Brief Genetics Report

The *HADHSC* Gene Encoding Short-Chain L-3-Hydroxyacyl-CoA Dehydrogenase (SCHAD) and Type 2 Diabetes Susceptibility

The DAMAGE Study

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The short-chain L-3-hydroxyacyl-CoA dehydrogenase (SCHAD) protein is involved in the penultimate step of mitochondrial fatty acid oxidation. Previously, it has been shown that mutations in the corresponding gene (*HADHSC*) are associated with hyperinsulinism in infancy. The presumed function of the SCHAD enzyme in glucose-stimulated insulin secretion led us to the hypothesis that common variants in *HADHSC* on chromosome 4q22-26 might be associated with development of type 2 diabetes. In this study, we have performed a large-scale association study in four different cohorts from the Netherlands and Denmark (n = 7,365). Direct sequencing of *HADHSC* cDNA and databank analysis identified four tagging single nucleotide polymorphisms (SNPs) including one missense variant (P86L). Neither the SNPs nor haplotypes investigated were associated with the disease, enzyme function, or any relevant quantitative measure (all P > 0.1). The present study provides no evidence that the specific *HADHSC* variants or haplotypes examined do influence susceptibility to develop type 2 diabetes. We conclude that it is unlikely that variation in *HADHSC* plays a major role in the pathogenesis of type 2 diabetes in the examined cohorts. *Diabetes* 55:3193–3196, 2006

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NGT, normal glucose tolerance; SCHAD, short-chain L-3-hydroxyacyl-CoA dehydrogenase; SNP, single nucleotide polymorphism.

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Previously, it was shown that variation in *HADHSC* is associated with the development of hyperinsulinism of infancy (OMIM 601820) (1–3). *HADHSC* encodes the short-chain L-3-hydroxyacyl-CoA dehydrogenase enzyme (SCHAD; EC 1.1.1.35), an important enzyme in mitochondrial fatty acid oxidation that is highly expressed in pancreatic β-cells (National Center for Biotechnology Information gene no. 3033). Disease-associated mutations in the gene result in severely reduced enzyme levels and activity in different tissues. Patients suffer from unregulated insulin hypersecretion resulting in hypoglycemia. The exact mechanisms behind this association remain, however, unclear. The fact that patients are responsive to the effects of the drug diazoxide on the closure of the pancreatic ATP-sensitive K^+ channels suggests involvement of the ATP-sensitive K^+ channel–dependent pathway of glucose-stimulated insulin secretion. Whether this is via increased ATP levels in the cytosol or other (mitochondrial) factors affecting channel function and membrane depolarization of the β-cell remains unclear.

Previously, it has been shown that (common) variants in other hyperinsulinism in infancy-associated genes are associated with enhanced susceptibility to develop type 2 diabetes (4–7). Glucose intolerance in these cases is most likely caused by defects in glucose-stimulated insulin secretion (8). Most prominent examples are variants in the genes encoding the two subunits of the ATP-sensitive potassium channel, *ABCC8* and *KCNJ11* and glucokinase (*GCK*) (4–9). Several other studies (10–13) have furthermore shown the importance of correct mitochondrial function in relation to insulin secretion, insulin resistance, glucose homeostasis, and type 2 diabetes.

Together, this has led us to the hypothesis that polymorphisms in *HADHSC* might be associated with enhanced type 2 diabetes susceptibility. Therefore, we have performed an association study with *HADHSC* in four population cohorts from the Netherlands and Denmark involving in total 7,365 subjects.

RESEARCH DESIGN AND METHODS

Subjects participating in the association studies were selected from four study populations in the Netherlands and Denmark. The first cohort, HS1, consisted

TABLE 1
Allele frequencies for the P86L (C→T, rs4956145) SNP in *HADHSC* in four different cohorts

Cohort	NGT		Type 2 diabetes		*P*	OR (95% CI)*
	C	T	C	T		
Hoorn Study 1	472	48 (0.092)	391	29 (0.069)	0.23	1.37 (0.85–2.22)
Hoorn Study 2	520	46 (0.081)	555	39 (0.066)	0.31	1.26 (0.81–1.96)
DK1, Steno case-control study	957	97 (0.092)	1124	82 (0.068)	0.04	1.39 (1.02–1.89)
DK2, Inter99 case-control study	8155	703 (0.079)	1381	131 (0.087)	0.33	0.91 (0.75–1.10)
Common OR					0.29	1.08 (0.93–1.26)

*For C- vs. T-alleles. *P* values were calculated using Fisher's exact tests. Common OR was calculated using Mantel-Haenszel statistics.

of 470 participants of the Hoorn Study, a population-based study in the Netherlands (14). Subjects were classified as having type 2 diabetes (n = 210, aged 65 ± 8 years, 48% men) or as glucose-tolerant control subjects (n = 260, aged 60 ± 7 years, 42% men) based on the baseline fasting oral glucose tolerance test according World Health Organization criterion.

