Variants in the Calpain-10 Gene Predispose to Insulin Resistance and Elevated Free Fatty Acid Levels

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The calpain-10 gene (*CAPN10*) has been associated with type 2 diabetes, but information on molecular and physiological mechanisms explaining this association is limited. Here we addressed this question by studying the role of *CAPN10* for phenotypes associated with type 2 diabetes and free fatty acid (FFA) metabolism. Among 395 type 2 diabetic patients and 298 nondiabetic control subjects from Finland, the SNP-43 allele 1 ($P = 0.011$), SNP-63 allele 2 ($P = 0.010$), and the haplotype combination SNP-44/43/19/63 1121/1121 ($P = 0.028$) were associated with type 2 diabetes. The SNP-43 genotypes 11 and 12 were associated with higher fasting insulin and homeostasis model assessment (HOMA) insulin resistance index among control subjects ($P = 0.021$ and $P = 0.0076$) and with elevated FFA among both control subjects ($P = 0.0040$) and type 2 diabetic patients ($P = 0.0025$). Multiple regression analysis further indicated that SNP-43 is an independent predictor of FFA levels ($P = 0.0037$). Among 80 genotype discordant sibling pairs, the SNP-43 allele 1 was associated with elevated fasting serum insulin and HOMA index ($P = 0.013$ and $P = 0.0068$). None of the four SNPs showed distorted transmission of alleles to patients with type 2 diabetes in a qualitative transmission disequilibrium test, including 108 trios. Because FFA and insulin resistance are known to predict type 2 diabetes, the finding that variation in the *CAPN10* gene influences FFA levels and insulin resistance may provide an explanation for how the *CAPN10* gene increases susceptibility to type 2 diabetes. *Diabetes* 51:2658–2664, 2002

The first type 2 diabetes susceptibility gene revealed by a genome-wide scan and positional cloning was an ubiquitously expressed cystein protease calpain-10 (*CAPN10*), located on chromosome 2q37 (1,2). Intronic variation in *CAPN10* has been associated with type 2 diabetes in Mexican-Americans, Finns, Germans, and the British (2,3). Although *CAPN10* was not associated with type 2 diabetes in Pima Indians, the common allele 1 (G) of SNP-43 was associated with decreased glucose disposal in individuals with normal glucose tolerance (NGT) (3). In skeletal muscle, the genotype 11 (GG) was associated with reduced *CAPN10* mRNA (4). However, the questions of how this cystein protease increases susceptibility to type 2 diabetes, and particularly how intronic variations in *CAPN10* translate into deterioration of insulin sensitivity, have mostly remained unanswered.

The calpains are a family of calcium-activated, neutral, nonlysosomal proteases (5–7). Calpains have been implicated in many cellular functions, including intracellular signal transduction, neuronal functions, and cytoskeletal rearrangements. A number of calpain substrates are known from in vitro studies (8), and given their association with diabetes, protein kinase Cβ, phospholipase Cβ3, glycogen synthase (9), pyruvate kinase, and even insulin (10), are attractive candidate substrates for calpains. In addition, calpains have been proposed to have a role in terminal adipocyte differentiation through transcriptional activation of C/EBPα (11), as well as in downregulation of insulin receptor substrate 1 (12).

In Pima Indians, the SNP43 genotype 11 (GG) has been associated with decreased glucose disposal and increased lipid oxidation (4). Lipid oxidation occurs predominantly in the liver and skeletal muscle, where free fatty acids (FFAs) compete with glucose as energy substrate, so that an increased FFA concentration could inhibit glucose uptake (13,14). Although lipolysis is highly sensitive to the suppressive effect of insulin, patients with type 2 diabetes maintain slightly elevated rates of FFA turnover throughout the day (15). It is thus possible that insulin resistance of glucose disposal is a result of, or at least is promoted by, insulin resistance of lipolysis.

A *CAPN10* risk haplotype similar to that in Mexican-Americans was earlier found to affect susceptibility to type 2 diabetes in the population from the Botnia region in Western Finland (2). In the present study, we expanded our studies on *CAPN10* by extending the case-control material and applying family-based approaches (i.e., qualitative and quantitative transmission disequilibrium tests [TDT and QTDT, respectively]) and analysis of genotype discordant sibling pairs. In particular, we investigated the hypothesis that the association of *CAPN10* with insulin resistance is explained by effects on FFA metabolism.

RESEARCH DESIGN AND METHODS

Study subjects. Three different designs and study samples were used in the present studies (Table 1). First, we performed a case-control association study in a random sample of 395 unrelated type 2 diabetic patients and 298

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FFA, free fatty acid; HOMA, homeostasis model assessment; IGT, impaired glucose tolerance; NGT, normal glucose tolerance; OGTT, oral glucose tolerance test; OR, odds ratio; PKC, protein kinase C; QTDT, quantitative transmission disequilibrium test; SBE, single base pair extension; TDT, qualitative transmission disequilibrium test; WHR, waist-to-hip ratio.

