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Studies of Association between the Gene for Calpain-10 and Type 2 Diabetes Mellitus in the United Kingdom

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Variation in *CAPN10*, the gene encoding the ubiquitously expressed cysteine protease calpain-10, has been associated with type 2 diabetes in Mexican Americans and in two northern-European populations, from Finland and Germany. We have studied *CAPN10* in white subjects of British/Irish ancestry, using both family-based and case-control studies. In 743 sib pairs, there was no evidence of linkage at the *CAPN10* locus, which thereby excluded it as a diabetes-susceptibility gene, with an overall sib recurrence risk, λ_S, of 1.25. We examined four single-nucleotide polymorphisms (SNP-44, -43, -19, and -63) previously either associated with type 2 diabetes or implicated in transcriptional regulation of calpain-10 expression. We did not find any association between SNP-43, -19, and -63, either individually or as part of the previously described risk haplotypes. We did, however, observe significantly increased ($P = .033$) transmission of the less common C allele at SNP-44, to affected offspring in parents-offspring trios (odds ratio 1.6). An independent U.K. case-control study and a small discordant-sib study did not show significant association individually. In a combined analysis of all U.K. studies ($P = .015$) and in combination with a Mexican American study ($P = .004$), the C allele at SNP-44 is associated with type 2 diabetes. Sequencing of the coding region of *CAPN10* in a group of U.K. subjects revealed four coding polymorphisms—L34V, T504A, R555C, and V666I. The T504A polymorphism was in perfect linkage disequilibrium with the diabetes-associated C allele at SNP-44, suggesting that the synthesis of a mutant protein and/or altered transcriptional regulation could contribute to diabetes risk. In conclusion, we were not able to replicate the association of the specific calpain-10 alleles identified by Horikawa et al. but suggest that other alleles at this locus may increase type 2 diabetes risk in the U.K. population.

Introduction

Type 2 diabetes mellitus is a common chronic disorder affecting >135 million people worldwide (King et al. 1998). It is characterized by three major metabolic abnormalities: impaired insulin-stimulated glucose uptake in muscle and fat, alterations in glucose-stimulated insulin secretion, and increased hepatic glucose production (American Diabetes Association 1997). Both genetic and nongenetic factors contribute to its development. Recent studies have shown that the variation in *CAPN10* (MIM 605286), the gene encoding calpain-10, affects susceptibility to type 2 diabetes mellitus in Mexican Americans and in two northern-European populations: the Swedish-speaking population of the Botnia region of Finland and the German population of Saxony (Horikawa et al. 2000). The inheritance of a specific haplotype combination defined by three single-nucleotide polymorphisms—SNP-43, -19, and -63—was found to be associated with a threefold increased risk. These polymorphisms are in noncod-

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Table 1

Clinical Characteristics of Subjects

	No. of Subjects (M:F)	AAD[a] (years)	BMI[a] (kg/m^2)	Treatment: Diet/OHA/Insulin[b] (%)
Sib-pair linkage study	1,223 (659:564)	55.6 ± 8.6	28.7 ± 5.4	20/64/16
Case-control study 1:				
Trios probands	153 (96:57)	40.1 ± 7.1	32.0 ± 6.8	21/64/15
Controls[c]	411(213:198)	N/A	N/A	N/A
Case-control study 2:				
Diabetic probands	222 (106:116)	56.0 ± 8.0	28.1 ± 5.4	20/60/20
Adult controls	212 (71:141)	50.4 ± 13.7[d]	27.0 ± 4.5	N/A
Discordant-sib study:				
Diabetic subjects	49 (23:26)	53.5 ± 8.1	30.2 ± 6.3	18/74/8
Unaffected sibs	49 (17:32)	61.0 ± 9.5[d]	28.0 ± 4.1	N/A

NOTE.— The probands for case-control study 2, as well as the diabetic and nondiabetic sibs in the discordant-sib study, were taken from the sib-pair families, but, to avoid replication, no individual was included in more than one association study.

[a] Data are as mean ± SD.

[b] OHA = oral hypoglycemic agents; N/A = not applicable.

[c] Birth cohort.

[d] Age when studied.

ing regions of *CAPN10* and are believed to alter risk by affecting the transcriptional regulation of calpain-10 (Horikawa et al. 2000). The presence of SNP-43 and an adjacent polymorphism, SNP-44, in an enhancer-like element, as well as the association between the SNP-43 genotype and calpain-10 mRNA levels in skeletal muscle, is consistent with this hypothesis (Baier et al. 2000; Horikawa et al. 2000). SNP-43 was also found to be associated with measures of insulin action in Pima Indians with normal glucose tolerance, suggesting that calpain-10 increases susceptibility to type 2 diabetes through its effects on the oxidation of glucose in skeletal muscle (Baier et al. 2000).

