Brief Genetics Report

Variation in Three Single Nucleotide Polymorphisms in the Calpain-10 Gene Not Associated With Type 2 Diabetes in a Large Finnish Cohort

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Variations in the calpain-10 gene have recently been reported to be associated with type 2 diabetes in a Mexican-American population. We typed three single nucleotide polymorphisms (SNPs) in the calpain-10 gene (SNPs 43, 56, and 63) to test for association between variation at these loci and type 2 diabetes and diabetes-related traits in 1,603 Finnish subjects: two samples of 526 (Finland-U.S. Investigation of NIDDM Genetics [FUSION] 1) and 255 (FUSION 2) index case subjects with type 2 diabetes, 185 and 414 unaffected spouses and offspring of FUSION 1 index case subjects or their affected siblings, and 223 elderly normal glucose-tolerant control subjects. We found no significant differences in allele, genotype, haplotype, or haplogenotype frequencies between index case subjects with diabetes and the elderly and spouse control populations (all $P > 0.087$). Although variation in these three SNPs was associated with variation in some type 2 diabetes-related traits within each of the case and control groups, no consistent pattern of the implicated variant or combination of variants was discerned. We conclude that variation in these three SNPs in the calpain-10 gene is unlikely to confer susceptibility to type 2 diabetes in this Finnish cohort. *Diabetes* 51:1644–1648, 2002

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AIR, acute insulin response; FUSION, Finland-U.S. Investigation of NIDDM Genetics; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; NPL, nonparametric linkage; OR, odds ratio; S_I, insulin sensitivity; SNP, single nucleotide polymorphism.

In 1996, Hanis et al. (1) reported that a genome-wide screen in Mexican-American sibling pairs with type 2 diabetes localized a susceptibility gene to chromosome 2qter. After further refining the region harboring the locus (2), they found an intronic single nucleotide polymorphism (SNP) in the calpain-10 gene, SNP 43, that showed evidence for linkage and association with type 2 diabetes (3). Further, they identified a pair of three-polymorphism (SNPs 43, 19, and 63) haplotypes that, in combination, were associated with a 2.8-fold increased risk of type 2 diabetes (3). In the same report, similar, though not significant, trends were found in samples from Germany and the Botnia area of Finland. They attributed the lack of statistically significant results in the German and Finnish samples to the small number of individuals with the high-risk haplotype combination (haplogenotype) in those samples. When the two European samples were combined, the haplogenotype of interest was significantly associated with a 3.16-fold increase in the risk of type 2 diabetes. Given the importance of the calpain-10 finding in the Mexican-Americans, we examined the role of SNPs 43, 56, and 63 in conferring susceptibility to type 2 diabetes in a large Finnish cohort. Specifically, we tested for association between these polymorphisms and type 2 diabetes affection status and diabetes-related quantitative traits in the Finland-U.S. Investigation of NIDDM Genetics (FUSION) study samples (4).

We genotyped SNPs 43, 56, and 63 in 1,603 Finnish subjects, including two samples of 526 FUSION 1 (F1) and 255 FUSION 2 (F2) index case subjects with type 2 diabetes, 185 nondiabetic spouses and 414 nondiabetic offspring of F1 index case subjects or their affected siblings, and 223 normal glucose-tolerant elderly (70 years of age) control subjects. SNP 56 was more easily typed than SNP 19 using our genotyping platform (see RESEARCH DESIGN AND METHODS) and was reportedly in complete linkage disequilibrium with SNP 19 (verified; see RESEARCH DESIGN AND METHODS). Table 1 shows selected phenotypic traits for each FUSION study group. A similar table for all phenotypic traits discussed here can be found on-line on our website (http://www.sph.umich.edu/csg/CAPN10).

Table 2 shows the frequency of the common allele for each SNP for our sample and those reported by Horikawa

TABLE 1
Phenotype characteristics

Trait	F1 index cases	F2 index cases	Unaffected spouses	Elderly control subjects	Unaffected offspring
Sex (male:female)	289:237	149:106	60:125	108:115	214:200
Age at enrollment (years)	63.6 ± 7.5	64.5 ± 8.1	61.7 ± 7.7	70.0 ± 0.3	34.9 ± 7.3
Age at diagnosis (years)	50.0 ± 7.9	51.8 ± 9.2	—	—	—
Duration of diabetes (years)	13.6 ± 7.0	12.7 ± 7.7	—	—	—
BMI (kg/m^2)	30.0 ± 4.8	30.1 ± 4.5	28.5 ± 4.5	27.0 ± 4.0	26.1 ± 4.5
Waist-to-hip ratio	0.94 ± 0.08	0.95 ± 0.07	0.89 ± 0.08	0.88 ± 0.08	0.86 ± 0.09
Fasting glucose (mmol/l)	10.6 ± 3.4	9.6 ± 2.9	5.2 ± 0.6	5.0 ± 0.5	5.0 ± 0.6
Fasting insulin (pmol/l)	116.2 ± 101.8	110.6 ± 81.6	77.5 ± 50.3	66.3 ± 35.1	67.6 ± 33.5