TABLE 1
Clinical characteristics of study subjects

Study group	*n* (M/F)	Age (years)	Age at diagnosis (years)	BMI (kg/m^2)
Case-control				
Type 2 diabetes	395 (171/224)	61 ± 10	53 ± 11	29.2 ± 4.6
Control subjects	298 (140/158)	60 ± 9	—	26.7 ± 3.9
TDT				
Probands	108 (53/55)	42 ± 9	38 ± 8	29.7 ± 5.4
Parents	216 (108/108)	68 ± 8	—	28.2 ± 5.1
Siblings				
Type 2 diabetes	206 (86/120)	68 ± 11	60 ± 13	28.5 ± 5.1
NGT	384 (178/206)	42 ± 14	—	25.0 ± 3.8

Data are means ± SD.

normoglycemic spouses (Table 2) to obtain an estimate of allele and haplotype frequencies of *CAPN10* SNP-44, -43, -19, and -63 and to study their role for phenotypes associated with type 2 diabetes and FFA metabolism. Second, 108 parent-offspring trios, where an offspring had type 2 diabetes or severe impaired glucose tolerance (IGT) were studied to follow transmission of *CAPN10* alleles and haplotypes. Finally, 590 individuals comprising 295 sibling pairs were genotyped for SNP-43 to identify genotype discordant sibling pairs for comparison of type 2 diabetes–associated phenotypes. In all, 1,561 individuals were genotyped for SNP-43 and 991 individuals were genotyped for SNP-44, -19, and -63. Altogether, 46 individuals participated in two of the studies. The following phenotypes were compared among the different genotype/haplotype carriers in the case-control study, included in QTDT analysis, and compared among the genotype discordant sibling pairs: fasting serum insulin, fasting blood glucose, homeostasis model assessment (HOMA) insulin resistance index (16), waist-to-hip ratio (WHR), BMI, and fasting FFA.

Case-control association study. The 395 type 2 diabetic patients were randomly chosen, one patient per family. Control subjects were comprised of 298 spouses age 40 years or older, with NGT during an oral glucose tolerance test (OGTT) and without known family history of diabetes. The case-control study material was composed of two subgroups, the first of which originates from the Botnia region in Western Finland (referred to as Botnia I) and contains 192 type 2 diabetic patients (87 men/105 women, age 61 ± 9 years, fasting plasma glucose 10.1 ± 3.3 mmol/l, BMI 29 ± 5 kg/m^2) and 192 control subjects (88 men/104 women, age 61 ± 9 years, fasting plasma glucose 5.6 ± 0.6 mmol/l, BMI 27 ± 4 kg/m^2) (Table 2). Allele and haplotype frequencies of SNP-43, -19, and -63 in Botnia I have been reported earlier (2). The second sample (referred to as Botnia II) originated from other regions of Finland and included 203 type 2 diabetic patients (84 men/119 women, age 61 ± 12 years, fasting plasma glucose 10.1 ± 3.4 mmol/l, BMI 30 ± 5 kg/m^2) and 106 control subjects (52 men/54 women, age 60 ± 10 years, fasting plasma glucose 5.3 ± 0.5 mmol/l, BMI 27 ± 4 kg/m^2) (Table 2).

TABLE 2
Clinical characteristics of study subjects in the case-control association study

Variable	Type 2 diabetic patients	Control subjects
n	395	298
Sex (M/F)	171/224	140/158
Age (years)	61 ± 10	60 ± 9
BMI (kg/m^2)	29.2 ± 4.6	26.7 ± 3.9
WHR (males)	0.99 ± 0.06	0.95 ± 0.06
WHR (females)	0.89 ± 0.07	0.83 ± 0.06
Fasting plasma glucose (mmol/l)	10.0 ± 3.4	5.5 ± 0.6
Fasting serum insulin (mU/l)	18.2 ± 22.9	8.3 ± 4.8
HOMA	8.9 ± 14.3	2.1 ± 1.3
Fasting FFA (μmol/l)	841 ± 300	664 ± 241
Insulin treatment (%)	23.3	—

Data are means ± SD. The study material is composed of two subgroups, Botnia I and Botnia II. Data on FFA levels were available for a subset of individuals (212 control subjects [102 men, 110 women] and 274 type 2 diabetic patients [113 men, 161 women]). (See RESEARCH DESIGN AND METHODS for description of groups.)

TDT analyses. For TDT and QTDT, one offspring with type 2 diabetes or severe IGT per family was randomly selected from a cohort of 196 parent-offspring trios with one or more offspring (17). Severe IGT was defined as plasma glucose >9.6 mmol/l at 2 h of an OGTT and fasting plasma glucose <7.0 mmol/l. This identified 108 trios with one diabetic offspring (Table 1). The trios originated from the Botnia region in Western Finland (n = 46), from other parts of Finland (n = 32), and from Southern Sweden (n = 30). Data on FFA levels were available for a subset of individuals (64 mothers, 61 fathers, and 70 affected individuals).

Genotype discordant sibling pair study. Siblings were identified from a sample of 295 sibling pairs matched for age, sex, and glucose tolerance (Table 1). Siblings originated from 272 sibships from 198 extended families; both one male and one female pair were included from 23 sibships, whereas only one pair was included from the other sibships. In all, 80 sex-matched sibling pairs discordant for the SNP-43 genotypes were identified, including 160 unique individuals (45 male and 35 female pairs; 21 type 2 diabetes and 59 NGT pairs). Data on FFA levels were available for a subset of individuals (39 pairs, of which 28 were nondiabetic and 11 were diabetic).

