td>111/112</td>
<td>3</td>
<td>2.14 ± 0.14</td>
<td>0.54</td>
<td>171 ± 24</td>
<td>0.52</td>
</tr>
<tr>
<td>111/121</td>
<td>8</td>
<td>2.05 ± 0.19</td>
<td>0.69</td>
<td>200 ± 30</td>
<td>0.04*</td>
</tr>
<tr>
<td>111/122</td>
<td>3</td>
<td>2.15 ± 0.10</td>
<td>0.50</td>
<td>198 ± 20</td>
<td>0.31</td>
</tr>
<tr>
<td>111/221</td>
<td>5</td>
<td>2.16 ± 0.12</td>
<td>0.31</td>
<td>174 ± 18</td>
<td>0.55</td>
</tr>
<tr>
<td>121/121</td>
<td>14</td>
<td>2.04 ± 0.24</td>
<td>0.43</td>
<td>172 ± 22</td>
<td>0.19</td>
</tr>
<tr>
<td>121/221</td>
<td>6</td>
<td>2.09 ± 0.35</td>
<td>0.94</td>
<td>189 ± 47</td>
<td>0.66</td>
</tr>
<tr>
<td>121/122</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**p ≤ 0.01.**

**p ≤ 0.05.**

SNP-43, del/ins-19 and SNP-63 did not show a statistically significant difference in triglyceride levels and different genotypes in all SNPs or in del/ins-19 for both patients and controls. For SNP-43, total cholesterol levels in heterozygous patients were higher than in homozygous patients for the G allele (p < 0.01). T2DM patients with G/A genotype have higher total cholesterol levels in comparison to those homozygous for allele 1 (G/G) (p < 0.01). For del/ins-19, total cholesterol levels were highest in patients with the homozygous allele.

**Discussion**

We have studied the effect of calpain-10 on the risk of T2DM and on the levels of triglycerides and total cholesterol in patients and controls living in the Gaza Strip. We tested 4 polymorphisms in calpain-10, SNP-44, SNP-43, del/ins-19 and SNP-63, for association with T2DM using a case-control design. We selected these polymorphisms because of their prior association with either T2DM, either individually or in combination, or with the levels of triglycerides or total cholesterol.
Based on the results of our study, no significant association between T2DM and allele frequencies of SNP-43, del/ins-19 or SNP-63 exists ($p = 0.40, 0.66$ and $1.0$, respectively). However, there is a significant difference in the allele frequency of SNP-44 ($p = 0.01$), where the presence of allele 2 (C) was associated with a 2.7-fold increased risk of diabetes. Functional studies by Horikawa et al. [7] suggested that SNP-44 is located in an enhancer element and might affect calpain-10 expression. Our result confirmed previous studies [13, 15] indicating that the C-allele at SNP-44 was associated with an increased risk of T2DM.

The haplotypes found in the Gaza Strip are consistent with earlier reports which showed that only 3–4 of 8 possible haplotypes occur in appreciable frequency [16]. The most common haplotype in this study was 121, which is in agreement with other previous studies [7, 14, 17–19]. Our results further confirmed previous studies [12–14, 18–20] indicating that there is no significant difference in the distribution of haplotypes among T2DM patients and control subjects.

It has been demonstrated that the haplotypes comprising the SNP-43, del/ins-19 and SNP-63 polymorphisms define the risk of T2DM better than individual SNPs; for example the haplotype combination 112/121 was found to be associated with an increased risk of T2DM in Mexican-Americans [7], but neither the 112/121 haplotype combination nor any other haplotype combination had significant impact on increasing the risk of T2DM in the Gaza Strip (table 3). Apparently, our results are similar to those of Tsai et al. [20], Fingerlin et al. [19] and Horikawa et al. [21]. Although the human chromosomes and the loci that they contain are identical throughout the species, the nature of different alleles and their frequencies at many loci vary widely among population groups. This can be due to selection, mutation or migration, but usually occurs slowly, in small increments. Gene-gene or gene-environmental interactions could lead to the varying genetic effects of calpain-10 observed in different populations [22].

Our results showed that there is an association between the heterozygosity of SNP-43 in T2DM patients and total cholesterol levels. This result is in contradiction with that of Daimon et al. [9], who showed that genotype combinations of SNP-43 G/G and SNP-44 T/T had significantly increased serum total cholesterol levels.

Our results also showed that T2DM patients homozygous for the del/ins-19 allele 2 (3 repeats of 32 bp) have total cholesterol levels higher than those heterozygous or those homozygous for allele 1 (2 repeats of 32 bp), but this finding was not similar to other studies [8, 9]. The difference could be due to the limited number of studies that included triglycerides and total cholesterol levels and their associations with calpain-10.

Our other finding showing that T2DM patients with the 121/221 haplotype combination have higher total cholesterol levels in comparison to other haplotype combinations ($p = 0.005$) was not reported in other studies [9, 18]. The reason could also be due to the limited number of studies that tested the association between haplotype combinations and triglyceride/total cholesterol levels in T2DM patients.

Our finding that control subjects with the haplotype combination 111/121 have higher serum total cholesterol levels than those with other haplotype combinations is different from the results of Wu et al. [18], who showed that the control subjects with the haplotype combination 112/121 had the highest total cholesterol level in the Chinese population. Disease association with certain haplotypes found in certain populations may not be applicable for other populations, since not all the expected haplotypes can be found in all populations. Increased or decreased frequency of particular haplotypes is determined by genetic and environmental factors, such as linkage disequilibrium, founder effect and selection [23].

**Limitations of the Study**

The sample size was small; in genetic association studies it is better to have a large sample size. The control individuals were on average younger than the patients, but the genetic structure is our main outcome and this is independent of age.

**Conclusion**

The presence of allele 2 (C-allele) in SNP-44 was associated with a 2.7-fold increased risk of T2DM in the Gaza Strip. There was an association between the heterozygosity of SNP-43 in T2DM patients and total cholesterol levels in the Gaza Strip. In addition, T2DM patients who are homozygous for del/ins-19 allele 2 have higher total cholesterol levels than those heterozygous or homozygous for allele 1. T2DM patients with the 121/221 haplotype combination have higher plasma total cholesterol levels than those with other haplotype combinations. Control subjects with the haplotype combination 111/121 have higher plasma total cholesterol levels than those with other haplotype combinations.
References