Brief Genetics Report

Association of the Diabetes Gene Calpain-10 With Subclinical Atherosclerosis

The Mexican-American Coronary Artery Disease Study

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The powerful relation between atherosclerosis and diabetes may have a common genetic basis. However, few genes predisposing to both have been identified. Calpain-10 (*CAPN10*) was the first gene for type 2 diabetes identified by positional cloning, wherein a combination of haplotypes conferred increased risk of diabetes. We sought to determine whether *CAPN10* influences subclinical atherosclerosis. Among nondiabetic subjects from 85 Mexican-American families with a history of coronary artery disease, subclinical atherosclerosis was assessed by common carotid artery intima-media thickness (IMT), insulin sensitivity was assessed by hyperinsulinemic-euglycemic clamp, and insulin secretion was estimated by the oral glucose tolerance test. These phenotypes were tested for association with *CAPN10* haplotypes. Haplotype 1112 (of single nucleotide polymorphisms [SNPs] 44, 43, 56, and 63) was associated with increased IMT, while haplotype 1221 was associated with decreased IMT. The 112/121 haplotype combination (of SNPs 43, 56, and 63), originally found to confer increased risk for diabetes, was associated with the largest IMT in our study population. *CAPN10* was also associated with both insulin sensitivity and insulin secretion. Covariate analysis suggested that *CAPN10* affects IMT independently of these diabetes-related phenotypes. The fact that the diabetes gene *CAPN10* also influences the risk for atherosclerosis shows that inherited factors may underlie the frequent co-occurrence of these two conditions. *Diabetes* 54:1228–1232, 2005

Both coronary artery disease (CAD) and type 2 diabetes have a strong genetic basis (1). They often occur together, and epidemiologic evidence suggests that this co-occurrence has a genetic basis (2). However, few genes have been identified that affect both diabetes and atherosclerosis. We demonstrate that the diabetes gene calpain-10 (*CAPN10*, chromosome 2q37), which encodes a nonlysosomal cysteine protease of unknown function (3), plays a role in atherosclerosis in a population enriched for both atherosclerosis and insulin resistance. We also show in the same population that *CAPN10* is associated with the fundamental processes underlying diabetes itself: insulin sensitivity and insulin secretion.

RESULTS

The clinical characteristics of the phenotyped offspring generation are shown in Table 1. Weight and insulin-to-glucose ratio at 30 min (IGR30) were significantly different between the men and women but not when standardized to BMI and disposition index (DI)-1, respectively. Carotid intima-media thickness (IMT) was significantly greater in the men.

Association of *CAPN10* with carotid IMT. The overall mean carotid IMT was 0.66 ± 0.12 mm. Haplotype 1221 was significantly associated with decreased carotid IMT, with and without covariate adjustment (Fig. 1*A*). Haplotype 1112 showed a significant association with increased carotid IMT and marginal significance when age, sex, and BMI were analyzed as covariates.

Given previous reports of the association of the 43-19-63 haplotype combination 112/121 with diabetes (3,4) and diabetes-related traits (5), we specifically analyzed the equivalent 44-43-56-63 haplotype combination 1112/1121 for phenotype associations. Haplotype combination 1112/1121 was significantly associated with increased IMT (P = 0.012). A total of 34 subjects with 1112/1121 had a mean IMT of 0.72 ± 0.16 mm, whereas 275 subjects with any

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CAD, coronary artery disease; DI, disposition index; IGR30, insulin-to-glucose ratio at 30 min; IMT, intima-media thickness; INSR30, insulin at 30 min–to–insulin at baseline ratio; OGTT, oral glucose tolerance test; SI, sensitivity index; SNP, single nucleotide polymorphism.

