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Association of calpain 10 gene polymorphisms with type 2 diabetes mellitus in Southern Indians

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Abstract

The aim was to investigate the association between the *CAPN10* gene single nucleotide polymorphisms (SNPs) −44 (rs2975760), −43 (rs3792267), −19 (rs3842570), and −63 (rs5030952) and type 2 diabetes mellitus in an Asian Indian population in Southern India. A total of 1443 subjects, 794 normal glucose tolerant (NGT) and 649 type 2 diabetes mellitus subjects, were randomly selected from the Chennai Urban Rural Epidemiology Study. These subjects were genotyped for the 4 *CAPN10* SNPs using polymerase chain reaction–restriction fragment length polymorphism and validated by direct sequencing. None of the 4 SNPs showed any significant differences in the genotypic distribution among the NGT and type 2 diabetes mellitus subjects ($P = .20$, .86, .34, and .39 for SNPs −44, −43, −19, and −63, respectively). The NGT subjects with the 11 genotype of the SNP −63 had significantly higher 2-hour postload plasma glucose (mean ± SD, 5.66 ± 1.05 mmol/L) levels compared with the combined 12 and 22 genotype group (5.33 ± 1.11 mmol/L, $P = .004$). The P value remained significant even after adjusting for age, sex, body mass index, smoking, and alcohol consumption (nominal $P = .008$). No significant difference in the biochemical parameters was observed when the subjects were stratified according to the other SNPs. The 2111 haplotype corresponding to SNPs −44, −43, −19, and −63 showed a significant difference in the proportion among NGT (0.18) and type 2 diabetes mellitus subjects (0.22, nominal $P = .014$). Although the Bonferroni correction based on the asymptotic test does not preserve this significance, the test based on the empirical distribution remained significant. In conclusion, our study raises the possibility that the 2111 haplotype of SNPs −44, −43, −19, and −63 may be associated with type 2 diabetes mellitus, although none of these SNPs may be individually associated with diabetes.

1. Introduction

CAPN10 located on chromosome 2q37, encoding calpain-10, an ubiquitously expressed member of the calpain-like cysteine protease family, was the first type 2 diabetes mellitus gene to be identified in a genomewide scan followed by positional cloning [1,2]. Individuals carrying the haplotype combination 112/121 defined by 3 single nucleotide polymorphisms (SNPs), SNP −43 (G/A), SNP −19 (an insertion-deletion polymorphism with either 2 or 3 repeats), and SNP −63 (C/T), were at a greater risk of type 2 diabetes mellitus in the original publication [2]. Since this initial work, there have been a number of follow-up studies on a diverse range of ethnic populations. Whereas some studies confirmed the association of these SNPs with type 2 diabetes mellitus individually or along with another SNP, SNP −44 (T/C) [3-5], other studies challenged the original findings [6-15]. In addition, a series of pooled and meta-analyses of *CAPN10* data have also been performed, but with inconsistent results [5,16-18].

A number of factors, environmental, statistical, and genetic, have been proposed to underlie the variation in results observed in different populations [19,20]. Nonreplication of the association of specific locus alleles between populations of different ethnic backgrounds could be simply due to statistical bias from factors such as underlying population stratification. The frequency of individual

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CAPN10 allelic variants, haplotypes, and haplotype combinations has been shown to be both geographically and ethnically divergent [21]. Alternatively, association with the same variant allele may be undetectable or different between ethnic groups because of differences in relative risks or pleiotropic genotype effects on phenotypic traits. In this respect, replication studies within the same ethnic group are a more robust choice especially in populations such as Asian Indians, who have greater insulin resistance and increased susceptibility to type 2 diabetes mellitus [22,23]. Both genetic and functional data have indicated that calpain-10 has an important role in insulin resistance and intermediate phenotypes, and there is emerging evidence of a role for calpain-10 in glucose transporter 4 translocation and insulin secretion [24]. Hence, the present study was designed to investigate whether the *CAPN10* gene polymorphisms SNP −44 (rs2975760), −43 (rs3792267), −19 (rs3842570), and −63 (rs5030952) are associated with type 2 diabetes mellitus in an Asian Indian population in Southern India.