Phenotypic characterization of the subjects. All laboratory specimens were taken after a 12-h overnight fast. Glucose tolerance was assessed by a 75-g OGTT. Diagnosis of diabetes was based upon World Health Organization criteria from 1998. Waist was measured with a soft tape midway between the lowest rib and the iliac crest, and the hip circumference was measured at the widest part of the gluteal region. Fasting concentrations of serum insulin and plasma glucose were measured as earlier described (18). Fasting serum FFA levels were analyzed using an enzymatic colorimetric method (NEFAC ACS-ACOD Method, Wako Chemicals, Richmond, VA). The HOMA index (fasting serum insulin multiplied by fasting plasma glucose divided by 22.5) was used to estimate the degree of insulin resistance (16).

Genotyping

SNP-43 and -44 [CAPN10-g.4852G (allele 1)/A (allele 2) and CAPN10-g.4841T (allele 1)/C (allele 2)]. Genotyping was performed using the SNaPShot kit (Applied Biosystems, Stockholm, Sweden) for single base pair extension (SBE) analysis on an ABI377 (Perkin Elmer, La Jolla, CA). A 479-bp fragment containing both SNPs was amplified with primers 5′-GCTGGCTG GTGACATCAGTGC and 5′-TCAGGTTCCATCTTTCTGCCAG. PCR was carried out in 20-μl volume containing 1 × PCR buffer (Amersham Pharmacia Biotech, Uppsala, Sweden), 10 μmol/l each dNTP, 3 mmol/l $MgCl_2$, 0.5 units *Taq* polymerase (Amersham Pharmacia Biotech), 10 pmol of each primer, and 25 ng genomic DNA. The cycling conditions were 94°C for 5 min, 30 cycles of 94°C for 30 s, 64°C for 30 s, and 72°C for 60 s, followed by final extension at 72°C for 10 min. SBE reactions were performed according to the manufacturer's instructions with detection primers 5′-GGCTTAGCCTCACCTTCAAA and 5′-GACTGCAGGGCGCTCACGCTTGCTG. Then 0.8 μl of SBE products were loaded onto 10% polyacrylamide gels and run for 2 h at 51°C, 200 W. Genotypes were analyzed using the Genescan 3.1 and Genotyper 2.0 (Applied Biosystems) and by manual detection from the gels.

SNP-19 (CAPN10-g.7920indel132bp). This insertion-deletion polymorphism was amplified using primers 5′-GTTTGGTTCTCTTCAGCGTGGAG and 5′-CATGAACCCTGGCAGGGTCTAAG. PCR was carried out in 20-μl volume containing 1 × PCR buffer, 10 μmol/l each dNTP, 1.5 mmol/l $MgCl_2$, 0.5 units *Taq* polymerase, 10 pmol of each primer, and 25 ng genomic DNA. The cycling conditions were 94°C for 5 min, 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 10 min. PCR products

TABLE 3
Allele and haplotype frequencies of *CAPN10* SNPs in Finland

		Botnia I			Botnia II			Pooled		
A. Marker	Allele	Type 2 diabetes	Control subjects	*P*	Type 2 diabetes	Control subjects	*P*	Type 2 diabetes	Control subjects	*P*
SNP-44	1 (T)	293 (78.3)	288 (75.0)	0.28	327 (80.5)	173 (81.6)	0.75	620 (79.5)	461 (77.3)	0.34
	2 (C)	81 (21.7)	96 (25.0)		79 (19.5)	39 (18.4)		160 (20.5)	135 (22.7)	
SNP-43	1 (G)	294 (76.6)	259 (67.4)	0.0049	301 (74.5)	153 (72.9)	0.66	595 (75.5)	412 (69.4)	0.011
	2 (A)	90 (23.4)	125 (32.6)		103 (25.5)	57 (27.1)		193 (24.5)	182 (30.6)	
SNP-19	1 (ins)	217 (56.5)	236 (61.5)	0.16	229 (56.4)	112 (52.8)	0.40	446 (56.5)	348 (58.4)	0.47
	2 (del)	167 (43.5)	148 (38.5)		177 (43.6)	100 (47.2)		344 (43.5)	248 (41.6)	
SNP-63	1 (C)	356 (92.7)	369 (96.6)	0.017	346 (85.2)	183 (86.3)	0.71	702 (88.9)	552 (92.9)	0.010
	2 (T)	28 (7.3)	13 (3.4)		60 (14.8)	29 (13.7)		88 (11.1)	42 (7.1)	

Data are *n* (%). All SNPs were genotyped in 395 patients with type 2 diabetes and 298 control subjects. In all, 0.3% of the genotypes could not be provided despite repeated genotyping. Allele frequencies of SNP-43 and -44 did not significantly differ between Botnia I and II samples, whereas SNP-19 allele 2 was more common among control subjects from Botnia II compared with control subjects from Botnia I (47.2 vs. 38.5%; $P = 0.041$). The SNP-63 allele 2 was substantially more common among both type 2 diabetic patients and control subjects in Botnia II than in Botnia I (14.8 vs 7.3, [$P = 0.00080$] and 13.7 vs. 3.4% [$P = 0.000013$] for type 2 diabetes patients and control subjects in Botnia II and I, respectively). All genotype frequencies were in Hardy-Weinberg equilibrium, and those of SNP-43 and -63 differed significantly between type 2 diabetic patients and healthy control subjects (SNP-43: 57.6, 35.8, and 6.6 vs. 48.2, 42.4, and 9.4%; $P = 0.039$; SNP-63: 79.8, 18.2, and 2.0 vs. 87.2, 11.5, and 1.4%, $P = 0.036$ for genotypes 11, 12, and 22, respectively).

were separated onto 3% agarose gels. Allele 1 was detected as a 155-bp fragment and allele 2 as a 187-bp fragment.