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TABLE 1
Clinical characteristics of phenotyped individuals

	Men (n = 128)	Women (n = 199)
Age (years)	35.6 ± 9.5 (19–62)	35.7 ± 8.5 (18–60)
Weight (kg)*	83.9 ± 15.9 (52.5–126.6)	72.1 ± 14.3 (38.6–128.5)
BMI (kg/m^2)	28.9 ± 4.8 (17.8–45.4)	29.1 ± 5.5 (18.1–54.8)
Carotid IMT (mm)†	0.68 ± 0.13 (0.4–1.19)	0.65 ± 0.11 (0.37–1.18)
SI	2.0 ± 1.2 (0.3–5.7)	1.8 ± 1.0 (0.1–6.3)
IGR30‡	0.64 ± 0.45 (0.05–2.46)	0.71 ± 0.43 (0.07–2.55)
INSR30	6.6 ± 4.2 (0.6–24.3)	7.1 ± 4.2 (0.7–29.4)
DI1	1.1 ± 0.9 (0.04–6.6)	1.1 ± 0.8 (0.02–4.8)
DI2	12.6 ± 10.7 (0.5–53.7)	12.4 ± 10.0 (0.2–68.1)
SBP (mmHg)*	123.5 ± 13.1 (101.3–166.7)	113.2 ± 14.5 (84.3–153.0)
DBP (mmHg)*	73.1 ± 9.4 (49.0–99.7)	66.6 ± 10.2 (45.0–97.7)
Total cholesterol (mg/dl)	186.2 ± 38.7 (102.0–297.0)	179.7 ± 34.8 (107.0–309.0)
LDL cholesterol (mg/dl)	110.5 ± 37.0 (23.0–227.0)	106.6 ± 31.5 (50.0–250.0)
HDL cholesterol (mg/dl)*	42.6 ± 12.5 (23.0–86.0)	51.2 ± 12.4 (25.0–94.0)
Triglycerides (mg/dl)*	181.0 ± 115.5 (23.0–974.0)	112.6 ± 65.6 (31.0–425.0)

Data are mean ± SD (range). *$P < 0.001$, †$P = 0.014$, and ‡$P = 0.031$ for men vs. women. IMT, n = 309; insulin SI, n = 294.

other haplotype combination had a mean IMT of 0.65 ± 0.11 mm (Fig. 1*A*).

The robust associations of haplotype 1221 and the haplogenotype 1112/1121 with carotid IMT remained significant even when blood pressure or lipid parameters were included as covariates (data not shown).

Association of *CAPN10* with insulin sensitivity and insulin secretion. Haplotype 1112 demonstrated association with sensitivity index (SI) (Table 2). Mean levels indicated that 1112 was associated with significantly reduced insulin sensitivity (Fig. 1*B*). This association with insulin sensitivity remained significant even when IGR30 or insulin at 30 min–to–insulin at baseline ratio (INSR30) were included as covariates (data not shown).

Haplotype 1112 was associated with IGR30, DI1, and DI2 ($P = 0.029$, $P = 0.0046$, and $P = 0.0066$, respectively), and haplotype 1221 was associated with INSR30 ($P = 0.029$) (Table 2). Figure 1*C* illustrates that haplotype 1112 was associated with lower insulin secretion and that 1221 was associated with higher insulin secretion. To further assess *CAPN10* association with insulin secretion indexes correcting for insulin sensitivity, besides using DI, we also evaluated the association of *CAPN10* with IGR30 and INSR30 by including SI as a covariate. In these models, the *CAPN10* associations with IGR30 and INSR30 remained significant (Table 2).

***CAPN10* association with carotid IMT is independent of diabetes-related phenotypes.** Because diabetes itself and its preclinical abnormalities may predispose to atherosclerosis, we sought to determine whether the association of variation in *CAPN10* with carotid IMT was independent of diabetes-related phenotypes. Table 3 shows that the association of haplotype 1221 with carotid IMT was still significant when SI, IGR30, and INSR30, alone or in combination, were analyzed as covariates. The association of the haplotype combination 1112/1121 with carotid IMT was consistently significant under all covariate models (Table 3).