2. Methods

2.1. Study subjects

A total of 1443 unrelated subjects were chosen from the Chennai Urban Rural Epidemiology Study (CURES). The methodology of the study has been published elsewhere [25]. In Phase 1, 26 001 subjects were recruited that included 1529 self-reported diabetic subjects. In Phase 2 of CURES, all self-reported diabetic subjects were invited to our center for detailed studies, of whom 1382 responded (response rate, 90.4%; 1382/1529). In Phase 3 of the CURES, every 10th individual from Phase 1 (26001/10 = 2600) were invited to the center. Out of the 2600, there were 2350 responders; and among those who responded, there were about 143 self-reported diabetic subjects. Excluding these 143 self-reported diabetic subjects, the rest of the subjects (2350 − 143 = 2207) underwent an oral glucose tolerance test (OGTT) to rule out diabetes. Those who were confirmed by OGTT to have 2-hour plasma glucose value of at least 11.1 mmol/L (based on World Health Organization consulting group criteria) were labeled as *newly detected diabetic subjects* (n = 222). Subjects who were confirmed by OGTT to have fasting plasma glucose less than 5.6 mmol/L (100 mg/dL) and 2-hour plasma glucose value not exceeding 7.8 mmol/L (140 mg/dL) were categorized as *normal glucose tolerant* (NGT) subjects (n = 1736) [26]. The total number of diabetic subjects in the CURES study population is 1604 (1382 self-reported diabetic subjects from Phase 2 + 222 newly detected diabetic subjects from Phase 3). From these 1604 diabetic subjects, 649 subjects were randomly selected; and from the 1736 NGT subjects in Phase 3, 794 subjects were randomly selected for the present study.

Anthropometric measurements including weight, height, and waist were obtained using standardized techniques. The body mass index (BMI) was calculated as the weight in kilograms divided by the square of height in meters. Biochemical analyses were carried out on a Hitachi-912 Autoanalyzer (Hitachi, Mannheim, Germany) using commercial kits (Roche Diagnostics, Mannheim, Germany). Fasting plasma glucose was estimated using the glucose oxidase-peroxidase method [27]. Serum cholesterol was estimated using the cholesterol oxidase-phenol 4-amino antipyrene peroxidase method [28]. Serum triglyceride was estimated using the glycerol phosphatase oxidase-phenol 4-amino antipyrene peroxidase method [29]. High-density lipoprotein (HDL) cholesterol was estimated using the polyethylene glycol-pretreated enzymes method [30], and low-density lipoprotein (LDL) cholesterol was calculated using the formula of Friedewald et al [31]. Glycated hemoglobin was estimated by high-performance liquid chromatography [32] using the Variant machine (Bio-Rad, Hercules, CA).

2.2. Genotyping

DNA was isolated from whole blood using the phenol-chloroform method. Single nucleotide polymorphisms −43, −19, and −63 were genotyped by mutagenically separated-polymerase chain reaction (MS-PCR), PCR, and PCR–restriction fragment length polymorphism, respectively, using protocols published previously [6]. Single nucleotide polymorphism −44 was genotyped using the following primers: forward 5′-ATG TGG GCA TCC ATA GCT TC-3′ and reverse 5′-ATG GTC TGT AGC ACC CCA AA-3′. A final reaction volume of 15 μL of PCR contained 100 ng genomic DNA, 5 pmol of each primer, PCR buffer with 1 mmol/L of $MgCl_2$, 100 μmol/L of each dNTP, and 0.5 unit of Taq polymerase (Life Technologies, Carlsbad, CA). The PCR was carried out on a Peltier Thermal Cycler (MJ Research, Waltham, MA) using the following conditions: 95°C for 5 minutes, followed by 34 cycles of 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds, and a final extension of 72°C for 9 minutes. The PCR products were digested with 2 units of *Fnu*4HI enzyme and electrophoresed on a 3% agarose gel. To ensure that the genotyping was of adequate quality, we performed random duplicates in 20% of the samples. The assays were performed by a technician who was masked to the phenotype, and there was 99% concordance in the genotyping. Furthermore, a few variants were confirmed by direct sequencing with an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA). Informed consent was obtained from all the subjects who participated in this study, and the study was approved by the institutional ethical committee.

2.3. Statistical analysis

SPSS Windows, version 10.0 (SPSS, Chicago, IL), was used for statistical analysis. One-way analysis of

variance was used to compare groups for continuous variables. Data for continuous variables were expressed as mean ± SD. All the quantitative traits except for serum triglycerides had a normal distribution; the latter values were therefore log transformed before analysis. χ^2 test was used to compare the proportions of genotypes or alleles. Regression analysis was done using diabetes as dependent variable and the genotypes as independent variables. P values < .05 were considered statistically significant. Linkage disequilibrium (LD) pattern between the SNPs was analyzed using the software HAPLOVIEW [33]. The HAPLOPOP program [34] was used to determine the frequencies of the 4-locus haplotypes. A Bonferroni correction was carried out for multiple testing comparisons in haplotype analysis. Power was estimated using an online post hoc power computation tool (http://www.dssresearch.com/toolkit/spcalc/power_p2.asp).

3. Results

3.1. Subject characteristics

Table 1 shows the clinical and biochemical characteristics of the study subjects by sex. Diabetic subjects (both male and female) had higher BMI (P < .01), waist circumference (P < .01), total cholesterol (P < .01), LDL cholesterol (P < .01), and serum triglycerides (P < .01) as compared with the NGT subjects.