SNP-63 [CAPN10-g.16378C (allele 1)/T (allele 2)]. The SNP-63 was amplified with primers 5′-AAGGGGGGCCAGGGCCTGACGGGGGTGGCG and 5′-AGCACTCCCAGCTCCTGATC. PCR conditions were the same as for SNP-19, except that annealing was performed at 62°C. PCR products were digested with two units of *Hha*I (New England Biolabs) according to the manufacturer's instructions for 2 h at 37°C. The digested products were separated onto 4.5% agarose gels. Allele 1 (C) was detected as a 162-bp fragment and allele 2 (T) as a 192-bp fragment.

Two independent readers read all genotypes and at least 5% of all genotypes were randomly repeated to exclude genotyping errors.

Statistical analyses. Allele and haplotype frequency comparisons among groups were performed by the χ^2 test. The significance of differences among clinical characteristics was assessed by Student's *t* test or by the Mann-Whitney test if the variable was not normally distributed. Because our previous association studies have indicated that the *CAPN10* SNP-43 allele 1 is associated with type 2 diabetes, we hypothesized that the phenotypic differences among the sibling pairs discordant for this SNP would differ significantly from zero. The genotype discordant sibling pairs were independent (only one sex-specific pair per family), and phenotypic differences were compared by a paired *t* test or matched signed rank test. The differences in clinical characteristics were computed as the value in the sibling with the greater number of allele 1 of SNP-43 minus the value in the sibling with less or no allele 1 (i.e., sibling pairs with genotypes 11–12, 11–22, or 12–22). Multiple regression analysis for FFA as a dependent variable was performed using age, sex, and diabetes status as independent covariates. For all the analyses above, BMDP Statistical Software, Version 1.12 (BMDP Statistical Software, Los Angeles, CA) or the NCSS Statistical Package (NCSS, Kaysville, UT) was used.

TDT was performed using Genehunter 2.1 implementing the TDT4 function for analysis of haplotype transmissions (http://linkage.rockefeller.edu/soft/gh/) (19). For TDT4, Genehunter first performs a traditional TDT (20) followed by a four-locus TDT, which follows identical rules for counting transmissions and nontransmissions as the single-marker TDT and produces four-locus TDT for markers in map order, assuming no recombination between adjacent markers. QTDT (21) was performed using a linear model with age, sex, and BMI as covariates.

Haplotypes were constructed based on haplotype frequencies of TDT trios. In agreement with earlier published data on SNP-44, -43, -19, and 63 (2,3), our TDT trios indicated a strong linkage disequilibrium among the SNPs, with the five most common haplotypes accounting for 99.2% of all haplotypes. The possibility of the rare haplotypes (1211, 1212, 2121, 2112, and 2221) accounting for <0.2% each was ignored while constructing the haplotype combinations in the case-control association study.

Data are shown as means ± SD. $P < 0.05$ was considered statistically significant. *P* values are reported uncorrected or corrected (P_c) for multiple comparisons. For correction, *P* values were multiplied by the number of phenotypes ($n = 5$), which may be considered overly conservative as all the phenotypes are interrelated. Haplotype analysis of SNP-44/43 was corrected for the number of haplotypes ($n = 6$). *P* values were not corrected for the number of analyzed SNPs or for the number of SNP-44/43/19/63 haplotype combinations, as the SNP-43/19/63 haplotypes and SNP-44 have been shown to be associated with type 2 diabetes (2,3).

RESULTS

Allele and haplotype frequencies of *CAPN10* SNPs in Finland. Of the genotyped SNPs, SNP-43 (allele 1; 75.5 vs. 69.4%; $P = 0.011$) and SNP-63 (allele 2; 11.3 vs. 7.1%; $P = 0.010$) were associated with type 2 diabetes in the case-control association study (Table 3). A separate analysis of Botnia I and II samples revealed that these associations were confined to the Botnia I sample from Western Finland (Table 3). In addition, the SNP-44/43/19/63 haplotype combination 1121/1121 was associated with increased risk for type 2 diabetes (10.5 vs. 5.7%; odds ratio [OR] 1.93, $P = 0.028$) (Table 4). In contrast to individual SNPs, support for association between type 2 diabetes and haplotype 1121/1121 was obtained from both Botnia I and Botnia II samples (Botnia I 11.2 vs. 6.3%, $P = 0.085$; Botnia II 9.9 vs. 4.8%, $P = 0.12$), although the difference did not reach statistical significance in any of the subgroups alone. Further, one of the haplotype combinations of SNP-44 and -43 11/11 was found to be associated with type 2 diabetes in the Botnia I sample (Botnia I 32.1 vs. 17.6%, $P = 0.0011$; Botnia II 31.3 vs. 27.9%, $P = 0.54$; pooled groups 31.6 vs. 20.9%; OR 1.75 [1.23–2.48], $P = 0.0019$, $P_c = 0.011$).