The identification of *CAPN10* as a candidate gene for type 2 diabetes susceptibility—and of specific variants that alter risk—allows us to examine the contribution of this gene to diabetes risk in other populations. Here, we examine the contribution of *CAPN10* to the development of type 2 diabetes in white subjects of English/Irish ancestry, using family-based and case-control studies.

Subjects and Methods

Subjects

The clinical characteristics of the British/Irish type 2–diabetic and control subjects used in the linkage and association studies are summarized in table 1. Linkage studies were performed on 743 sib pairs from 573 families in the Diabetes UK Warren 2 Repository, which consists of families of British/Irish origin, each of which has at least two sibs diagnosed with type 2 diabetes who

Table 2

Sequences of Primers for PCR and Sequencing Exons 1–7 and 9–13 of *CAPN10*

Exon(s)	Primer (5′→3′) Forward	Primer (5′→3′) Reverse	Product Size (bp)
1	GATTGGGCCGCCTGTCACGTG	AACGGCGGACCCTGCGTTCG	501
2	TTCGAAGCCAACATTTAGCTG	AGGGCGAACCTCCCTGATGGAGC	647
3	GTGTGCGTTAGAGTTCTCTGCAG	AGAATGAGCTGCCAGACCCTC	469
4	GGCTGGCTGGTGACATCAGTG	CTTAGTCCTGACAGGGCTGTG	715
5	GTGGGCTGGAGCATCTCCAG	AGCAGGGACTCAGGGGCCTTG	378
6	TGCAGAGCTGCTTCGGGTGTGG	CAGAGCACGTGCCCAGCACAG	369
7	ACCCTGCCAGGGTTCATGAGG	AAGGCAGAAAGACCTGCAGTC	577
9/10	TGGGCCACGGTGCCTTTGTGG	CCTCTGAGAGGTTGGAGATGG	1,065
11	GGCTGTATGTGACTCAAGAGG	GTGAGGGGCAGGCAGGAGAC	402
12/13	TCTGAGCCTGGAAGGAGAGTC	ACCAGCCCCCACAGTGCAAG	528

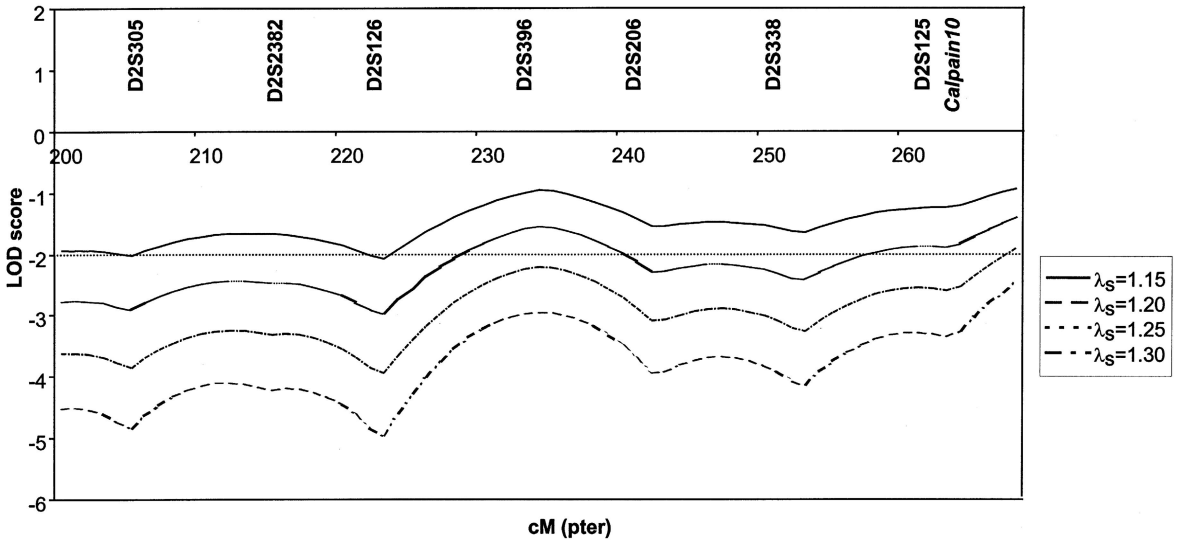

Figure 1 Studies of linkage between *CAPN10* region of chromosome 2 and type 2 diabetes in affected sib pairs. The LOD score at various values of λ_S is shown. *D2S125* is assumed to be ~3 cM proximal to *CAPN10*.

are 35–70 years of age (Frayling et al. 2000). Transmission distortion was examined in 153 parents-offspring trios from the Diabetes UK Warren 2 Trios Collection (Frayling et al. 1999). Appropriate numbers of microsatellite markers had been typed in both the sib-pair and parent-offspring collections to allow confirmation of family relationships and to exclude half-sibs.