3.2. Genotype and allele frequency of CAPN10 SNPs

Table 2 shows the genotype and allele frequencies of the 4 SNPs in the study subjects. The genotypic distributions of the SNPs −44 and −19 in both the NGT and the type 2 diabetes mellitus groups were in Hardy-Weinberg equilibrium (HWE). The HWE P values were not computed for SNPs −43 and −63, as the asymptotic HWE approximations are not valid in SNPs with very low minor homozygous genotype frequency. There was no significant difference in the genotypic distribution of SNPs −44, −43, −19, and −63 among the NGT and type 2 diabetes mellitus subjects (P = .20, .86, .34, and .39, respectively). Although the frequency of the minor allele (allele 2, "C") of SNP −44 was higher among the type 2 diabetes mellitus subjects (24%) when compared with the NGT subjects (21%), the difference was not statistically significant (P = .08).

3.3. Association of CAPN10 SNPs with different biochemical parameters

A comparison of the clinical and biochemical characteristics of the study subjects stratified based on SNPs −44, −43, −19, and −63 showed that the NGT subjects with the 11 genotype of the SNP −63 had significantly higher 2-hour postload plasma glucose (mean ± SD, 5.66 ± 1.05 mmol/L) levels compared with the combined 12 and 22 genotype group (5.33 ± 1.11 mmol/L, P = .004) (Table 3). The finding remained significant even after adjusting for age, sex, BMI, smoking, and alcohol consumption (nominal P = .008). We note that, even if we correct for 4 tests of association corresponding to the 4 SNPs considered in our analyses using Bonferroni, the nominal P value is lower than the Bonferroni threshold of .05/4, that is, .0125. Using the online power calculator http://www.dssresearch.com/toolkit/spcalc/power_a2.asp, we find that the power to detect the observed difference in mean plasma glucose levels at level .0125 is approximately .5. No significant difference in the

Table 1
Clinical and biochemical characteristics of the study subjects

	NGT subjects (n = 794)				Type 2 diabetes mellitus subjects (n = 649)			
	Male		Female		Male		Female	
	Mean ± SD	95% CI of mean	Mean ± SD	95% CI of mean	Mean ± SD	95% CI of mean	Mean ± SD	95% CI of mean
n	310	–	484	–	295	–	354	–
Age (y)	46 ± 13	45-48	45 ± 12	44-46	51 ± 11	50-52	51 ± 11	49-52
BMI (kg/m^2)	22.7 ± 4.0	22.2-23.1	24.2 ± 4.9	23.8-24.7	24.1 ± 3.7	23.7-24.6	26.5 ± 4.7	26.0-27.0
Waist circumference (cm)	85.7 ± 11.2	84.4-87.0	83.8 ± 11.6	82.8-84.9	91.5 ± 9.7	90.4-92.7	91.4 ± 10.5	90.3-92.6
Fasting plasma glucose (mmol/L)	4.67 ± 0.46	4.62-4.73	4.70 ± 0.44	4.66-4.74	8.95 ± 3.93	8.50-9.41	9.11 ± 3.80	8.71-9.50
2-h postload plasma glucose (mmol/L)	5.47 ± 1.13	5.34-5.60	5.70 ± 1.01	5.62-5.79	-	-	-	-
Fasting serum insulin (μIU/mL)	8.14 ± 5.93	7.45-8.84	9.52 ± 6.61	8.93-10.1	-	-	-	-
Glycated hemoglobin (%)	5.72 ± 0.60	5.65-5.79	5.74 ± 0.59	5.69-5.79	8.88 ± 2.19	8.63-9.13	8.78 ± 2.05	8.56-8.99
Total cholesterol (mmol/L)	4.70 ± 0.94	4.59-4.81	4.91 ± 0.96	4.83-5.00	5.09 ± 1.07	4.97-5.22	5.36 ± 1.05	5.25-5.47
HDL cholesterol (mmol/L)	1.07 ± 0.27	1.04-1.10	1.22 ± 0.26	1.19-1.24	1.03 ± 0.21	1.01-1.05	1.15 ± 0.23	1.13-1.18
LDL cholesterol (mmol/L)	2.96 ± 0.82	2.86-3.04	3.09 ± 0.82	3.01-3.17	3.03 ± 0.94	2.93-3.14	3.29 ± 0.94	3.19-3.38
Serum triglycerides (mmol/L)	1.26 ± 0.60	1.21-1.31	1.32 ± 0.59	1.25-1.39	1.70 ± 0.81	1.64-1.77	1.87 ± 0.88	1.76-1.97

Two-hour postload plasma glucose and fasting serum insulin were not done for the type 2 diabetes mellitus subjects. P < .01 for all biochemical traits in both male and female subjects.