Data are means ± SD.

et al. (3) for the Mexican-American and Botnian-Finnish samples. In our sample, we compared the allele frequencies for each group of index case subjects to each of the spouse and elderly control samples separately and as combined case and control samples. There were no significant differences in allele frequencies between either of the two samples of index case subjects and spouse and elderly control subjects for any of the three SNPs (all $P > 0.087$). The lack of association persisted when the two index case samples (F1 and F2) were combined (all $P > 0.487$). In addition, when we compared genotype frequencies in the same manner as above, we found no significant differences (all $P > 0.208$) between case subjects and control subjects.

Similarly, there were no significant differences in haplotype or haplogenotype frequencies between case subjects and spouse and elderly control subjects (all $P > 0.126$). We estimated haplotype frequencies via the EM algorithm for case subjects and spouse and elderly control subjects using the three SNPs. Haplotypes could be unambiguously assigned for 70% of the individuals in the sample. Because only four of the eight possible haplotypes were estimated to have a frequency >0.1%, and obligate carriers of the rare haplotypes could account for the estimated frequencies of such, we were able to assign haplotypes with near certainty for the remaining individuals. Table 3 shows the odds ratio (OR) and 95% CI associated with each common (>1%) haplogenotype for our sample and those reported by Horikawa et al. (3). In contrast to the Botnian-Finnish sample, we did not observe any striking trends for higher risk in case subjects versus control subjects (significant or otherwise), even when the two index case groups (F1 and F2) were combined (data not shown).

Within each study group and for SNPs 43 and 56, we compared diabetes-related traits between subjects with and without each allele and among subjects with each of the three genotypes after adjusting for age and sex and, for nonanthropometric measures, BMI. Because of the small number of homozygotes for the rare T-allele at SNP 43, only those with and without the rare allele were compared. Finally, we compared the same traits between subjects with and without each haplotype or haplogenotype within each study group. A total of 20 traits were analyzed in diabetic subjects, 16 traits in elderly control subjects, and 23 traits in unaffected spouses and offspring (see REASEARCH DESIGN AND METHODS). Tables of the means, medians, SDs, and P values for each of the comparisons with $P \leq 0.01$ and $P \leq 0.05$, respectively, are available on our website (http://www.sph.umich.edu/csg/CAPN10).

Although there were many phenotypic comparisons that reached the nominal significance levels of $P \leq 0.05$ and $P \leq 0.01$, the number of dependent comparisons made within each group for each trait was large. Across all comparisons, these findings were consistent with random chance. Specifically, 4% of the comparisons were significant at the 5% level and 1% at the 1% level. As such, many of these differences are not likely to be physiologically meaningful. Nonetheless, we note a few items of potential interest. First, weight-related traits were consistently nominally significant across many of the different comparisons. Specifically, for three of the four haplotypes and four of the nine haplogenotypes, the comparisons for one or more of these traits had P values ≤0.05 in at least two FUSION study groups. Although a definite phenotypic pattern associated with a specific variant or combination of variants was not clear and many of the P-values were quite modest, it is possible that variation at this locus influences adiposity in some way that we currently cannot discern.

Second, among elderly control subjects, variation at

TABLE 2
Comparison of SNP allele frequency estimates for SNPs 43, 19 (56), and 63

		Mexican-American sample*		FUSION sample			Botnia, Finland sample*	
SNP	Allele†	All patients (n = 110)	Random sample (n = 112)	F1 index cases (n = 526)	F2 index cases (n = 255)	Control‡ subjects (n = 408)	Diabetic subjects (n = 192)	Control subjects (n = 191)
43	1	0.80	0.75	0.74	0.74	0.75	0.77	0.67§
19 (56)	2	0.58	0.57	0.55	0.59	0.58	0.56	0.62
63	1	0.77	0.77	0.88	0.91	0.88	0.93	0.97§

*Horikawa et al. (3); †allele designations: 43, 1 = G, 2 = A; 19, 1 = 2 unit-repeats, 2 = 3 unit-repeats; 56, 1 = A, 2 = G; 63, 1 = C, 2 = T; ‡elderly and spouse control subjects combined; §difference between two groups significant at $P < 0.05$.