For case-control association studies, we used 222 diabetic probands taken from the sib-pair collection and two control groups. The first group consisted of a birth cohort of 411 babies born in Plymouth, England (Macfarlane et al. 1999); the second control group consisted of 212 nondiabetic adults of British/Irish origin who had normal glucose tolerance as shown by fasting plasma glucose (<5.5 mmol/liter) and/or an HbA1c within the normal range (<6%).

A discordant-sib analysis was performed in a subgroup of 49 families from the Diabetes UK Warren 2 Repository sib-pair collection in whom DNA was available from nondiabetic sibs. Family members were defined as nondiabetic when (*a*) a clinical diagnosis of diabetes had not been made and (*b*) they had an HbA1c within the normal range (<6%). This subgroup was taken from the same families that contributed the 222 probands. To avoid duplication, we used a nonproband diabetic sib for the discordant-sib analysis, whereas the diabetic probands were used in the case-control association study.

Linkage Analysis

Microsatellites of type $(CA)_n$ were used for the linkage analysis. These were genotyped by PCR using fluorescently labeled primers, with detection by an ABI 377

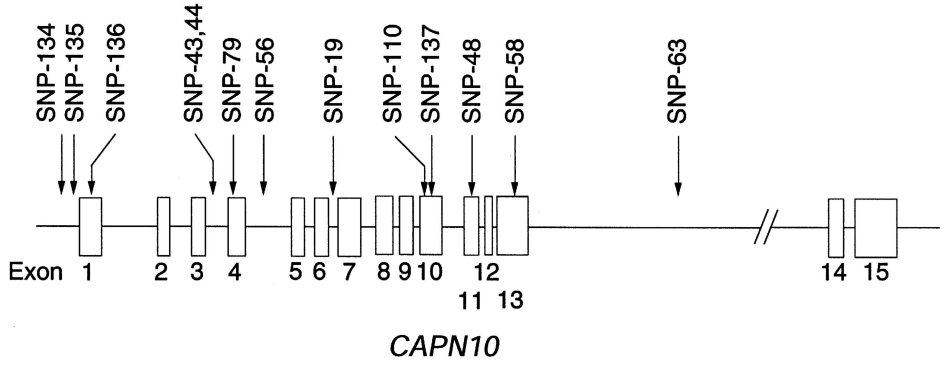

Figure 2 Exon-intron organization of *CAPN10*. The locations of the SNPs described in the text are shown.

Table 3

Transmission of *CAPN10* SNP Alleles from Heterozygous Parents to Diabetic Offspring

	No.		
Polymorphism	Transmitted	Not Transmitted	χ^2 (*P*)
SNP-44	54	34	4.55 (.033)
SNP-43	59	48	1.13 (.29)
SNP-19	70	63	.37 (.54)
SNP-63	19	27	1.39 (.24)

Note.—Data shown are for allele 2 of each polymorphism.

DNA sequencer (Applied Biosystems). Thirty markers spanning chromosome 2 that were from Applied Biosystems linkage mapping set 2 were used, the closest to *CAPN10* being *D2S125*. PCR, electrophoresis, and analysis of the markers were performed according to the manufacturer's protocol.

Genotyping

SNP-43 (CAPN10-*g.4852G/A*).—Subjects were genotyped for this SNP by a mutagenically separated PCR (MS-PCR) method, which uses a common forward primer and two allele-specific reverse primers of different lengths: forward primer, 5′-CATCCATAGCTTCCACGCCTC-3′; reverse primer allele 1 (G), 5′-GCTTAGCCTCACCTTCAATC-3′; and reverse primer allele 2 (A), 5′-ATCCTCACCAAGTCAAGCGTTAGCCTCACCTTCAAGT-3′. The underlined nucleotides are mismatched to the template, to improve the allele specificity (Newton et al. 1989). PCR was performed in a 10-μl volume containing 1 × PCR buffer (Applied Biosystems), 200 μmol of each dNTP/liter, 1.5 mmol of $MgCl_2$/liter, 0.25 U Ampli*Taq* Gold (Applied Biosystems), and 40 ng of genomic DNA. Primer concentrations were 1,000 nmol of common primer/liter, 1,000 nmol of allele 1 primer/liter, and 67 nmol of allele 2 primer/liter, giving a 15:1 ratio of short primers to long primer. The cycling conditions were 96°C for 12 min; 35 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s; and 72°Cfor 10 min. The PCR products were separated on a 3% NuSieve agarose gel (Flowgen) and were visualized by ethidium bromide staining: allele 1 (G) is 134 bp and, allele 2 (A) is 152 bp. We validated the MS-PCR method by genotyping 30 samples, using this method and DNA sequencing; no inconsistencies were found. In addition, we typed eight samples in 10 separate reactions, and all samples were typed correctly on all occasions.