Table 2
Genotype and allele frequency of the polymorphisms studied

Genotypes	NGT subjects (n = 794)	Type 2 diabetes mellitus subjects (n = 649)	*P* value
SNP −44 (rs2975760)			
11	499 (62.9%)	383 (59.0%)	.20
12	259 (32.6%)	226 (34.8%)	
22	36 (4.5%)	40 (6.2%)	
P for HWE	.74	.39	
MAF	331 (21%)	306 (24%)	
OR (95% CI)	1.17 (0.98-1.40)		.08
SNP −43 (rs3792267)[a]			
11	760 (95.7%)	620 (95.5%)	.86
12 + 22	34 (4.3%)	29 (4.5%)	
MAF	35 (2%)	30 (2%)	
OR (95% CI)	1.05 (0.62-1.77)		.94
SNP −19 (rs3842570)			
11	151 (19%)	135 (21%)	.34
12	405 (51%)	306 (47%)	
22	238 (30%)	208 (32%)	
P for HWE	.35	.25	
MAF	881 (55%)	722 (55%)	
OR (95% CI)	1.01 (0.87-1.17)		.96
SNP −63 (rs5030952)[a]			
11	721 (90.8%)	599 (92.3%)	.39
12 + 22	73 (9.2%)	50 (7.7%)	
MAF	75 (4.7%)	53 (4%)	
OR (95% CI)	0.86 (0.59-1.25)		.45

SNP 44: allele 1, T; allele 2, C. SNP 43: allele 1, G; allele 2, A. SNP 19: allele 1, 2 repeats of 32 base pairs (bp) (Del); allele 2, 3 repeats of 32 bp (Ins). SNP 63: allele 1, C; allele 2, T. MAF indicates minor allele frequency; OR, odds ratio; CI, confidence interval.

[a] Because the frequencies of the minor homozygous genotypes are very low, they have been combined with the heterozygous genotypes. The HWE *P* values are not presented, as the asymptotic HWE approximations are not valid.

biochemical parameters was observed when the subjects were stratified according to the −44, −43, and −19 SNPs.

3.4. LD estimation between CAPN10 SNPs and haplotype analysis

Linkage disequilibrium estimation between these SNPs showed that the pairwise LD between any of these SNPs was not high (r^2 values in Appendix A–Table 1). To perform haplotype-based analyses, 4 locus haplotypes were constructed; and analysis was restricted to those haplotypes that have frequency of at least 0.01 in either cases or controls. There was a significant difference in the proportion of the 2111 haplotype of the −44, −43, −19, and −63 SNPs between NGT and type 2 diabetes mellitus groups (nominal *P* value = .014) (Table 4). Because 6 tests were performed corresponding to 6 haplotypes satisfying the selection criterion, a multiple correction was done using Bonferroni. The association finding did not remain significant, as the nominal *P* value is higher than the Bonferroni threshold of .05/6, that is, .0083.

In view of the fact that the association of the haplotype 2111 could not be ascertained after correction for multiple testing, we performed power computation to evaluate whether our sample size had sufficient power to detect the observed difference in the proportion of the haplotype 2111 in the 2 groups and found that the power was approximately 0.6. Different haplotype combinations were also analyzed for association with type 2 diabetes mellitus (Table 4), and the analysis was restricted to those combinations with a frequency higher than 0.01. None of the haplotype combinations showed any significant association with diabetes.

Given that the association of the 2111 haplotype with type 2 diabetes mellitus is a novel finding in South India, we wanted to validate it using permutation tests instead of asymptotic χ^2 tests. We permuted the genotypic combinations at the 4 SNPs arbitrarily between the cases and controls, thus preserving the genotypic, allelic, and haplotypic frequencies in the combined sample. We generated 10 000 such permuted data sets to obtain the empirical distribution of the difference in proportion of the 2111 haplotype between cases and controls. We found that the empirical *P* value of our observed difference is .0013, which would remain significant even after correcting for 6 haplotype tests. This raises the possibility of the association finding to be a true positive.

3.5. Association of CAPN10 haplotypes with different biochemical parameters

Analysis of the clinical and biochemical characteristics of the study subjects carrying different haplotype combinations

Table 3
Two-hour plasma glucose levels of the NGT subjects stratified based on *CAPN10* SNPs

	n	2-h postload plasma glucose (mmol/L)
SNP −44		
11	499	5.66 ± 1.05
12	259	5.66 ± 1.05
22	36	5.61 ± 1.22
SNP −43		
11	760	5.66 ± 1.05
12 + 22[a]	34	5.94 ± 0.94
SNP −19		
11	151	5.66 ± 1.05
12	405	5.66 ± 1.0
22	238	5.61 ± 1.16
SNP −63		
11	721	5.66 ± 1.05
12 + 22[a]	73	5.33 ± 1.11*

Data presented as mean ± SD.

[a] Because the frequencies of the minor homozygous genotypes are very low, they have been combined with the heterozygous genotypes.

* *P* = .008 after adjusting for age, sex, BMI, smoking, and alcohol consumption.