TABLE 3
Comparison of risks of type 2 diabetes associated with haplotype combinations

Haplotype combination	Mexican-American sample*		FUSION Sample		Botnia, Finland sample*
	Patient set 1†	Patient set 2†	F1 index cases‡	F2 index cases‡	Patients§
111/111	0.85 (0.26–2.76)	0.89 (0.20–3.84)	1.15 (0.73–1.80)	1.26 (0.75–2.12)	1.61 (0.88–2.97)
111/121	1.60 (0.76–3.39)	1.41 (0.56–3.53)	1.08 (0.77–1.51)	0.94 (0.62–1.41)	0.96 (0.59–1.54)
111/112	1.17 (0.50–2.74)	0.47 (0.12–1.81)	1.12 (0.64–1.95)	0.96 (0.48–1.90)	2.02 (0.60–6.83)
111/221	1.02 (0.43–2.43)	0.99 (0.34–2.92)	1.03 (0.71–1.51)	1.02 (0.65–1.60)	0.60 (0.36–1.00)
112/112	0.29 (0.08–0.99)	0.92 (0.29–2.95)	0.64 (0.17–2.38)	0.63 (0.12–3.27)	1.99 (0.18–22.13)
112/121	2.80 (1.23–6.34)	3.58 (1.43–8.92)	0.86 (0.52–1.42)	0.72 (0.38–1.37)	2.55 (0.79–8.29)
112/221	0.36 (0.15–0.86)	0.26 (0.07–1.04)	1.36 (0.81–2.30)	0.64 (0.30–1.37)	2.01 (0.50–8.16)
121/121	0.67 (0.31–1.43)	1.07 (0.45–2.59)	0.70 (0.44–1.11)	1.09 (0.65–1.82)	1.92 (0.92–4.00)
121/221	1.27 (0.65–2.51)	0.99 (0.42–2.35)	0.91 (0.63–1.32)	1.18 (0.77–1.80)	0.69 (0.41–1.17)
221/221	0.59 (0.22–1.63)	0.35 (0.07–1.71)	1.01 (0.60–1.73)	1.10 (0.60–2.06)	0.68 (0.31–1.46)

Data are OR (95% CI). *Horikawa et al. (3); †patients compared with random sample; ‡index case subjects compared with elderly and spouse control subjects; §patients compared with control subjects.

SNP 56 was associated with fasting and 2-h serum insulin levels. The mean fasting insulin level for the AG genotypic group was higher than that for each of the AA and GG groups (76.1 ± 44.4, 56.9 ± 27.9, and 58.0 ± 19.8 for AG, AA, and GG groups, respectively; genotypic $P = 0.011$). Those with either the GG or AG genotype had higher 2-h insulin levels (402.1 ± 235.7) than those with the AA genotype (311.0 ± 173.4; G-allele $P = 0.037$). After adjustment for BMI, the genotypic differences in fasting insulin levels remained significant ($P = 0.012$). In addition, the presence of at least one copy of haplotype 121 (which includes the G-allele at SNP 56) was associated with higher 2-h insulin levels (430.1 ± 244.9 vs. 330.5 ± 197.0; $P = 0.001$; BMI-adjusted $P = 0.007$). There were no consistent patterns of association (both in terms of direction of the association and combination of variants) between these two insulin traits and other haplotypes or haplogenotypes, but we mention the above because of the obvious importance of insulin levels in the pathogenesis of type 2 diabetes.

The fact that we were not able to replicate the association results of Horikawa et al. (3) for Mexican-Americans in our Finnish sample is not particularly surprising given the previously reported lack of linkage evidence for this region in our sample (5) and the likely genetic heterogeneity between Mexican-American and Finnish subjects. Indeed, based on their samples, Horikawa et al. estimated that the population-attributable risk associated with variation in calpain-10 is only 4% in Europeans compared with 14% in Mexican-American populations (1). Perhaps the more intriguing question is why our Finnish sample is so dissimilar to the Botnian-Finnish sample in terms of the risk of type 2 diabetes associated with variation in the three polymorphisms. Whereas in the Botnian sample the G-allele at SNP 43 and the T-allele at SNP 63 were significantly associated with diabetes status and a trend in increased risk was demonstrated for those with the implicated haplotype combination (112/121) first identified in the Mexican-Americans, we found no evidence for this in our sample. One plausible explanation is that Swedish admixture in the Botnian-Finnish sample is sufficient such that the disease-predisposing variants differ between the two populations (6). However, the allele frequencies for all three polymorphisms in the Botnian *patient* sample are very similar to those in each of our case and control (elderly and spouse) samples. Consequently, differences in allele frequencies between the Botnian and FUSION *control* samples are responsible for the observed differences in risk associated with these polymorphisms in each population. Hence, Swedish admixture that led to the presence of different disease susceptibility loci in the two populations does not appear to be a satisfactory explanation for differences in risk between the two samples.