SNP-44 (CAPN10-*g.4841T/C*).—We also typed this SNP by an MS-PCR method. The primers used were as follows: common reverse primer, 5′-CTCATCCTCACCAAGTCAAGGC-3′; allele 1 (T) primer, 5′-CAGGGCGCTCACGCTTGCTAT-3′; and allele 2 (C) primer, 5′-GTGGGCAGAGGACTGGTGGGCGCTCACGCTTGCTTC-3′. The reaction mixture was the same as that used for SNP-43; cycling conditions were also the same, except for the annealing temperature, which was 60°C. The PCR products were separated on 3% NuSieve agarose gel: allele 1 (T) is 60 bp, and allele 2 (C) is 75 bp. We compared the genotypes of 18 samples, by both this method and DNA sequencing, and we found no differences. We also typed six samples 10 times, using MS-PCR, and all were typed correctly.

SNP-19 (CAPN10-*g.7920indel32bp*).—This insertion/deletion polymorphism was amplified by forward and reverse primers—5′- GTTTGGTTCTCTTCAGCGTGGAG-3′ and 5′- CATGAACCCTGGCAGGGTCTAAG-3′, respectively. PCR was performed in a 10-μl volume containing 1 × PCR buffer, 200 μmol of each dNTP/liter, 1.5 mmol of $MgCl_2$/liter, 5% dimethyl sulfoxide, 250 nmol of each primer/liter, 0.25 U of Ampli*Taq* Gold, and 40 ng of genomic DNA. The cycling conditions were 94°C for 12 min; 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and 72°C for 10 min. The PCR products were separated on a 3% NuSieve agarose gel (Flowgen): allele 1 (two repeats of 32-bp sequence) is 155 bp, and allele 2 (three repeats) is 187 bp.

SNP-63 (CAPN10-*g.16378C/T*).—This SNP was typed by a protocol provided by Dr. Marju Orho-Melander (Malmo University Hospital, Lund, Sweden). The forward and reverse primers were 5′-AAGGGGGGCCAGGGCCTGACGGGGGTGGCG-3′ and 5′-AGCACTCCCAGCTCCTGATC-3′, respectively. The PCR conditions were the same as those for SNP-19, except that the annealing temperature was 62°C. PCR products were digested with 2 units of *Hha*I (NEB) in 1 × NE4 buffer (NEB) plus 1 × bovine serum albumin, at 37°C for 2 h. The digested products were separated on 3% NuSieve agarose gel. Alleles 1 (C) and 2 (T) are 162 and 192 bp, respectively.

SNP-110 (CAPN10-*g.9803A/G*).—This SNP, which generates the polymorphism T504A, was amplified by

Table 4

Transmission of *CAPN10* SNP-44, -43, -19, and -63 Haplotypes from Heterozygous Parents to Diabetic Offspring

		No.		
Haplotype	Frequency in Trios	Transmitted	Not Transmitted	χ^2 (*P*)
1111	.16	31	42	1.64 (.20)
1121	.06	50	55	.23 (.63)
1221	.30	47	38	.95 (.33)
2111	.26	45	28	3.95 (.047)
1112	.22	13	24	3.28 (.07)
2112	.004	1	0	... (...)

Note.—It was possible to assign unequivocal haplotypes for 427 of the 459 members of the trios. The allele frequencies and transmission data are for only those subjects in whom unequivocal haplotypes were assigned.

Table 5

Allele Frequencies of *CAPN10* Polymorphisms

	Frequency[a]			
	Case-Control Study 1		Case-Control Study 2	
Polymorphism	Trios Probands (n = 153)	Population Controls (n = 411)	Type 2–Diabetic Probands (n = 222)	Adult Controls (n = 212)
SNP-44	.77	.84	.84	.86
SNP-43	.74	.75	.73	.72
SNP-19	.45	.39	.38	ND
SNP-63	.93	.93	.92	ND

[a] All frequency are for allele 1. ND = not determined.

forward and reverse primers—5′-CGCCATCAGGGCAGTGGCCAAGAACAGC-3 and 5′-CAGAGTGATGCGGACGCAGCG, respectively. The PCR conditions were the same as those for SNP-19, and the PCR product was digested with *Hha*I, as described for SNP-63. The digestion products were separated on 3% Metaphor agarose gel (Flowgen). Allele 1 (A [Thr]) is 196 bp, and allele 2 (G [Ala]) is digested to 172 bp + 24 bp.