Table 4
Comparison of frequencies of haplotypes and haplotype combinations in NGT and type 2 diabetes mellitus subjects

Haplotypes for SNPs 44/43/19/63 (>1% frequency)	NGT subjects n (%)	Type 2 diabetes mellitus subjects n (%)	Observed value of test statistics (*z*)	*P* value
1111	319 (0.201)	232 (0.179)	−1.495	.136
1121	830 (0.523)	676 (0.521)	−0.107	.912
2111	300 (0.189)	293 (0.226)	2.446	**.014***
1112	73 (0.046)	50 (0.039)	0.83	.406
1221	35 (0.022)	30 (0.023)	0.054	.957
2121	14 (0.009)	13 (0.010)	0.010	.934
Haplotype combinations (>1% frequency)	**NGT subjects n (%)**	**Type 2 diabetes mellitus subjects n (%)**	**OR[a] (95% CI)**	***P* value**
2111/2111	31 (0.039)	39 (0.060)	1.57 (0.97-2.55)	.066
2111/1111	62 (0.078)	50 (0.077)	0.98 (0.66-1.45)	.941
2111/1121	177 (0.223)	149 (0.230)	1.03 (0.81-1.33)	.763
2111/1221	6 (0.008)	9 (0.015)	1.88 (0.69-5.15)	.208
2111/1112	6 (0.008)	7 (0.012)	1.50 (0.52-4.31)	.443

SNP 44: allele 1, T; allele 2, C. SNP 43: allele 1, G; allele 2, A. SNP 19: allele 1, 2 repeats of 32 bp (Del); allele 2, 3 repeats of 32 bp (Ins). SNP 63: allele 1, C; allele 2, T.

[a] Odds ratio was computed taking all other haplotype combinations as reference.

* Proportion of 2111 haplotype significantly different between NGT and type 2 diabetic groups.

(2111/2111, 2111/1111, and 2111/1121) was performed (Appendix A–Table 2). The NGT subjects with the 2111/1121 combination of genotypes had significantly higher cholesterol levels (mean ± SD, 4.96 ± 1.04 mmol/L) compared with the subjects with the rest of the genotypes (4.78 ± 0.93 mmol/L, $P = .036$). The P value remained significant (.031) after adjusting for age, sex, BMI, smoking, and alcohol consumption. However, this significant finding with total cholesterol does not stand up after correction for multiple tests with respect to either the number of haplotype combinations tested or the number of quantitative phenotypes considered.

We also performed a post hoc computation to evaluate the power to detect the observed difference in the means of the total cholesterol levels between the 2 genotypic groups. We found that using a Bonferroni-corrected level of 0.016 corresponding to the 3 genotypic tests, the power to detect the observed difference is approximately 0.45 and hence would ideally require a larger sample size to confirm the association finding. Subjects with the 2111/1111 and 2111/2111 haplotype combinations did not show any significant difference in the biochemical parameters as compared with subjects with the rest of the genotypes.

4. Discussion

In this study, we examined the association of the −44, −43, −19, and −63 SNPs in the *CAPN10* gene with type 2 diabetes mellitus using a case-control method. We selected these polymorphisms because of their prior association with either type 2 diabetes mellitus (SNPs −43, −19, and −63, either individually or in combination), insulin resistance (SNP −43), or transcriptional regulation of calpain-10 expression (SNPs −44 and −43). Neither the genotypic frequencies nor the allelic frequencies of any of the 4 SNPs were found to be associated with type 2 diabetes mellitus. This is in accordance with studies from the British [6], Oji-Cree [7], Samoan [8], Chinese [9], European Americans [11], Finnish [12], Danish/Swedish [13], and Japanese [14] populations. A previous study from South India also failed to find an association of these SNPs individually with diabetes except that the rare allele of −63 SNP was associated with impaired fasting glucose/impaired glucose tolerance [3].

Because haplotypes describe diabetes risk better than when the SNPs are analyzed individually, we constructed 4 locus haplotypes; and the 2111 haplotype of SNPs −44, −43, −19, and −63 was found to be associated with type 2 diabetes mellitus with a nominal significance ($P = .014$). Although the Bonferroni correction based on the asymptotic test does not preserve this significance, the test based on the empirical distribution remained significant. Association of the 2111 haplotype with diabetes has been previously shown in the British transmission disequilibrium test (TDT) study, where the SNP −44 allele 2 as well as the 2111 haplotype of SNPs −44, −43, −19, and −63 showed association with type 2 diabetes mellitus [6]. An analysis pooling the British TDT data with the Finnish TDT data also suggested the 2111 haplotype to be associated with diabetes [4]. The 2111 haplotype was not shown to be associated with diabetes in the previous report from South Indians [3]. Although the frequency of the 2111 haplotype in the NGT subjects was similar in both studies, the frequency in type 2 diabetes mellitus subjects was 22.6% in our study as compared with 18.4% in the previous report.