DISCUSSION

In a nondiabetic population at risk for CAD, we demonstrated an association of variation in the *CAPN10* gene with carotid IMT, a measure of subclinical atherosclerosis, and also with insulin sensitivity and insulin secretion, the critical factors contributing to diabetes. Adjusting each haplotype-phenotype association for the other phenotypes showed that *CAPN10* was independently associated with each trait. Haplotype 1112 was associated with increased common carotid IMT, insulin resistance, and impaired insulin secretion, whereas haplotype 1221 was associated with favorable levels of these phenotypes. Of note, the haplotype combination 112/121, found by others to increase risk of diabetes and diabetes-related traits (3–5), was also associated with the greatest degree of subclinical atherosclerosis in our population.

Our results provide an additional genetic link between atherosclerosis and diabetes. To date, there has been little evidence for such common genetic determinants identified in the same study population (2). Single nucleotide polymorphisms (SNPs) in the genes for IGF-I, methylenetetrahydrofolate reductase, adiponectin, glucokinase, and the platelet-surface integrin GPIIB-IIIa have been found to be associated with type 2 diabetes and CAD (6–9). In our Mexican-American population, we previously demonstrated an association of *LPL* haplotypes with CAD and insulin sensitivity (10,11). The present study is unique in that it identifies in healthy adults a gene underlying both subclinical atherosclerosis and pre-diabetic phenotypes.

To our knowledge this is the first report associating *CAPN10* with atherosclerosis. Demonstration of an effect of *CAPN10* haplotypes on development of overt atherosclerosis in a longitudinal study would provide convincing support to our cross-sectional results. Our results may not apply to other ethnic groups, especially those with a low frequency of haplotype 1112 or 1221. Replication in other populations will be necessary to substantiate *CAPN10* as a common genetic determinant of diabetes and atherosclerosis. Our data suggest that *CAPN10* affects these conditions independently. *CAPN10* haplotypes, in particular 1221 but also 1112, were associated with carotid IMT. These associations persisted even after adjustment for insulin sensitivity and insulin secretion, showing that at least some portion of the *CAPN10* effect on carotid IMT is independent of diabetes-related phenotypes. The observed

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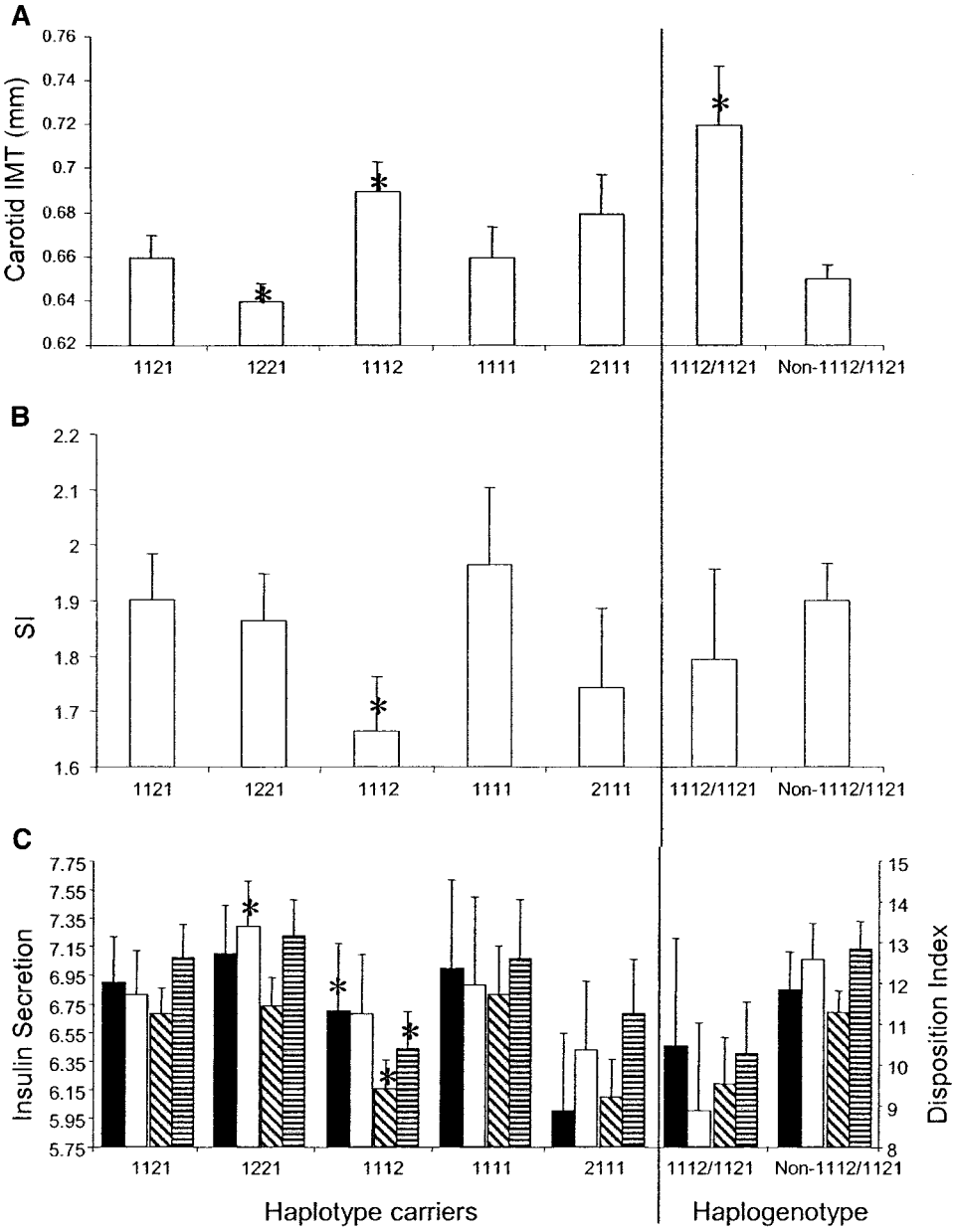