Resequencing of CAPN10

All the coding-sequence encoding exons (i.e., exons 1–7 and 9–13 [GenBank accession number AF158748]) were sequenced. Each exon was amplified by flanking primers (table 2) and an Expand long-template PCR kit (Roche) and were sequenced using a BigDye terminator kit (Applied Biosystems).

Statistical Analyses

Linkage analyses.—Multipoint nonparametric linkage analysis was performed by GENEHUNTER (Kruglyak et al. 1996) and GENEHUNTER-PLUS software (Kong and Cox 1997). Exclusion analysis was performed by the "exclude" command of GENEHUNTER, under the assumption that there was no dominance variance. Allele frequencies were derived from the family data by the RECODE program. The position of *CAPN10* was taken to be 3 cM distal to *D2S125*.

Allele-frequency comparisons.—Allele frequencies were compared, between groups, by a χ^2 test.

Transmission/disequilibrium test (TDT).—We used the TDT (Spielman et al. 1993) to test for linkage disequilibrium between polymorphisms and type 2 diabetes in the trios. The P values for the number of allele transmissions versus the number of nontransmissions were calculated by χ^2 tests.

Discordant-sibs analysis.—We performed a discordant-sib analysis by using a second, nonproband affected sib and a single (the eldest, when there were more than one) nondiabetic sib from the 49 families in whom there was at least one unaffected sib. Allele frequencies were compared by a χ^2 test.

Results

Linkage Studies

We tested for linkage between the *CAPN10* region of chromosome 2 and type 2 diabetes in a group of affected sib pairs from the Diabetes UK Warren 2 Repository, which consists of 573 families with a maximum 743 affected sib pairs. We did not find any evidence for linkage: the GENEHUNTER-PLUS maximum LOD score was 0.02, and we were able to exclude an effect, with a LOD score of 1.25, for the *CAPN10* region (fig. 1).

Family-Based Studies: Allele and Haplotype TDT

We typed SNP-44, -43, -19, and -63 (fig. 2) in the 153 trios. There was no significant departure from the expected Mendelian 50:50 transmission ratio, at either SNP-43, -19, or -63 (table 3). However, the C allele (allele 2) at SNP-44 was transmitted more often than expected to affected offspring (P = .03).

Horikawa et. al. (2000) have shown that haplotypes formed by SNP-43, -19, and -63 are better able to define the risk of type 2 diabetes than are individual SNPs. We constructed haplotypes from these polymorphisms and SNP-44, using the trios. Unequivocal haplotypes could be constructed in 427 of the 459 members of the trios. We found strong linkage disequilibrium between the four SNPs, with five haplotypes accounting for 99.6% of haplotypes (table 4). The C allele (allele 2) at SNP-44 occurred on only one of the common haplotypes (2111). The transmission of the haplotypes is shown in table 4. Only the 2111 haplotype, containing the SNP-44 rare allele, showed greater-than-expected transmission to diabetic offspring.

Table 6

Haplotype Frequencies: SNP-44, -43, -19, and -63 Combinations

	Frequency			
Haplotype	Trios Probands (n = 153)	Population Controls (n = 411)	Type 2–Diabetic Probands (n = 222)	Mexican Americans[a] (n = 98)
1111	.16	.16	.14	.12
1112	.06	.07	.08	.23
1121	.30	.36	.35	.32
1221	.26	.25	.27	.27
2111	.22	.16	.15	.06

[a] Frequencies are those reported by Horikawa et al. (2000).

Genotyping of Cases of Type 2 Diabetes and of Controls

We typed SNP-44, -43, -19, and -63 in 222 type 2–diabetic probands and in 411 population controls, and we typed SNP-44 and -43 in 212 adult controls. The allelic frequencies from these analyses are shown in table 5. The only significant difference was an excess of allele 2 of SNP-44 in the trios probands, compared with that in the population controls (.23 vs. .16; $P = .005$).

Haplotypes were constructed under the assumption that the five principle haplotypes seen in the trios were the principle haplotypes in both the cases and the controls. The calculated haplotype frequencies are shown in table 6. There was a clear difference between the U.K. haplotype frequency and that in Mexican American controls (table 6) previously reported by Horikawa et al. (2000): U.K. subjects had a lower frequency of the 1112 haplotype (.08 vs. .23; $P < .0001$) and a higher frequency of the 2111 haplotype (.15 vs. .06; $P = .0003$).