None of the SNPs or haplotype combinations showed any significant association with any of the biochemical

parameters investigated. However, NGT subjects with the 11 genotype of the SNP −63 had significantly higher 2-hour postload plasma glucose levels compared with the combined 12 and 22 genotype groups. This complements our finding based on the permutation test that the haplotype 2111 with the allele 1 of −63 SNP may be associated with diabetes. Interestingly, a meta-analysis in subjects of European ancestry that showed a significant effect of the121/121 haplogenotype of SNPs −43, −19, and −63 on risk of type 2 diabetes mellitus also consisted of the 11 genotype of SNP −63 [17]. However, we note that, although the permutation test of the 2111 haplotype provided a significant association even after correction for multiple testing, the regular χ^2 association test did not survive the Bonferroni correction for multiple testing; and hence, we need to interpret the association finding with caution, and an independent validation on a similar sample would strengthen our inference.

The haplotypes constructed in our study differ from the Mexican Americans [2] by the inclusion of the −44 SNP. The Mexican American 112 haplotype of SNPs −43, −19, and −63 is equivalent to our 1112 haplotype of SNPs −44, −43, −19, and −63, as 2112 haplotype was not found in our population. The Mexican American 121 haplotype is equivalent to our 1121 haplotype, as the 2121 haplotype had a very low frequency. Therefore, 1112/1121 haplotype combination in our population would be equivalent to the 112/121 "at risk" haplotype combination reported in the Mexican Americans. The 1112/1121 haplotype combination was not found to be associated with diabetes in this study population. Its frequency was found to be 0.05 (n = 41) in NGTs and 0.04 (n = 27) in type 2 diabetes mellitus subjects. This is in contrast to the previous report in South Indians [3] that reported an association of the 1112/1121 haplotype with diabetes, its frequency being 0.009 (n = 3) in NGTs and 0.06 (n = 5) in unrelated diabetic subjects (P = .015). The reason for this discrepancy is not known. One possibility could be the issue of population stratification, as it has been shown that there is significant diversity in allele frequencies at many autosomal loci within different castes in South India [35].

To minimize the risk of population stratification, we did a cross validation using genomic controls [36]. We performed a case-control study at 5 unlinked marker loci believed to be unrelated to diabetes but known to have allelic diversity among different populations. These loci were as follows: *Alu* repeat *TPA-25* (subfamily: HS-2) on chromosome 8, *Alu* repeat *PV-92* (subfamily: HS-1) on chromosome 16, *Alu* repeat *FXIIIB* (subfamily: HS-1) on chromosome 1, *Alu* repeat *ACE* (subfamily: HS-1) on chromosome 17, and *Alu* repeat *D1* (subfamily: HS-1) on chromosome 3. The allele frequency difference between diabetic and NGT subjects was not statistically significant at any of the 5 loci studied. This indicates that the findings in this study are not likely to be an artifact of population substructuring.

The novel 111/121 diplotype of SNPs −43, −19, and −63 that has been shown to be associated with diabetes in the Korean population [5] was found at a frequency of 0.43 in NGTs and 0.40 in type 2 diabetes mellitus subjects and was not associated with diabetes in this study population (P = .27). Although variation in *CAPN10* has been associated with type 2 diabetes mellitus in different populations, the polymorphisms and haplotypes associated with diabetes differ between populations. This may be due to the presence of different patterns of LD between these polymorphisms and a common causal variant(s).

In conclusion, our study raises the possibility that the 2111 haplotype of SNPs −44, −43, −19, and −63 may be associated with type 2 diabetes mellitus, although none of these SNPs may be individually associated with diabetes.

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Appendix A

Table 1
Linkage disequilibrium between the *CAPN10* polymorphisms

Locus 1	Locus 2	r^2 values	
		NGT	Type 2 diabetes mellitus
SNP −44	SNP −43	0.006	0.007
SNP −44	SNP −19	0.277	0.326
SNP −44	SNP −63	0.011	0.013
SNP −43	SNP −19	0.018	0.019
SNP −43	SNP −63	0.001	0.001
SNP −19	SNP −63	0.057	0.042

Table 2
Clinical and biochemical characteristics of the NGT subjects carrying the 2111/2111, 2111/1111, and 2111/1121 haplotype combinations of SNPs −44, −43, −19, and −63 of the *CAPN10* gene compared with the rest of the haplotype combinations