Furthermore, we used a second sample from the same study, the HS2 cohort (15). Subjects with either normal glucose tolerance (NGT) or type 2 diabetes at the year 2000 survey of the Hoorn Study were included in this cohort. To enhance power, the type 2 diabetes group was supplemented by subjects with screen-detected type 2 diabetes from the same geographic area (n = 217) (16). There was no overlap between the subjects in either cohort. In the type 2 diabetes group (n = 297), the mean age was 68 ± 8 years (48% men) and 69 ± 6 years for participants with NGT (n = 283, 50% men).

Both Dutch cohorts are part of the DAMAGE (Diabetes and Mitochondrial Aging Genes) study. Furthermore, we genotyped two case-control study samples from Denmark, DK1 and DK2. DK1 was a group of unrelated type 2 diabetic patients recruited from the Steno Diabetes Center, and a group of unrelated subjects with NGT sampled at random through public registers at the Steno Diabetes Center and the Research Centre for Prevention and Health (17). In the group of type 2 diabetic patients (n = 603, 59% men), the mean age was 60 ± 10 years and 58 ± 10 years for participants with NGT (n = 527, 46% men) (17).

DK2 consisted of unrelated type 2 diabetic patients recruited from the Steno Diabetes Center and the Research Centre for Prevention and Health (n = 756, aged 54 ± 10 years, 61% men), and a group of unrelated subjects with NGT sampled from the prospective Inter99 Study at the Research Centre for Prevention and Health (n = 4,429, aged 45 ± 8 years, 47% men) (18,19). All control subjects participating in the Danish studies underwent an oral glucose tolerance test according to World Health Organization criteria. Questionnaires were used to obtain other relevant information.

All participants were Caucasian by self-report. Informed written consent was obtained from all subjects before participation. The study was approved by the appropriate medical ethical committees and was in accordance with the principles of the Declaration of Helsinki.

***HADHSC* sequencing.** *HADHSC* is mapped to the chromosome 4q22–26 region and spans ~50 kb. Its eight exons encode a 314 aa protein (gene no. 3033). Total RNA was isolated from leukocytes (n = 25) and/or pancreas tissue (n = 7) from 25 Dutch volunteers with familial late-onset type 2 diabetes using standard procedures, which was subsequently converted into cDNA using a first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA). The coding region of *HADHSC* was amplified in two overlapping segments (primer sequences and assay conditions available from the authors on request). The resulting segments encompassing 1,036 bp of the coding region (including 32 and 59 bp 5′ and 3′, respectively, of the gene) were subsequently sequenced on an automated sequencer (Applied Biosystems, Foster City, CA).

Genotyping. Tagging single nucleotide polymorphisms (SNPs) in *HADHSC* were identified using the Haploview/Tagger software based on Hapmap Phase I CEPH data (multimarker method, $r^2 > 0.8$ and logarithm of odds threshold 3.0). Four tagging SNPs (rs4521419, rs4590125, rs4956145, and rs141066; databank accessed June 2005) (online appendix Fig. 1 [available at http://diabetes.diabetesjournals.org]), including one SNP in the coding region (P86L, rs4956145), were genotyped based on Taqman SNP genotyping technology (Applied Biosystems). Upon genotyping, two of the SNPs (rs4521419 and rs4956145) appeared to be in perfect linkage disequilibrium. Genotyping success rates were >95% for all SNPs. The P86L variant (rs4956145) was additionally tested via a PCR/restriction fragment–length polymorphism–based method in a subset of the samples (n = 188). Furthermore we confirmed the presence of the four variants by direct sequencing of DNA segments in 40 subjects. No discrepancies between the different methods were identified. Replication samples (n >350) for all SNPs showed an error rate <0.3%. Haplotypes were reconstructed using the Phase (version 2.1.1) program (20).

Enzyme activity. SCHAD enzyme activity was determined in duplicate in crude lysates and mitochondrial fractions from fibroblast cell lines and is given as the SCHAD/Citrate synthase activity ratio (units per Citrate synthase units [CSU]) as described by Clayton et al. (1). We had 80% power (α = 0.05) to detect a difference of at least 20–25%.