Assessment of Previously Described Allele and Haplotype Associations Seen between Calpain 10 and Type 2 Diabetes

Horikawa et al. (2000) have shown that the 112/121-haplotype combination of SNP-43, -19, and -63 is associated with type 2 diabetes both in Mexican Americans (odds ratio [OR] 3.02 [95% confidence interval {95%CI} 1.37–6.64]) and in a Finnish-and-German group (OR 3.16 [95%CI 1.19–8.40). In our study, there was no preferential transmission, in the trios, of either allele 1 at SNP-43 (table 3) or the 112 or 121 haplotypes (table 4). The common allele at SNP-43 and the high-risk haplotypes were of similar frequencies in type 2–diabetic cases and in controls (table 5 and 6). The 112/121-haplotype combination was less common than in Mexican Americans (genotype frequency 4.2% in the trios probands, 5.0% in the 222 type 2–diabetic probands, and 6.1% in the population controls) and was not associated with increased risk in the diabetic probands in the trios. We estimate that our study had >90% power, at $\alpha = .05$, to detect an OR of 3.0 for the 112/121-haplotype combination of SNP-43, -19, and -63.

Association between SNP-44 and Type 2 Diabetes

Allele 2 at SNP-44 showed transmission distortion in the trios, with the C allele being transmitted to 54 offspring and not being transmitted to 34 offspring ($P < .03$) (table 3). In case-control study 1, the population controls showed an allele frequency similar to that of the nontransmitted parental alleles; therefore, there was a significant difference, in the allele frequency of SNP-44, between the trios probands and the population controls ($\chi^2 = 8.01$; $P = .0047$) (table 5). The inheritance of the C allele at SNP-44 was associated with increased risk of type 2 diabetes (OR 1.59 [95%CI 1.15–2.2]).

Haplotype analysis showed that allele 2 was in a haplotype (2111) with the common alleles at SNPs –43, -19, and -63 in >98% of chromosomes and that this haplotype showed both transmission distortion and association in a manner similar to that seen for allele 2 at SNP-44 (tables 4 and 6). The 111/111-haplotype combination of SNP-43, -19, and -63 was associated with increased risk, compared to all other haplotypes (OR 2.04 [95%CI 1.22–3.39]), and this is entirely attributable to the increased risk associated with the SNP-44, -43, -19, and -63 haplotype, 2111. The highest-risk haplotype combinations when the trios probands were compared to the controls were 2111/2111 (OR 2.52 [95%CI 1.06–5.97]) and 2111/1111 (OR 2.36 [1.19–4.66]).

Further Studies of SNP44: Case-Control 2 and Discordant-Sib Analysis

To further examine the potential role of SNP-44, we used two additional independent studies—another case-control study and a discordant-sib analysis of 49 families. In a second independent group of cases and controls (case-control study 2), we compared the frequencies of SNP-44 (tables 5 and 6). The 222 type 2–diabetic probands were taken from the type 2–diabetic sib pairs that were used in the genomewide screen, and the 212 controls were a group of nondiabetic adults (table 1). There were no significant differences, in allele frequencies at SNP-44, between these two groups: the frequency of allele 2 in the cases was .158, versus .144 the controls ($P = .67$). Discordant-sib analysis, using only a single affected member and a single unaffected sib for each family, was possible in 49 families. The C allele occurred at a frequency of .20 in the cases, versus .17 in the nondiabetic-sib controls (OR 1.3; $P = .53$).

Sequencing of Coding Region of CAPN10 *in U.K. Subjects with Type 2 Diabetes*

CAPN10 consists of 15 exons spanning 31 kb. A complex pattern of alternative splicing generates at least eight transcripts, with calpain-10a mRNA being the most abundant in the tissues that have been examined (Horikawa et al. 2000). We sequenced the calpain-10a–encoding region (exons 1–7 and 9–13; fig. 2) to identify coding variants that might be in linkage disequilibrium with SNP-44. We selected 10 subjects for these studies, including, for each of the five common haplotypes, a homozygous parent and his or her heterozygous child. This enabled us to identify sequence changes and to assign them to a specific haplotype. We found four coding polymorphisms: L34V, T504A, R555C, and V666I. (L34V and R555C were not ob-

Table 7

***CAPN10* Polymorphisms Found, in 10 U.K. Type 2–Diabetic Subjects, by Direct Sequencing**

Polymorphism	Location	Nucleotide Change[a]	Amino Acid Change	Haplotype(s)	Allele Frequency[b] (n = 32)
SNP-134	5′ UTR	−162G/A		2111	.16
SNP-135	5′ UTR	−70T/A		2111	.16
SNP-136	Exon 1, codon 34	100C/T	L34V (CTG→GTG)	2111	.03
SNP-79	Exon 4, codon 200	5157A/G	P200 (CCA→CCG)	2111	ND
SNP-110	Exon 10, codon 504	9803A/G	T504A (ACC→GCC)	2111	.16
SNP-137	Exon 10, codon 555	9956C/T	R555C (CGC→TGC)	1221	.02
SNP-48	Exon 11, codon 620	11098A/G	A620 (GCA→GCG)	1111, 1112, 1221	ND
SNP-58	Exon 13, codon 661	11751G/A	V666I (GTC→ATC)	1111	.08

