**Homozygous combination of calpain 10 gene haplotypes is associated with type 2 diabetes mellitus in a Polish population**

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**Abstract**

**Objective:** The polymorphisms of two genes have recently been associated with complex forms of type 2 diabetes mellitus (T2DM): calpain 10 and peroxisome proliferator-activated receptor-γ (PPARγ). Calpain 10 is a member of a large family of intracellular proteases. It was shown in Mexican-Americans and other populations that variants of three single nucleotide polymorphisms (SNPs), -43, -19, and -63, of this ubiquitously expressed protein influence susceptibility to T2DM. However, substantial differences were shown between ethnic groups in at risk alleles and haplotypes as well as in their attributable risk. Thus, it is important to determine the role of calpain 10 in various populations.

**Aim:** To examine the role of calpain 10 SNPs -43, -19, and -63 in genetic susceptibility to T2DM in a Polish population.

**Methods:** Overall, 377 individuals were examined: 229 T2DM patients and 148 control individuals. The groups were genotyped for calpain 10 SNP-43, SNP-19, and SNP-63. SNP-19 was examined by electrophoresis of the PCR product on agarose gel by size, while the restriction fragment length polymorphism (RFLP) method was used for the two other markers. Differences in allele, genotype, haplotype, and haplotype combination distribution between the groups were examined by χ² test.

**Results:** Distributions of alleles, genotypes, and haplotypes at three loci defined by examined SNPs were not significantly different between the groups. However, the homozygote combination of 121 haplotype was more prevalent in the T2DM group than in the controls (17.9% vs 10.1%, P = 0.039). No difference was observed in the 112/121 haplotype distribution. This heterozygous haplotype combination was associated with increased risk of T2DM in several populations.

**Conclusion:** The results of our study suggest the association of calpain 10 121/121 haplotype combination created by SNPs -43, -19, and -63 with T2DM in a Polish population. However, we were not able to confirm the previously described role of the heterozygous 112/121 haplotype combination in susceptibility to T2DM.

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**Introduction**

Substantial scientific evidence exists for the role of genetic factors in the pathogenesis of type 2 diabetes mellitus (T2DM). For example, T2DM clusters in families, its concordance rate in monozygotic twins is higher than in dizygotic ones, and there are ethnic groups with a very high prevalence of this disease (1). Two major pathophysiological defects coexist in this disease: impairment of insulin secretion by β-cells and decreased insulin sensitivity in peripheral tissues (2). The knowledge of T2DM genetic background becomes very important due to its scientific, prognostic, prophylactic and also its therapeutic significance. Mutations in six genes were associated with the development of autosomal dominant T2DM called MODY (maturity onset diabetes of the young) (3, 4). The other monogenic form of T2DM is maternally inherited diabetes caused by mutations of mitochondrial DNA (MIDD – maternally inherited diabetes with deafness) (5). Impaired insulin secretion is a common clinical feature of both autosomal dominant and maternally inherited T2DM (3, 5).

Recently, two susceptibility genes for the common, polygenic forms of middle/late onset T2DM have been identified: calpain 10 (6) and peroxisome proliferator-activated receptor-γ (7). Calpain 10 is a member of a large family of intracellular proteases. They are essential for multiple cellular functions, for example, the processing property of these enzymes makes it possible to directly modulate the activities and structures of other proteins (8). They also influence the apoptosis...
process. The activity of calpains may be influenced directly (through specific receptor) and indirectly (through intracellular calcium level) by vitamin D (8–10). The molecular mechanisms of how some polymorphisms in calpain 10 genes increase the risk of T2DM development have yet to be discovered; however, most likely, the DNA variants of this gene affect both insulin sensitivity and insulin secretion (10–12). The risk of developing T2DM is not fully attributed to a single polymorphism of the calpain 10 gene, but, as was described in Mexican-Americans, results from the combination of haplotypes created by alleles of three single nucleotide polymorphisms (SNPs): SNP-43, SNP-19, and SNP-63 (6). All these three SNPs are located within introns and are very likely to influence the gene expression.

The aim of this study was to examine the role of calpain 10 SNPs -43, -19, and -63 in genetic susceptibility to T2DM in a Polish population.

Subjects and methods

We included 377 unrelated individuals in this study: 229 T2DM patients and 148 non-diabetic controls. All the study individuals were Caucasians and residents of Southern-Eastern Poland. During ascertainment, the WHO definitions and criteria were used (13). The patients received a standard questionnaire that contained questions regarding the age at T2DM diagnosis, family history, the treatment method and other medical issues. Only patients with a clinical diagnosis of T2DM and with no insulin therapy for at least two years soon after diagnosis were recruited. The study individuals underwent a basic physical examination that included the measurement of height, weight, and blood pressure. The control group contained only individuals with normal fasting glucose and negative family history of T2DM among first degree relatives. This group consisted mainly of the spouses of T2DM patients and volunteers from the medical personnel. This study was performed according to the Helsinki Declaration and was accepted by the Ethical Committee of the Jagiellonian University Medical College.