Clearly, inter-population heterogeneity is a possible explanation for our findings. It is also possible that there exists heterogeneity within our population with respect to risk associated with variation in the three SNPs. We carried out two types of analyses that investigate the most obvious subgroups of our index case samples for whom variation at these loci may be associated with type 2 diabetes status. First, despite the overall evidence against linkage to this region in our F1 sample (5), there are individual families that demonstrate evidence for linkage (defined here as a nonparametric linkage [NPL] score >0). We identified index case subjects from families with NPL scores >0 at 258.5 cM on the FUSION map, approximately corresponding to the linkage peak in the Mexican-American sample (3). We compared allele, genotype, haplotype, and haplogenotype frequencies between these case subjects (F1 $n = 178$, F2 $n = 95$, and F1 + F2 $n = 273$) and control subjects (spouses and elderly control subjects combined). Across these 57 comparisons, all P values were >0.062.

Second, for each of the index case groups (F1, F2, and F1 + F2), we also carried out logistic regression analyses to test for interactions between variation at SNP 43 and age, sex, and BMI in models that included each of these factors as main effects. There were no statistically significant ($P < 0.05$) interactions between any of these three factors and either genotypic or allelic variation at SNP 43. Similarly, we tested for interactions between the indicator of having the putative at-risk haplogenotype 112/121 and age, sex, and BMI. The only interaction that was statistically significant ($P < 0.05$) was that with sex in the F2 index case analysis ($P = 0.012$). However, the parameter estimates indicate that having haplogenotype 112/121 is

protective for men and is not associated with disease for women (OR and 95% CI for men and women, respectively: 0.16 [0.03–0.73] and 1.61 [0.63–4.11]).

We conclude that the lack of association between both SNP 43 and haplogenotype 112/121 and type 2 diabetes remains after considering interaction effects with age, sex, and BMI. We limited our analyses to those described because, in the absence of obvious a priori hypotheses, the number of competing analyses to be computed is very large. As such, the results would likely be very difficult, if not impossible, to interpret. In particular, we chose not to test for interactions in each of the continuous trait analyses because this would triple the very large number of comparisons already done for those traits.

Recently, a study in the U.K. also found no evidence for linkage between type 2 diabetes and the *NIDDM-1* region on chromosome 2, nor any evidence for any association between SNP 43, -19, or -63 and type 2 diabetes using family-based and case-control studies (7). However, the rare C-allele at UCSNP-44 was associated with type 2 diabetes in one of two case-control samples ($P = 0.005$) and was found to be in perfect linkage disequilibrium with a coding polymorphism, T504A, suggesting that other polymorphisms in the calpain-10 gene may be relevant in different study populations. Therefore, we cannot exclude the possibility that one or more other polymorphisms in the calpain-10 gene may be associated with type 2 diabetes in our sample.

Further study by these investigators in the U.K. found that the GG genotype at SNP 43 was associated with higher 2-h plasma glucose levels ($P = 0.05$) and that the 112/121 haplogenotype was associated with measures of insulin secretion and action in nondiabetic British subjects (8). Although we did not find any association between either variation at SNP 43 or the 112/121 haplogenotype and these traits, we did observe at least nominal associations with insulin levels at SNP 56 and with haplotype 121. More studies are needed to fully understand how variation at these loci may impact insulin secretion and action in different populations.

We conclude that variation in SNP 43, -56, and -63 in the calpain-10 gene does not appear to either confer susceptibility to type 2 diabetes or strongly influence diabetes-related traits in this Finnish cohort.