[a] The reference sequence is the gene sequence (GenBank accession number AF158748); the A of the ATG of the initiator Met codon is considered to be nucleotide 1.
[b] ND = not done (silent polymorphism).

served in Mexican Americans with type 2 diabetes, whereas T504A and V666I had been found in this population) (fig. 2 and table 7). The frequency of the coding polymorphisms was estimated by sequencing an additional 32 subjects (table 7). The only coding polymorphism that was present in >8% of the population was T504A, which appeared to be in linkage disequilibrium with SNP-44. To assess this further, all members of the trios were tested for the T504A coding polymorphism. This confirmed that the Ala504 allele of the polymorphism T504A (SNP-110) was in perfect linkage disequilibrium with the C allele (allele 2) at SNP-44. The rare Val34 allele was found on the same haplotype but at much lower frequency (3%), which made our sample size inadequate for more-detailed study of this variant.

Discussion

We have studied the effect that *CAPN10* has on the risk of type 2 diabetes in white subjects of British/Irish ancestry in the United Kingdom. We have tested four polymorphisms in *CAPN10*—SNP-44, -43, -19, and -63 (fig. 2)—for linkage and association with type 2 diabetes, using family-based and case-control methods. We have selected these polymorphisms because of their prior association with either type 2 diabetes (SNP-43, -19, and -63, either individually or in combination), insulin resistance (SNP-43), or transcriptional regulation of calpain-10 expression (SNP-44 and -43). We did not confirm, in our U.K. subjects, the previously described haplotype associated with type 2 diabetes in Mexican Americans and in two northern-European populations (Horikawa et al. 2000). The C allele (allele 2) at SNP-44 was preferentially transmitted to patients in the trios, a group of young and obese diabetic subjects (age at diagnosis [AAD] 40.1 ± 7.1 years; body-mass index [BMI] 32.0 ± 6.8 kg/m^2 [table 1]). The C allele at SNP-44 was associated with a 1.5–2.5-fold- increased risk of diabetes in the trios probands, depending on the other haplotypes inherited with it. The association was observed in both family-based and case-control studies. However, there was no significant association between SNP-44 and type 2 diabetes either in a second group of subjects in a case-control study or in a small discordant-sib study.

Table 8

Replication of Association between *CAPN10* SNP-44 C-Allele and Type 2 Diabetes

	Case-Control Study 1	Case-Control Study 2	Discordant Sibs	Mexican Americans
No. of subjects:				
Probands	153	222	49	108
Controls	411	212	49	103
Frequency of C allele:				
Probands	.232	.158	.20	.102
Controls	.163	.144	.17	.058
OR (95%CI)	1.59 (1.15–2.2)	1.1 (.75–1.6)	1.3 (.57–2.9)	1.94 (.90–4.16)
P[a]	.005	.67	.53	.10

[a] All values are two tailed. In addition, combined P values were calculated using the method of Spielman and Ewens (1998; also see Altshuler et al. 2000), which enables pooling of data from familial and case-control association studies: for the three U.K. studies only, P = .015; for all four studies, P = .004. Mantel-Haenszel χ^2 analysis revealed similar results: for the three U.K. studies only, P = .029; for all four studies, P = .009.

Although not all individual U.K. studies showed significant association when analyzed alone, the trend in each study was toward the C allele being associated with type 2 diabetes (table 8). When the studies were combined, the association remained significant ($P = .015$) in U.K. cohorts (table 8). A similar result is obtained if all the results in the U.K. diabetic subjects ($n = 424$) and controls (including the nontransmitted parental alleles) ($n = 825$) are combined in a single 2×2 contingency table, with association between the C allele at SNP-44 and type 2 diabetes being characterized by an OR of 1.25 (95%CI 1.01–1.56) ($P = .041$). The association between SNP-44 and type 2 diabetes in some but not all data sets is similar to the observation by Altshuler et al. (2000), in their study of the Pro12Ala PPARγ2 polymorphism and type 2 diabetes. Studies consisting of several hundred individuals are unlikely to be consistently significant when (*a*) the risk allele is uncommon (i.e., frequency ~.15) in the population and (*b*) the relative risk is ~1.2–1.5. The finding that linkage at the *CAPN10* locus is excluded with power sufficient exclude a λ_S of 1.25 is consistent with *CAPN10* being a minor susceptibility gene in the United Kingdom. The C allele at SNP-44, although rare in Mexican Americans (frequency 5.8% in controls, vs. 10.2% in diabetic subjects), is also associated with type 2 diabetes in Mexican Americans ($P = .05$, one tailed) (table 8). When the Mexican SNP-44 study is combined with the U.K. studies, the diabetes remains associated with SNP-44 ($P = .004$, two tailed). The results in several studies are therefore consistent with a role for SNP-44 in the determination of susceptibility to type 2 diabetes, although additional studies will be needed to confirm this.