The earlier proposed type 2 diabetes risk haplotype combination SNP-43/19/63 (121/112) (2) was not associated with significantly increased risk for type 2 diabetes (6.7 vs. 3.7%; OR 1.92 [0.93–3.93], $P = 0.076$). Instead, the haplotype combination 121/121 was more common among type 2 diabetic patients than among control subjects (10.5 vs. 6.1%; OR 1.97 [1.09–3.53], $P = 0.023$).

Clinical characteristics of type 2 diabetic patients and control subjects in the case-control association study according to *CAPN10* genotypes. Among the control subjects, no significant differences were found among the different SNP-43 genotype carriers regarding age, BMI, WHR, or fasting plasma glucose. However, the SNP-43 genotypes 11 and 12 were associated with higher

TABLE 4
Constructed *CAPN10* haplotype combinations and the risk of type 2 diabetes

Constructed haplotype combination SNP-44, -43, -19, -63	Frequency (%)		OR (95% CI)	*P*
	Type 2 diabetes	Control subjects		
1111/1111	1.5	1.0	1.53 (0.38–6.15)	0.55
1111/1112	2.0	1.7	1.22 (0.39–3.76)	0.73
1111/1121	9.0	7.4	1.23 (0.70–2.14)	0.47
1111/2111	5.1	5.7	0.89 (0.46–1.72)	0.72
1111/1221	5.4	7.8	0.67 (0.37–1.24)	0.21
1112/1112	1.5	1.4	1.14 (0.32–4.08)	0.84
1112/2111	3.6	2.4	1.54 (0.61–3.86)	0.36
1121/1121	10.5	5.7	1.93 (1.07–3.47)	0.028*†
1112/1121	6.7	3.7	1.85 (0.90–3.81)	0.095†
1112/1221	5.7	3.7	1.55 (0.74–3.25)	0.25
1121/2111	11.1	14.2	0.75 (0.47–1.18)	0.21
2111/2111	6.2	4.7	1.32 (0.67–2.60)	0.42
1221/1221	6.4	8.8	0.71 (0.40–1.26)	0.24
1121/1221	15.4	18.2	0.81 (0.54–1.22)	0.32
2111/1221	8.7	12.2	0.67 (0.42–1.13)	0.14
Other	1.0	1.4	NA	NA
Total	389	296	—	—

*Frequencies of the 1121/1121 haplotype combination in type 2 diabetes patients vs. control subjects in Botnia I sample were 11.2 vs. 6.3% ($P = 0.085$) and in Botnia II were 9.9 vs. 4.8% ($P = 0.12$). †Frequency of the 1121/1121 or 1121/1112 haplotype combinations in all type 2 diabetes patients vs. all control subjects was 17.2 vs. 9.5% ($P = 0.0036$); for Botnia I, 16.0 vs. 8.4% ($P = 0.023$); and for Botnia II, 18.3 vs. 11.4% ($P = 0.12$). All SNPs were genotyped in 395 patients with type 2 diabetes and in 298 control subjects. As 0.3% of the genotypes could not be provided despite repeated genotyping, haplotypes are missing for 6 patients and 2 control subjects. Haplotypes were constructed using information on TDT trios with data on parental alleles. A strong linkage disequilibrium between the four SNPs was found, with the five most common haplotypes accounting for 99.2% of all haplotypes in our TDT material. The possibility of the rare haplotypes (1211, 1212, 2121, 2112, 2221) accounting for <0.2% each was ignored while constructing the presented haplotype combinations. NA, not analysed.

fasting serum insulin (8.5 ± 4.9 vs. 6.8 ± 3.4 mU/l; $P = 0.021$, $P_c = 0.11$), higher HOMA insulin resistance index (2.1 ± 1.3 vs. 1.6 ± 0.8; $P = 0.0076$, $P_c = 0.038$), and higher fasting FFA concentrations (689 ± 233 vs. 522 ± 186 μmol/l; $P = 0.0040$, $P_c = 0.033$) compared to genotype 22. In contrast, no significant differences were found among the SNP-43 genotype 11 and 12 carriers concerning fasting insulin (8.4 ± 5.1 vs. 8.6 ± 4.7 mU/l), HOMA (2.1 ± 1.4 vs. 2.2 ± 1.3), or fasting FFA (675 ± 261 vs. 681 ± 214 μmol/l).

Similar to the findings in control subjects, diabetic carriers of the SNP-43 genotypes 11 or 12 had higher fasting FFA compared to genotype 22 carriers (850 ± 305 vs. 704 ± 159 μmol/l; $P = 0.0025$, $P_c = 0.013$). Analysis of the whole study group (i.e., after pooling patients and control subjects) revealed a remarkable difference in FFA levels between genotype 11 and 12 compared to genotype 22 carriers (776 ± 292 vs. 613 ± 198 μmol/l; $P = 0.000022$, $P_c = 0.00011$), particularly among male subjects (714 ± 286 vs. 517 ± 174 μmol/l; $P = 0.000074$, $P_c = 0.00038$) (Fig. 1).