FIG. 1. ***A*: Mean levels of carotid IMT by *CAPN10* haplotype. Mean carotid IMT levels by haplotype carrier status are shown on the left. On the right, the mean carotid IMT of those with haplogenotype 1112/1121 is shown compared with the mean carotid IMT of subjects with all other haplogenotypes. *Significant genotype-phenotype associations. Error bars indicate SE. *B*: Mean levels of insulin sensitivity by *CAPN10* haplotype. The bars indicate SI (mg/m^2 · ml · min^{-1} · μIU^{-1}). *Significant genotype-phenotype associations. Error bars indicate SE. *C*: Mean levels of insulin secretion and DIs by *CAPN10* haplotype. ■, IGR30 (μIU · dl · mg^{-1} · ml^{-1}); □, INSR30; ▨, DI1 (dl · m^{-2} · min^{-1}); ▤, DI2 (mg · m^{-2} · ml · min^{-1} · μIU^{-1}). *Significant genotype-phenotype associations. The left *Y*-axis is labeled for IGR30 × 10 and INSR30; the right *Y*-axis is labeled for DI1 × 10 and DI2. Error bars indicate SE.**

pleiotropic effect of *CAPN10* on atherosclerosis and diabetes-related phenotypes is an important observation because diabetes itself would be expected to increase atherosclerosis. Therefore, *CAPN10* may affect atherosclerosis both directly and indirectly via diabetes.

Physiological studies using calpain inhibitors suggest that calpains may affect atherosclerosis. Experimental hyperglycemia in rats increased adhesion molecule expression, increased leukocyte adherence to vascular endothelium, and decreased nitric oxide (NO) production; calpain inhibition prevented all these effects and stabilized endothelial NO synthase (12). Calpains also play a role in vascular smooth muscle cell proliferation and migration (13), as well as platelet aggregation, degranulation, and spreading (14).

Experimental work also supports a role for calpains in insulin sensitivity and secretion. Specific inhibition of *CAPN10* with antisense expression reduced insulin-stimulated glucose uptake and glucose transporter GLUT4 translocation in adipocytes (15); calpain inhibitors also reduced insulin-mediated glucose transport and glycogen synthesis in mouse muscle strips (16). In mouse pancreatic islet cells, effects of calpain inhibition on insulin secretion depended on duration of exposure, with acute inhibition increasing insulin exocytosis and chronic inhibition decreasing insulin secretion (16,17).