	2111/2111 (n = 31)	Other haplotype combinations (n = 763)	2111/1111 (n = 62)	Other haplotype combinations (n = 732)	2111/1121 (n = 177)	Other haplotype combinations (n = 617)
BMI (kg/m^2)	22.9 ± 4.4	23.7 ± 4.7	24.0 ± 5.0	23.6 ± 4.6	23.4 ± 4.7	23.7 ± 4.6
Waist circumference (cm)	83.1 ± 12.3	84.6 ± 11.5	85.1 ± 12.1	84.5 ± 11.5	84.1 ± 11.6	84.6 ± 11.5
Fasting plasma glucose (mmol/L)	4.62 ± 0.49	4.67 ± 0.44	4.73 ± 0.38	4.67 ± 0.44	4.67 ± 0.44	4.67 ± 0.44
2-h postload plasma glucose (mmol/L)	5.44 ± 1.21	5.61 ± 1.04	5.55 ± 1.04	5.61 ± 1.04	5.66 ± 0.99	5.61 ± 1.04
Fasting serum insulin (μIU/mL)	7.1 ± 4.6	9.1 ± 6.4	9.1 ± 6.5	9.0 ± 6.4	8.7 ± 6.0	9.0 ± 6.5
HOMA-IR	1.8 ± 0.8	1.9 ± 1.4	1.9 ± 1.5	1.9 ± 1.4	1.8 ± 1.3	1.9 ± 1.4
Glycated hemoglobin (%)	5.6 ± 0.50	5.7 ± 0.60	5.8 ± 0.63	5.7 ± 0.59	5.7 ± 0.60	5.7 ± 0.60
Total cholesterol (mmol/L)	4.81 ± 0.96	4.83 ± 0.93	4.88 ± 0.98	4.83 ± 0.96	**4.96 ± 1.04***	**4.78 ± 0.93**
HDL cholesterol (mmol/L)	1.23 ± 0.30	1.16 ± 0.27	1.14 ± 0.29	1.17 ± 0.27	1.19 ± 0.28	1.16 ± 0.27
LDL cholesterol (mmol/L)	3.01 ± 0.78	3.04 ± 0.83	3.06 ± 0.85	3.04 ± 0.83	3.14 ± 0.93	3.01 ± 0.80
Serum triglycerides (mmol/L)	1.16 ± 0.50	1.32 ± 0.67	1.40 ± 0.66	1.30 ± 0.67	1.32 ± 0.67	1.30 ± 0.67

Data presented as mean ± SD. HOMA-IR indicates homeostasis model assessment of insulin resistance. *P = .031 after adjusting for age, sex, BMI, smoking, and alcohol consumption.

References

[1] Hanis CL, Boerwinkle E, Chakraborty R, et al. A genome-wide search for human non–insulin-dependent (type 2) diabetes genes reveals a major susceptibility locus on chromosome 2. Nat Genet 1996;13:161-6.

[2] Horikawa Y, Oda N, Cox NJ, et al. Genetic variation in the gene encoding calpain-10 is associated with type 2 diabetes mellitus. Nat Genet 2000;26:163-75.

[3] Cassell PG, Jackson AE, North BV, et al. Haplotype combinations of calpain 10 gene polymorphisms associate with increased risk of impaired glucose tolerance and type 2 diabetes in South Indians. Diabetes 2002;51:1622-8.

[4] Orho-Melander M, Klannemark M, Svensson MK, Ridderstråle M, Lindgren CM, Groop L. Variants in the calpain-10 gene predispose to insulin resistance and elevated free fatty acid levels. Diabetes 2002;51: 2658-64.

[5] Kang ES, Kim HJ, Nam M, et al. A novel 111/121 diplotype in the calpain-10 gene is associated with type 2 diabetes. J Hum Genet 2006;51:629-33.

[6] Evans JC, Frayling TM, Cassell PG, et al. Studies of association between the gene for calpain-10 and type 2 diabetes mellitus in the United Kingdom. Am J Hum Genet 2001;69:544-52.

[7] Hegele RA, Harris SB, Zinman B, Hanley AJ, Cao H. Absence of association of type 2 diabetes with *CAPN10* and PC-1 polymorphisms in Oji-Cree. Diabetes Care 2001;24:1498-9.

[8] Tsai HJ, Sun G, Weeks DE, et al. Type 2 diabetes and three calpain-10 gene polymorphisms in Samoans: no evidence of association. Am J Hum Genet 2001;69:1236-44.

[9] Sun HX, Zhang KX, Du WN, et al. Single nucleotide polymorphisms in *CAPN10* gene of Chinese people and its correlation with type 2 diabetes mellitus in Han people of northern China. Biomed Environ Sci 2002;15:75-82.

[10] Daimon M, Oizumi T, Saitoh T, et al. Calpain 10 gene polymorphisms are related, not to type 2 diabetes, but to increased serum cholesterol in Japanese. Diabetes Res Clin Pract 2002;56:147-52.

[11] Elbein SC, Chu W, Ren Q, et al. Role of calpain-10 gene variants in familial type 2 diabetes in Caucasians. J Clin Endocrinol Metab 2002;87:650-4.