DNA of the individuals from the study groups was isolated from peripheral blood lymphocytes using a guanidine detergent-based protocol (DNAzol Reagent, GIBCO, Bethesda, MD, USA). The study groups were genotyped for calpain 10 SNPs -43, -19, and -63. DNA from the study individuals was used for the PCR reactions. For SNP-19 the DNA fragment containing 32 bp insertion/deletion was amplified using previously published primers and conditions (14). Alternative alleles (allele 1: two repeats of 32-bp sequence and allele 2: three 32-bp repeats) were determined by electrophoresis on 3% regular agarose gel by size. Mismatch primer modification of the restriction fragment length polymorphism (RLFP) method was applied for calpain 10 SNP-43 (15), and SNP-63 (14). For SNP-43, the presence of the A allele (allele 2) was associated with the existence of a restriction site for NsiI enzyme and digestion of the 144 bp PCR product into 121 and 23 bp fragments; for the G allele (allele 1) no NsiI restriction site was present. For SNP-63, the C allele (allele 1) created an HhaI restriction site and digestion of the 192 bp PCR product into 162 and 30 bp fragments, while no HhaI restriction site was present for the T allele (allele 2). Digestion products were separated on ethidium bromide-stained, 3.2% low-melting agarose gel. The results were documented by digital camera and stored as computer files in Bioquant software (Vilbert-Lourmat, Marne LaValle, France).

The variants of calpain 10 SNPs -43, -19, and -63 jointly determine eight possible haplotypes. Haplotypes carried by each study individual were inferred by the method of gene counting (16, 17). Under the assumptions of Hardy-Weinberg equilibrium and random mating, this iterative procedure yields maximum likelihood estimates of haplotype frequencies in the study population based on the joint distribution of two polymorphic markers. For phase-unknown genotypes, the conditional probabilities of the alternative haplotype configurations are calculated from the estimated haplotype frequencies, and all individuals with that genotype are allocated to the more likely pair of haplotypes. For markers in significant linkage disequilibrium, the difference between the conditional probabilities may be so large, as in the case of examined calpain 10 SNPs, that haplotypes can be assigned to phase-unknown individuals with a high degree of confidence. In our study, for individuals heterozygous for two markers (52 T2DM cases and 33 controls) the conditional probability of the haplotype pair 11/22 for SNPs -43 and -19 was greater than 0.99 as estimated for the total group. Similarly, the conditional probabilities of the haplotype pair 12/21 for SNPs -19 and -63 and of haplotype pair 12/12 for SNPs -43 and -63 were both greater than 0.99. Therefore, phase-unknown individuals heterozygous for two markers were assumed to have these pairs of haplotypes. Consistently, individuals heterozygous for three markers (10 T2DM cases and 9 controls) were allocated to have 112/221 three loci haplotypes. For individuals homozygous at three loci (76 T2DM patients and 37 controls) or heterozygous at only one locus (91 T2DM and 69 controls) haplotypes can be determined unambiguously. Linkage disequilibrium between three calpain 10 loci was assessed using the EH program (ftp://linkage.rockefeller.edu/software/eh). Deviations from Hardy–Weinberg equilibrium were tested using $\chi^2$ goodness-of-fit test.

Differences in distribution of alleles, genotypes, haplotypes, and haplotype combinations distributions between the groups were assessed by $\chi^2$ test. As the descriptive measure of association between genotypes and outcomes, odds ratios (ORs) and their 95%
confidence intervals (CIs) have been calculated. The Bonferroni correction for multiple comparisons was applied to stratified analyses based on age of T2DM onset (<46 years and >46 years, 46 being the mean age in T2DM group), body mass index (BMI) (<30 and ≥30, 30 being the mean BMI in the T2DM group), and family history of T2DM (positive and negative family history of T2DM in first and/or second degree relatives).

Results

The clinical characteristics of T2DM patients and control subjects are summarized in Table 1. Out of 229 patients there were 110 individuals who reported a positive family history of T2DM in first or/and second degree relatives. The patients were more obese than the controls. Overall, the SNPs’ alleles were determined in 377 individuals. Genotypes for three loci were in Hardy-Weinberg equilibrium in each group separately and in the total group. All three pairs of polymorphisms were in strong linkage disequilibrium ($P < 10^{-4}$):