In summary, we demonstrate that variation in *CAPN10* is independently associated with subclinical atherosclerosis and physiological determinants of type 2 diabetes. This report of a common genetic determinant of both atherosclerosis and diabetes has the support of compelling functional studies.

RESEARCH DESIGN AND METHODS

The University of California at Los Angeles (UCLA)/Cedars-Sinai Mexican-American Coronary Artery Disease (MACAD) Study enrolled families ascertained through a proband (parent) with documented CAD (11). Two generations were enrolled: *1*) the proband and proband spouses (parental

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TABLE 2
CAPN10 haplotype association results for insulin sensitivity and insulin secretion

Haplotype carriers	SI	IGR30	INSR30	IGR30 (cov: SI)	INSR30 (cov: SI)	DI1	DI2
1121	0.34	0.33	0.83	0.36	0.99	0.10	0.33
1221*	0.69	0.19	**0.029**	0.24	**0.026**	0.31	0.096
1112†	**0.028**	**0.029**	0.062	**0.012**	**0.034**	**0.0046**	**0.0066**
1111	0.53	0.61	0.75	0.73	0.53	0.82	0.30
2111	0.34	0.18	0.35	0.18	0.44	0.12	0.28

Data are *P* values for association by GENMOD; significant *P* values in bold. Age, sex, and BMI were analyzed as covariates in all analyses; additional covariates (cov) are indicated. *Haplotype associated with increased insulin secretion. †Haplotype associated with decreased insulin sensitivity, decreased insulin secretion, and decreased DI.

generation) and *2*) their adult (aged 18 years or older) offspring and the spouses of those offspring (offspring generation). All subjects were genotyped, and only the offspring generation was phenotyped. By design, the offspring were free of diabetes and clinically manifest cardiovascular disease, thus avoiding secondary changes in phenotype caused by overt disease.

All studies were approved by the human subject protection institutional review boards at UCLA and Cedars-Sinai Medical Center. All subjects gave informed consent before participation.

Genotyping. We genotyped SNPs 44, 43, 56, and 63 of *CAPN10*. SNP-56 is in near-perfect linkage disequilibrium with Indel-19 and thus served as a surrogate for Indel-19 that was compatible with our genotyping technology (3,18). To maintain consistency with the literature in terms of haplotype designations, we represented SNP-56 A as allele 1 and SNP-56 G as allele 2; for the other SNPs, 1 = major allele and 2 = minor allele. Genotyping in 486 subjects from 85 MACAD families was performed using the 5′-exonuclease (Taqman MGB) assay (11,19). PCR primer and oligonucleotide probe sequences are available from the authors. The genotype frequencies for all four markers were in Hardy-Weinberg equilibrium. Linkage disequilibrium among the four markers (D′) ranged from 0.56 to 0.85 (average pairwise D′ of 0.74).

Phenotyping. Measurement of carotid IMT using B-mode ultrasound, an oral glucose tolerance test (OGTT), and a hyperinsulinemic-euglycemic clamp were performed on separate days. Of the 365 subjects from the offspring generation, 327 underwent the OGTT, 309 underwent carotid IMT, and 294 underwent the euglycemic clamp at the time of analyses reported herein.

B-mode carotid artery images of the right distal common carotid artery were obtained using a standardized technique (20). The distance between the blood-intima and the media-adventitia echoes, measured by automated computerized edge detection algorithm (Prowin, patent pending), was taken as the IMT measure. The measure of distal common carotid IMT represented the average of ~80–100 IMT measurements made over 1 cm.

The hyperinsulinemic-euglycemic clamp (21) was performed as previously described with an insulin infusion of 60 $mU \cdot m^{-2} \cdot min^{-1}$ (10). The clamp-derived insulin SI ($mg/m^2 \cdot ml \cdot min^{-1} \cdot \mu IU^{-1}$) is the mean steady-state glucose infusion rate divided by the body surface area and steady-state insulin level.