Using sex ($P = 0.000020$), age ($P = 0.0062$), and diabetes status ($P < 10^{-6}$) as covariates, a multiple regression analysis further suggested SNP-43 as an independent predictor of FFA levels ($P = 0.0037$). SNP-43 remained as an independent predictor of FFA levels when fasting insulin was added to the model ($P = 0.00032$ and $P = 0.017$ for fasting insulin and FFA, respectively).

Transmission disequilibrium tests (TDT, TDT4, and QTDT). None of the alleles or haplotypes of the four SNPs

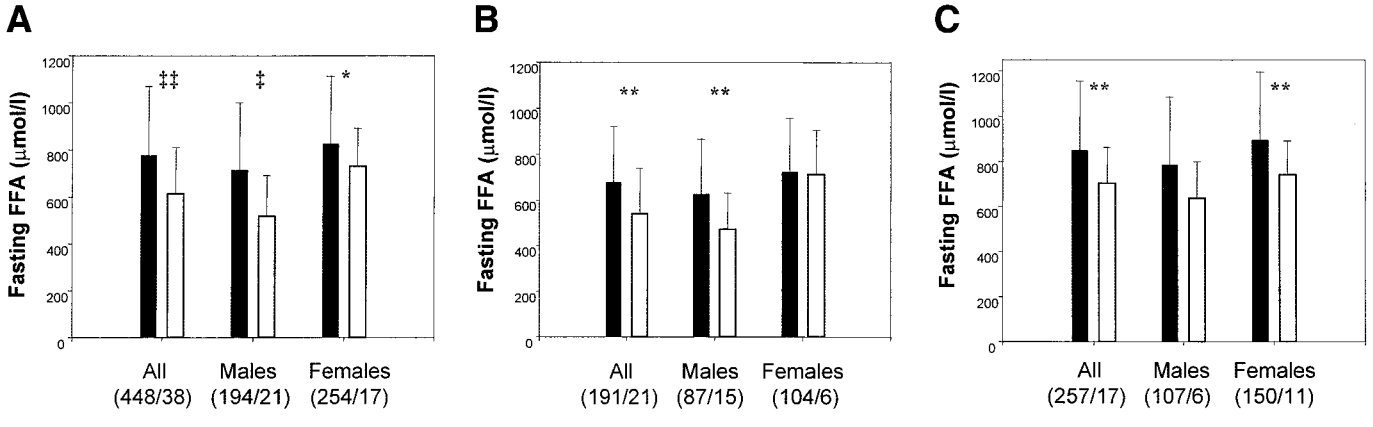

FIG. 1. Fasting FFA levels in all individuals (*A*), control subjects (*B*), and type 2 diabetic patients (*C*) participating in the case-control association study. Number of subjects in each genotype group are given in parentheses. FFA levels are given as means ± SD. Black bars indicate FFA levels in SNP-43 genotype 11 and 12 carriers and white bars indicate FFA levels in genotype 22 carriers. *$P < 0.05$; **$P < 0.01$; ‡$P < 0.0001$; ‡‡$P < 0.00005$.

TABLE 5
Transmission of *CAPN10* alleles and haplotypes (SNP-44/43/19/63) from heterozygous parents to offspring with type 2 diabetes or severe IGT

SNP/haplotype (-44/43/19/63)	Transmitted	Untransmitted	*P*
SNP-44 (allele 2)	32	22	0.17
SNP-43 (allele 2)	43	46	0.75
SNP-19 (allele 2)	48	49	0.32
SNP-63 (allele 2)	10	19	0.09
1111	15	14	0.85
1112	4	10	0.11
1121	25	24	0.89
1211	0	1	0.32
1221	26	26	—
1212	0	1	0.32
2111	21	14	0.24
2121	0	1	0.32
2112	1	0	0.32
2221	0	1	0.32

showed significant transmission disequilibrium from the expected mendelian 50:50 ratio in TDT (Table 5). In QTDT, the SNP-44 indicated weak association with fasting serum insulin (43 of 100 transmissions informative; $P = 0.049$, $P_c = 0.25$). Fasting plasma glucose and WHR were not associated with transmission of *CAPN10* alleles. The SNP-43 indicated some association with FFA levels (41 of 64 transmissions informative; $P = 0.14$).

Differences between siblings discordant for the SNP43. Altogether, 94.2% of the 590 siblings were found to be carriers of one or two SNP43 allele 1. Frequencies of SNP-43 allele 1 did not significantly differ among siblings with type 2 diabetes and NGT relatives (77.4 vs. 73.0%; $P = 0.12$) or among type 2 diabetic patients from the case-control and sibling study (76.4 vs. 77.1%; $P = 0.86$).