[12] Fingerlin TE, Erdos MR, Watanabe RM, et al. Variation in three single nucleotide polymorphisms in the calpain-10 gene not associated with type 2 diabetes in a large Finnish cohort. Diabetes 2002;51:1644-8.

[13] Rasmussen SK, Urhammer SA, Berglund L, et al. Variants within the calpain-10 gene on chromosome 2q37 (NIDDM1) and relationships to type 2 diabetes, insulin resistance, and impaired acute insulin secretion among Scandinavian Caucasians. Diabetes 2002; 51:561-7.

[14] Horikawa Y, Oda N, Yu L, et al. Genetic variations in calpain-10 gene are not a major factor in the occurrence of type 2 diabetes in Japanese. J Clin Endocrinol Metab 2003;88:244-7.

[15] Song Y, You N, Hsu Y, et al. Common genetic variation in calpain-10 gene (*CAPN10*) and diabetes risk in a multi-ethnic cohort of American postmenopausal women. Hum Mol Gen 2007;16:2960-71.

[16] Song Y, Niu T, Manson JE, Kwiatkowski DJ, Liu S. Are variants in the *CAPN10* gene related to risk of type 2 diabetes? A quantitative assessment of population and family-based association studies. Am J Hum Genet 2004;74:208-22.

[17] Tsuchiya T, Schwarz EH, Bosque-Plata LD, et al. Association of the calpain-10 gene with type 2 diabetes in Europeans: results of pooled and meta analyses. Mol Genet Metab 2006;89:174-84.

[18] Jensen DP, Urhammer SA, Eiberg H, et al. Variation in *CAPN10* in relation to type 2 diabetes, obesity and quantitative metabolic traits: studies in 6018 whites. Mol Genet Metab 2006;89:360-7.

[19] Turner MD, Cassell PG, Hitman GA. Calpain-10: from genome search to function. Diabetes Metab Res Rev 2005;21:505-14.

[20] Cox NJ, Hayes MG, Roe CA, Tsuchiya T, Bell GI. Linkage of calpain-10 to type 2 diabetes: the biological rationale. Diabetes 2004;53: S19-25.
[21] Fullerton SM, Bartoszewicz A, Ybazeta G, et al. Geographic and haplotype structure of candidate type 2 diabetes susceptibility variants at the calpain-10 locus. Am J Hum Genet 2002;70:1096-106.
[22] Sharp PS, Mohan V, Levy JC, Mather HM, Kohner EM. Insulin resistance in patients of Asian Indian and European origin with non-insulin dependent diabetes. Horm Metab Res 1987;19:84-5.
[23] Abate N, Chandalia M. The impact of ethnicity on type 2 diabetes. J Diabetes Complications 2003;17:39-58.
[24] Turner MD. Coordinated control of both insulin secretion and insulin action through calpain-10-mediated regulation of exocytosis? Mol Gen Metab 2007;91:305-7.
[25] Deepa M, Pradeepa R, Rema M, et al. The Chennai Urban Rural Epidemiology Study (CURES)—study design and methodology (urban component) (CURES1). J Assoc Phys India 2003;51: 863-70.
[26] Alberti KG, Zimmet PZ. Definition diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus, provisional report of a WHO consultation. Diabet Med 1998;15:539-53.
[27] Trinder P. Determination of glucose in blood using glucose oxidase with alternative oxygen acceptor. Ann Clin Biochem 1969;6:24-7.
[28] Allian CC, Poon LS, Chan CS, Richmond W. CHOD-PAP method for determination of total cholesterol. Clin Chem 1974;20:470.
[29] Wahefeld AW, Bergmeyer HU, editors. Methods of enzymatic analysis, 2nd English ed.New York (NY): Academic Press Inc; 1974. p. 1831.
[30] Sugiuchi H, Uji Y, Okabe H, et al. Direct measurement of high-density lipoprotein cholesterol in serum with polyethylene glycol–modified enzymes. Clin Chem 1995;41:717-23.
[31] Friedewald WT, Levy RI, Fredrickson DS. Estimation of low density lipoprotein cholesterol without the use of the preparative ultracentrifuge. Clin Chem 1972;18:499-502.
[32] Nathan DM, Raskin P. Convenient automated method for liquid-chromatographic measurement of glycated hemoglobin. Clin Chem 1984;30:813-4.
[33] Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 2005;21: 263-5.
[34] Majumdar P and Majumder PP. HAPLOPOP: a computer program to obtain maximum-likelihood estimates of haplotype frequencies from genotype data on a set of unrelated individuals via the EM algorithm. 1999; technical report # AHGU-1/1999 Indian Statistical Institute, Calcutta.
[35] Basu A, Mukherjee N, Roy S, et al. Ethnic India: a genomic view, with special reference to peopling and structure. Genome Res 2003;13:2277-90.
[36] Devlin B, Roeder K, Wasserman L. Genomic control, a new approach to genetic-based association studies. Theor Popul Biol 2001;60: 155-66.