The strongest evidence for SNP-44 contributing to susceptibility to type 2 diabetes comes from the diabetic subjects from the parents-offspring trios, rather than from the group of affected sib pairs; compared with the probands in the affected sib pairs, the probands in the trios were younger when diagnosed with type 2 diabetes and were more obese (AAD 40.1 ± 7.1 years vs. 56.0 ± 8.0 years; BMI 32.0 ± 6.8 vs. 28.1 ± 5.38 kg/m^2 [table 1]). This may reflect that, in the probands in the trios, genetic factors make a greater contribution to risk than do nongenetic factors. In subjects with type 2 diabetes, the frequency of missense mutations in the insulin-promoter–factor 1 gene is higher in younger individuals than in older individuals (Hani et al. 1999; Macfarlane et al. 1999). Large, population-based studies will be necessary to determine the magnitude of the effect that SNP-44 and other variations in *CAPN10* have on diabetes risk in U.K. and other European populations.

SNP-44 may either directly alter susceptibility to type 2 diabetes or be in linkage disequilibrium with the disease-predisposing variant. Functional studies suggest that SNP-44 plays a role in the transcriptional regulation of *CAPN10* (Horikawa et al. 2000). We have also shown that SNP-44 is in perfect linkage disequilibrium with the amino acid polymorphism T504A. This polymorphism is located in domain T of calpain-10, a region of unknown function. Thr504 is also not a conserved amino acid; this residue is Ser in mouse calpain-10. In addition, the polymorphism L34V, which is located in domain I, occurs in ~20% of Ala504 alleles and is a possible contributing factor (in both human and mouse calpain-10, this residue is Leu). Functional studies are necessary to assess the role that T504A and L34V play in calpain-10 activity.

Variation in *CAPN10* has been associated with 2–3-fold-increased risk of type 2 diabetes, both in Mexican Americans and, now, in three northern-European populations: Finns (Botnia), Germans (Saxony), and British/Irish (United Kingdom). However, the polymorphisms and haplotypes associated with diabetes differ between populations. This may be due to the presence of multiple susceptibility alleles at *CAPN10* and/or to different patterns of linkage disequilibrium between these polymorphisms and a common causal variant(s). The amino acid polymorphisms identified in the U.K. subjects were either rare (in the case of T540A) or absent (in the case of R555C) in the Mexican American population studied by Horikawa et al. (2000); these amino acid polymorphisms may alter the risks associated with haplotypes and haplotype combinations originally defined in the Mexican American population.

The results presented here highlight an important issue that needs to be addressed in replication studies—that is, replication of specific polymorphisms/alleles at a locus, versus replication of the locus. The results of our studies of *CAPN10* in the U.K. population do not provide replication of the polymorphisms or haplotypes at *CAPN10* that are associated with the highest risk of type 2 diabetes in the Mexican American, Finnish (Botnia), or German populations (Horikawa et al. 2000); however, they do provide replication at the level of the locus, *CAPN10,* with different alleles of this susceptibility gene being associated with increased risk in the U.K. population.

In summary, we were not able to replicate the association between the specific calpain-10 alleles identified by Horikawa et al. (2000) and type 2 diabetes in whites of British/Irish ancestry. There is evidence that the rare allele at SNP-44, which is in complete linkage disequilibrium with the coding polymorphism T504A, plays a possible role in the susceptibility to type 2 diabetes. Additional studies, including large, population-based case-control studies, will provide a better understanding of the contribution that *CAPN10* makes to diabetes risk in this population.

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Electronic-Database Information

The accession number and URLs for data in this article are as follows:

GenBank Overview, http://www.ncbi.nlm.nih.gov/Genbank/GenbankOverview.html (for exons 1–7 and 9–13 [accession number AF158748])
GENEHUNTER, http://waldo.wi.mit.edu/ftp/distribution/software/genehunter/gh2
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for *CAPN10* [MIM 605286])
RECODE, ftp://watson.hgen.pitt.edu/pub/

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