In all, 80 sibling pairs were discordant for the SNP-43 genotypes (Table 6). Within each sibling pair, the siblings with more allele 1 (i.e., 11 or 12) had significantly elevated fasting serum insulin and HOMA insulin resistance indexes compared with their siblings with more allele 2 (i.e., 12 or 22) (11.6 ± 13.4 vs. 8.1 ± 6.5 mU/l; $P = 0.013$, $P_c = 0.065$, and 3.6 ± 5.7 vs. 2.2 ± 2.3; $P = 0.0068$, $P_c = 0.034$, respectively) (Table 6). This was particularly seen among type 2 diabetic sibling pairs (25.1 ± 21.6 vs. 13.5 ± 10.4 mU/l, $P = 0.040$, and 9.9 ± 8.9 vs. 4.6 ± 3.6, $P = 0.012$). We also genotyped SNP-44, -19, and -63 among the siblings discordant for SNP-43 to identify sibling pairs discordant for the earlier defined risk-haplotype combinations (SNP-44/43/19/63 1121/1121 or 1112/1121). Among all the sibling pairs, 13 pairs were discordant for the risk-haplotype combinations. Compared to siblings without the risk haplotypes, siblings with risk haplotypes had an elevated fasting serum insulin (22.0 ± 27.1 vs. 7.6 ± 3.8 mU/l; $P = 0.028$) and HOMA index (8.0 ± 10.9 vs. 2.5 ± 1.8; $P = 0.051$). This difference did not reach statistical significance among the small number ($n = 6$) of type 2 diabetic pairs (insulin 43.2 ± 32.4 vs. 8.5 ± 2.6 mU/l, $P = 0.063$; HOMA 15.9 ± 3.8 vs. 3.8 ± 1.5, $P = 0.063$).

DISCUSSION

In the present study, we investigated the role of *CAPN10* in type 2 diabetes in a Finnish population using a case-control and two different family-based approaches. *CAPN10* gene was recently identified by positional cloning, and variants in the gene have been associated with a two- to threefold increased risk of type 2 diabetes in several different populations (2,3,22,23). However, the polymorphisms and haplotypes associated with type 2 diabetes, as well as their frequencies, differ remarkably among studies and populations (2,3,22,23). Here, we attempted to define the risk haplotypes in two groups of type 2 diabetic patients of different ages and with a different age at onset of type 2 diabetes (i.e., patients in the case-control study and the TDT probands). In addition, by using a genotype discordant sibling pair design, we examined whether features associated with type 2 diabetes are also associated with *CAPN10*.

The strongest risk haplotype in the Finnish population was SNP-44/43/19/63 1121/1121. The SNP-43 allele 1 was individually associated with type 2 diabetes and SNP-43 genotypes 11 and 12 were associated with higher fasting serum insulin, higher FFA concentrations, and higher HOMA insulin resistance index in normoglycemic control subjects and with higher fasting FFA levels in type 2 diabetic patients. The association with elevated fasting insulin and HOMA index was replicated in the genotype discordant sibling pair analysis; multiple regression analysis further suggested SNP-43 as an independent predictor of fasting FFA levels.

In the original study describing an association between

TABLE 6
Paired comparison of sibling pairs discordant for the *CALP10* SNP43

	All ($n = 80$)		Type 2 diabetes ($n = 21$)		NGT ($n = 59$)	
Variable	Sib 1	Sib 2	Sib 1	Sib 2	Sib 1	Sib 2
Sex (M/F)	45/35		13/8		32/27	
Age (years)	47 ± 16	47 ± 16	64 ± 10	64 ± 10	40 ± 13	40 ± 12
Age at onset (years)	54 ± 7	52 ± 16	54 ± 7	52 ± 16	—	—
BMI (kg/m^2)	26.5 ± 4.0	26.2 ± 5.3	30.0 ± 5.1	29.2 ± 7.3	25.3 ± 2.6	25.2 ± 3.9
WHR	0.90 ± 0.08	0.90 ± 0.09	0.94 ± 0.09	0.93 ± 0.10	0.89 ± 0.08	0.88 ± 0.08
Fasting plasma glucose (mmol/l)	6.7 ± 2.5	6.5 ± 2.5	9.9 ± 3.0	9.2 ± 3.7	5.5 ± 0.5	5.5 ± 0.3
Fasting serum insulin (mU/l)	11.6 ± 13.4	8.1 ± 6.5*	25.1 ± 21.6	13.5 ± 10.4†	7.1 ± 3.1	6.3 ± 3.08
HOMA	3.6 ± 5.7	2.2 ± 2.3‡	9.9 ± 8.9	4.6 ± 3.6§	1.5 ± 0.7	1.4 ± 0.7

Data are means ± SD. Sib 1 refers to SNP-43 genotype 11 and 12 carriers and Sib 2 refers to genotype 12 and 22 carriers. *$P = 0.013$ (for non–insulin-treated: 9.7 ± 9.7 vs. 7.8 ± 5.9 mU/l; $P = 0.038$); †$P = 0.040$ (for non–insulin-treated: 20.3 ± 17.7 vs. 13.2 ± 10.5; $P = 0.18$); ‡$P = 0.0068$ (for non–insulin-treated [$n = 67$]: 2.7 ± 3.7 vs. 2.0 ± 2.1; $P = 0.050$); §$P = 0.012$ (for non–insulin-treated: 7.4 ± 6.6 vs. 4.5 ± 3.7; $P = 0.13$).