The OGTT consisted of baseline glucose and insulin measurements followed by administration of 75-g oral glucose with blood drawn at 30, 60, 90, 120, and 180 min. Insulin secretion indexes were obtained from the OGTT 30-min time-point glucose and insulin measurements to estimate early insulin secretion. The insulin-to-glucose ratio (IGR30, $\mu IU \cdot dL \cdot mg^{-1} \cdot ml^{-1}$) is the 30-min insulin value divided by the 30-min glucose value, and the insulin ratio (INSR30) is the insulin value at 30 min divided by the baseline insulin value (22). We also used the DI, defined as the product of an insulin secretion index with an insulin SI, a measure of β-cell compensation for insulin resistance, based on the demonstrated hyperbolic relationship between insulin sensitivity and insulin secretion (23). We defined DI1 as IGR30 × SI and DI2 as INSR30 × SI.

Data analysis. Haplotypes were determined by the maximum likelihood method, using the simulated annealing algorithm implemented in the program Simwalk2 (24). Five common haplotypes were observed; these were analyzed for genotype-phenotype associations. Haplotype (comprised of SNPs 44, 43, 56, and 63) frequencies were 34.6% for 1121, 31.7% for 1221, 16.7% for 1112, 9.7% for 1111, and 7.2% for 2111.

Log-transformed or square-root–transformed trait values were used to reduce skewness for all analyses. *T* tests were used to compare trait values between men and women. Mean trait values are given as arithmetical mean ± SD.

Because family data are correlated, we evaluated association using a robust variance estimation approach using the generalized estimating equation (GEE1 ref. 25) to test hypothesized associations between phenotypes and haplotypes, while accounting for familial correlations present in the data. The PROC GENMOD procedure in SAS (version 8.0; SAS Institute, Cary, NC) was used for the analysis using the GEE1 model. Family was taken as the cluster factor, i.e., members from the same family were assumed to be correlated. Analyses were conducted using age, sex, and BMI as covariates unless otherwise specified. Because insulin secretion is influenced by insulin sensitivity, we further assessed the association of *CAPN10* with insulin secretion in two ways. First, we evaluated the association of *CAPN10* with the DIs. Second, we evaluated *CAPN10* association with the insulin secretion indexes including insulin SIs as covariates. For *CAPN10* association with carotid IMT, we included SI, IGR30, INSR30, and their combination or products (DI1 or DI2) as covariates.

Given the focus on haplotypes in the literature on *CAPN10*, we reported the results of haplotype-phenotype associations. Statistical correction for multiple testing was not used because our primary goal was to test the specific hypothesis that diabetogenic *CAPN10* haplotypes were associated with carotid IMT. Subsequent analyses focused on elucidating the relationship of these haplotypes with diabetes-related traits and carotid IMT adjusted for these traits.

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TABLE 3
CAPN10 haplotype means and association results for carotid IMT

Haplotype carriers	*n*	Mean IMT ± SD (mm)	No covariates	Covariates: Age, sex, and BMI	SI	IGR30	INSR30	SI and IGR30	SI and INSR30	DI1	DI2
1121	171	0.66 ± 0.13	0.88	0.52	0.48	0.72	0.70	0.66	0.64	0.64	0.57
1221	156	0.64 ± 0.10	**<0.001**	**<0.001**	**0.0011**	**<0.001**	**0.001**	**0.0015**	**0.0031**	**0.0015**	**0.0022**
1112	96	0.69 ± 0.13	**0.012**	0.092	0.11	0.11	0.14	0.12	0.17	0.12	0.17
1111	62	0.66 ± 0.11	0.77	0.42	0.47	0.36	0.43	0.44	0.48	0.45	0.49
2111	51	0.68 ± 0.13	0.21	0.15	0.27	0.15	0.15	0.25	0.29	0.27	0.31
1112/1121	34	0.72 ± 0.16	**0.019**	**0.012**	**0.011**	**0.020**	**0.029**	**0.017**	**0.023**	**0.017**	**0.021**

Data are *P* values for association by GENMOD (significant *P* values in bold) unless otherwise indicated. Age, sex, and BMI were analyzed as covariates in all analyses (except that labeled "no covariates").

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