RESEARCH DESIGN AND METHODS

Subjects and measures. The FUSION study is a multi-center collaborative effort to map and positionally clone genes that predispose to type 2 diabetes and that influence intermediate quantitative traits in Finnish subjects (4). The design of the FUSION study and details regarding the determination of diabetes status have been described elsewhere (4). The following 14 traits were analyzed for all subjects: BMI, waist-to-hip ratio, waist circumference, weight at enrollment, maximum lifetime weight, fasting plasma glucose, fasting serum insulin, total cholesterol, HDL cholesterol, HDL ratio [HDL cholesterol/total cholesterol], LDL cholesterol, triglycerides, and systolic and diastolic blood pressure. In addition, weight at age 20 years, change in weight after age 20 years, and maximum lifetime weight change after age 20 years were analyzed for all but the elderly control group. Age at diagnosis of diabetes, duration of diabetes, and fasting C-peptide concentrations were also analyzed for the index case subjects. Two-hour fasting glucose and insulin measures were analyzed for the unaffected spouses, offspring, and elderly control subjects only. Four measures derived from a minimal model analysis (9,10) were analyzed for those spouses and offspring who underwent a tolbutamide-modified frequently sampled intravenous glucose tolerance test: glucose effectiveness (S_G), insulin sensitivity (S_I), acute insulin response (AIR) to glucose, and the disposition index (DI) (DI = S_I x AIR). The FUSION studies were approved by the Institutional Review Boards at the National Institutes of Health in Bethesda, MD (assurance number SPA S-5737-05), and the Ethical Committee of the National Public Health Institute in Helsinki, Finland.

Genotyping. We genotyped SNP 19 on 93 subjects representing all three genotypes at SNP 56 to verify that the two SNPs were in complete linkage disequilibrium and to determine phase. SNP 19 is a 32-base tandem repeat of either two or three units. PCR amplification of the region surrounding the repeats was performed using the forward oligonucleotide primer 5′-AGGCC CAGTTTGGTTCTCTT and the reverse primer 5′- AGCTACGGCCACAGACA GAG under standard thermocycling conditions. The reaction products were separated by electrophoresis in a 2% agarose gel and stained with ethidium bromide. The presence of a band 135 bp in size corresponded to the 2-unit repeat, and a band of 167 bp corresponded to the 3-unit repeat. In all 93 subjects, there was complete correlation between SNPs 19 and 56, indicating complete linkage disequilibrium.

The SNPs 43, 56, and 63 were amplified from genomic DNA by PCR followed by a primer extension reaction, and the resulting products were analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry as previously described (11). PCR amplification of DNA fragments containing the variant site were performed using the following primer sets: SNP 43, 5′-CTGTGTGTGGGCAGAGGAC, 5′-AGCGGATAA CAATTTCACACAGGCCTCATCCTCACCAAGTCAAG; SNP-56, 5′-CAAGGGT GGTGTCCTCAGTT, 5′-AGCGGATAACAATTTCACACAGGCCTCGCACTAGT GGAAAGGA; and SNP-63, 5′-AGCGGATAACAATTTCACACAGGCCCTGGTC ACTGGATGTTGC, 5′-AGCGGATAACCCTGAAGGTTCCACTCTCCA. A universal sequence biotinylated primer, 5′-biotin-AGCGGATAACAATTTCACACAGG, corresponding to the 23 nucleotides at the 5′ end of the longer primers in each reaction, was included to enable purification of single strand template for the primer extension assay. The genomic DNA was amplified in a thermocycling protocol of 95°C for 15 min followed by 55 cycles of 95°C for 5 s, 53°C for 20 s, and 72°C for 30 s. The primer extension reaction was performed using the SNP-specific primers abutting the polymorphic nucleotide. The extension products were applied to a SpectroChip (Sequenom, San Diego, CA) prespotted with a matrix of 3-hydroxypicolinic acid. The genotype of each sample was determined by analysis of the mass of the primer extension products using a modified Bruker Biflex III MALDI-TOF mass spectrometer (DNA MassArray; Sequenom). The sequence of the extension reaction oligoprimers and the expected masses of the products identifying each allele are available on our website (http://www.sph.umich.edu/csg/CAPN10).

Statistical analyses. Differences in allele, genotype, haplotype, and haplogenotype frequencies between index case subjects and control subjects were assessed by χ^2 tests of independence. The genotypic comparisons for SNP 63 were computed via Fisher's exact test because of the small number of homozygotes for the T-allele. The spouse and elderly control groups were combined for all haplotype and haplogenotype comparisons. Trait differences associated with variation at both the single-SNP (allele and genotype) and three-SNP (haplotype and haplogenotype) level within index case, spouse control, and elderly control subgroups were assessed using ANOVA. Because many of the offspring were related, we used a generalized estimating equation approach for this group (12). Each analysis was adjusted for age and sex and, for nonanthropometric measures, BMI. Traits were transformed to approximate normality when necessary. No adjustments for multiple comparisons were made. Excluded from all analyses were those with a first-degree relative with type 1 diabetes and those with uncertain affection status. In addition, those who on the day of examination took medication that might be expected to strongly affect the trait of interest were excluded from the analyses for that trait.

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