CAPN10 and type 2 diabetes in Mexican-Americans, Finns, and Germans, the greatest risk for type 2 diabetes was associated with the SNP-43/19/63 haplotype combination 121/112 (2). In the current study, we included SNP-44, which has been associated with type 2 diabetes in the British population (21). Although SNP-44 was not by itself associated with type 2 diabetes in our study population, it formed a putative risk haplotype combination together with SNP-43 (11/11) in the Botnia I sample from Western Finland. In contrast, in the Botnia II sample, none of the four SNPs were individually associated with type 2 diabetes. The Botnia I and Botnia II samples differed in some important aspects, which may explain at least some of these differences. Most importantly, although study subjects of both samples originate from the relatively homogenous and isolated Finnish population, Botnia I is assumed to represent a sample more homogenous and isolated than the Botnia II, as all individuals in this sample originate from the linguistically isolated, Swedish-speaking Western coast of Finland. Subjects in the Botnia II sample originate from other, geographically much larger and mainly Finnish-speaking parts of Finland. Second, the number of control subjects in the Botnia II sample was smaller than that in the Botnia I sample, which reduced the power to detect association solely in this subgroup.

Although none of the *CAPN10* variants or haplotypes were associated with type 2 diabetes in the TDT cohort, the frequency of the SNP-44/43 and the SNP-44/43/19/63 haplotype combinations (11/11 and 1121/1121) was similar among TDT probands compared to type 2 diabetic patients in the case-control study (29.6 vs. 31.6 and 10.5 vs. 11.1%, respectively). These results are particularly interesting, as the SNP-44 allele 2, as well as the 2111 haplotype of SNP-44/43/19/63, were associated with type 2 diabetes in the British TDT study (3). Among individuals in our case-control study, the SNP-44 allele 2 was found in 20.5% of type 2 diabetic patients and in 22.7% of control individuals, and the haplotype 2111 was found in 20.4% of type 2 diabetic patients and 22.1% of control subjects. However, pooling our TDT data with the British data reveals a significant association of the SNP-44 allele 2 to type 2 diabetes (transmitted 86, not transmitted 56; $P = 0.012$). In addition, pooled data suggests the 2111 haplotype as a risk haplotype for type 2 diabetes (transmitted 66, not transmitted 42; $P = 0.021$) and the 1112 haplotype as a protective haplotype (transmitted 17, not transmitted 34; $P = 0.017$). Clearly there is a discrepancy between our case-control and the TDT study results. We think that this could be explained by the fact that TDT follows the transmission of alleles (or haplotypes), but does not take into account the created genotype (or haplotype) combination. Another explanation could be the difference between the type 2 diabetic cohorts studied. First, the mean age of the TDT offspring was almost 20 years lower than that of the type 2 diabetic patients in the case-control study (42 ± 9 vs. 61 ± 10 years), and their diagnosis of diabetes had been made 15 years earlier (38 ± 8 vs. 53 ± 11 years). Similar to the affected offspring in our trios, the type 2 diabetic patients in the British sample had their type 2 diabetes diagnosed at an early age (40 ± 7 years). Also, in contrast to our case-control study subjects, who all originated from Finland, the TDT trios originated from both Finland and Sweden, and the risk haplotypes between the two populations may differ. Finally, Horikawa et al. (2) proposed a model in which certain risk haplotypes could affect expression of *CAPN10* in insulin-responsive tissues and others could affect expression in insulin-secreting cells; this hypothesis could explain differences in the effect of different risk haplotypes.

SNP-43 and -44 are located in intron three of *CAPN10*, and in vitro experiments have suggested these variants as regulators of *CAPN10* expression (2). *CAPN10* is alternatively spliced to a family of different proteins, but it is unknown how the different intronic polymorphisms in *CAPN10* affect alternative splicing or the transcription rate in different tissues. In normoglycemic Pima Indians, SNP-43 genotype 11 has been associated with reduced *CAPN10* mRNA expression in skeletal muscle (3). In addition, *CAPN10* expression levels correlated positively with carbohydrate oxidation, indicating that individuals with lower *CAPN10* expression oxidized less carbohydrates and more fat. We found that fasting FFA levels were significantly higher in carriers of the SNP-43 genotypes 11 or 12 compared to genotype 22 carriers. This difference was evident across both sexes in patients with type 2 diabetes as well as in normoglycemic individuals.

Why do carriers of the SNP-43 allele 1 have higher fasting FFA levels? One possibility could be that they are less sensitive to the antilipolytic effect of insulin, thereby leading to elevated FFA levels and a worsening of insulin resistance to glucose. Some support for this can be found from in vitro studies that have shown that calpains have several substrates, among them the protein kinase C (PKC)-β (7). PKC has been shown to be involved in skeletal muscle insulin resistance and obesity, and it has been proposed that elevated serine/threonine phosphorylation by PKC leads to decreased tyrosine kinase phosphorylation of the insulin receptor (24). As FFAs activate PKC, the unfavorable effect of FFAs on insulin sensitivity could be mediated by elevated PKC activity added to the direct effect of elevated FFA concentration (25). Whether the FFA-activated PKC isoforms are substrates of calpain-10 is not known, but if so, decreased *CAPN10* expression could translate into deteriorated insulin sensitivity by both elevated FFA and PKC, and be further strengthened by activation of PKC by FFA.

To summarize, we have reported an association of *CAPN10* with increased risk of type 2 diabetes, with features of insulin resistance and elevated FFA levels. Several studies have shown that FFA concentrations predict subsequent diabetes (26). Therefore, the findings that a genetic variation in the *CAPN10* gene is associated with elevated FFA levels and insulin resistance may be potentially important and explain why variants in the *CAPN10* gene increase susceptibility to type 2 diabetes.

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