Both groups had similar distribution of alleles and genotypes (Tables 2 and 3) created by variants of SNP-43, SNP-19, and SNP-63. There was no significant difference in distribution of haplotypes (Table 4). The gene counting results of haplotype determination are consistent with earlier reports that only four of eight possible haplotypes occur in appreciable frequency in Europeans and other ethnic groups (6, 14). In the analysis of the haplotype combination, the homozygotes for the 121 haplotype were more prevalent in the T2DM group than in controls (17.9% vs 10.1%, $P = 0.039$) (Table 5). No clinical difference between 121/121 haplotype combination carriers and the rest of the patient group was observed. We stratified the T2DM group in respect to BMI, age of disease onset and family history of T2DM. We observed that 121/121 homozygotes were more prevalent in the subgroup with a positive family history than in the controls (21.8% vs 10.1%, $P = 0.009$), the difference was statistically significant after Bonferroni correction ($P = 0.027$). No difference was observed in the 112/121 haplotype distribution. This heterozygous haplotype combination was associated with increased risk of T2DM in several populations (6). In general, the frequencies determined in our population were similar to other Caucasians (6, 14).

Discussion

There are a growing number of reports on the role of calpain 10 in the pathogenesis of T2DM. However,

### Table 2

<table>
<thead>
<tr>
<th>Alleles</th>
<th>T2DM</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNP-43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2DM</td>
<td>335</td>
<td>123</td>
</tr>
<tr>
<td>Controls</td>
<td>205</td>
<td>91</td>
</tr>
<tr>
<td>SNP-19</td>
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<td></td>
</tr>
<tr>
<td>T2DM</td>
<td>157</td>
<td>301</td>
</tr>
<tr>
<td>Controls</td>
<td>104</td>
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<tr>
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<td></td>
</tr>
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<td>T2DM</td>
<td>425</td>
<td>33</td>
</tr>
<tr>
<td>Controls</td>
<td>269</td>
<td>27</td>
</tr>
</tbody>
</table>

$p$-values: 1 degree of freedom.
The equivocal answer to the question which alleles, genotypes, haplotypes and haplotype combinations carry the risk of developing T2DM is lacking. Horikawa et al. reported an association between T2DM and the heterozygous combination of three locus haplotypes created by SNP-43, SNP-19 and SNP-63 in a Mexican-American population (6). Similar results were obtained in Germans and Finns but the population attributable risk in these European populations was lower than in Mexican-Americans (6). However, it should be pointed out that some other studies were not able to replicate these results (14, 18). We found that the homozygotic 121/121 combination of three point haplotypes created by SNPs -43, -19, and -63 may be associated with an increased risk of T2DM in a Polish population. This association with T2DM was also observed in a subset of individuals with a positive family history of T2DM. The frequency of this 121/121 combination and related odds are similar to those observed in a Finnish population (6). However, contrary to Mexican-Americans, Finns, and Germans, this Polish population did not show evidence of the 112/121 heterozygous combination being associated with T2DM. We did not genotype our study groups for other polymorphisms of calpain 10, for example SNP-44, that have recently been suggested to influence susceptibility to T2DM (14, 19). Our primary goal was to focus on the markers reported originally by Bell and his group (6). In addition, allele 2 of SNP-44 is exclusively associated with haplotype 111 created by the common alleles at SNP-43, SNP-19, and SNP-63, so this marker would not affect the significance of 121/121 association in this Polish population (14, 19). It should also be pointed out that the 111 haplotype is present with almost identical frequencies in both our study groups.

Finally, the shortcomings of our study design should be considered. First, in this case-control study we performed numerous tests and the problem of a correction for multiple comparisons arises. We stratified our patient group based on BMI, age of T2DM onset, and family history of T2DM, and subsequent comparisons were carried out. Bonferroni correction was used for these stratification analyses. For the non-stratified comparisons for alleles, genotypes, haplotypes and haplotype combinations, the correction was not made. However, it should be pointed out that we were testing a specific hypothesis that has been suggested by others. Moreover, the alleles, genotypes, haplotypes, and haplotype combinations of calpain 10 which were examined, are not independent of each other. The nature of biology (i.e. linkage disequilibrium among the sites) insures that the results of these comparisons are very highly correlated. In addition, we followed the approach accepted in other papers on calpain 10 and T2DM (6, 14). The second important issue is the size of our study groups. It is very important in case-control genetic studies that the size of the examined groups be large enough to detect a putative association. Our groups were similar in size to the European populations, Germans and Finns, which were examined previously for case-control comparisons (6). Those groups were large enough to detect a modest effect of calpain 10 in these populations.

### Conclusions

The results of the case-control study in a Polish population suggest a role of the 121/121 haplotype combination created by calpain 10 SNPs -43, -19, and -63 in the susceptibility to T2DM. We were not able to replicate the association that was reported earlier in several populations between calpain 10 and T2DM (6, 14). The second important issue is the size of our study groups. It is very important in case-control genetic studies that the size of the examined groups be large enough to detect a putative association. Our groups were similar in size to the European populations, Germans and Finns, which were examined previously for case-control comparisons (6). Those groups were large enough to detect a modest effect of calpain 10 in these populations.

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### References