SCHAD protein expression. SCHAD protein expression was determined by Western blot analysis using a rabbit polyclonal antibody raised against the protein (21). Total fibroblast lysates and mitochondrial fractions were separated on a polyacrylamide gel according to standard procedures. Enhanced chemiluminescence was used to visualize the 34 kd and corresponding to the SCHAD protein.

mRNA expression levels. *HADHSC* mRNA levels were determined in total RNA isolated from fibroblasts using quantitative real-time PCR on a ABI 7900ht. Glyceraldehyde-3-phosphate dehydrogenase, *HPRT1*, and *β-actin* were used as control genes.

Statistical analyses. Hardy-Weinberg equilibrium was confirmed in all cohorts before further analysis. Differences in allele or genotype frequencies were tested by Fisher's exact tests or logistic regression analysis with age and sex as covariates. A common odds ratio (OR) was calculated after testing the homogeneity of the population-specific ORs revealed no significant differences using a Mantel-Haenzel test. Differences in clinical variables were tested by ANOVA and/or linear regression analysis with adjustment for age, sex, and BMI. Variables were log transformed before analysis if necessary. The statistical software packages SPSS11 (SPSS, Chicago, IL) and StatXact6.0 (Cytel, Cambridge, MA) were used.

RESULTS

Databank analysis and direct sequencing of *HADHSC* cDNA revealed 17 common intronic SNPs (minor allele frequency >0.05) and three cSNPs in this locus. The three cSNPs were all nonsynonymous variants: P86L (rs4956145), H152Q (rs1051519), and D279E (rs17417368). The latter two seemed to be rare and were not detected in our (sequenced) samples. Therefore, we focused our research on the P86L variant and three tagging SNPs that capture all other alleles in the HapMap Phase I CEPH dataset (online appendix Fig. 1).

The minor allele frequency of the P86L variant varied between 6.6 and 9.2% in the different case-control groups from the Netherlands and Denmark (Table 1). Only in the DK1 cohort did the difference reach statistical significance (P = 0.04; Table 1). Meta-analysis was used to calculate a common OR after homogeneity testing revealed no significant differences between the studies (P > 0.05). The common OR was 1.08 (95% CI 0.93–1.26, P = 0.29) (Table 1 and online appendix Fig. 2). Adjustment for age and sex did not significantly alter the results. Analysis of the genotype distributions in the different cohorts assuming different genetic models and adjusted for age and sex yielded similar nonsignificant results (online appendix Table 1). Based on the observed allele frequency of the P86L variant (minor allele frequency 0.08), we had >80% power to detect effects with an OR ≥1.2 (α = 0.05) in our sample (>95% for an OR ≥1.25). Circulating glucose, insulin, and lipid levels, as well as other relevant diabetes-related clinical variables, were also not significantly differ-

TABLE 2
Clinical characteristics of NGT subjects in the different study populations in relation to *HADHSC* Pro[86]Leu polymorphism

	Hoorn Study 1		Hoorn Study 2		DK2, Inter99 Study	
	PP	PL + LL	PP	PL + LL	PP	PL + LL
n	215	45	238	42	3,753	676
Age (years)	60 ± 7	62 ± 7	69 ± 6	69 ± 6	45 ± 8	46 ± 8
Sex (male/female)	88/127	15/30	121/117	20/22	1,739/2,014	311/365
BMI (kg/m^2)	26.3 ± 3.0	25.4 ± 2.8	26.1 ± 3.2	26.2 ± 3.6	25.5 ± 4.0	25.6 ± 4.2
HbA_{1c} (%)	5.5 ± 0.4	5.5 ± 0.4	5.7 ± 0.4	5.7 ± 0.5	5.8 ± 0.4	5.8 ± 0.4
Fasting plasma glucose (mmol/l)	5.3 ± 0.5	5.4 ± 0.5	5.4 ± 0.4	5.5 ± 0.4	5.3 ± 0.4	5.3 ± 0.4
2-h post-OGTT glucose (mmol/l)	5.0 ± 1.3	5.3 ± 1.2	5.6 ± 1.2	5.9 ± 1.0	5.5 ± 1.1	5.5 ± 1.1
Fasting serum insulin (pmol/l)	80 ± 36	73 ± 25	51 ± 24	52 ± 23	38 ± 23	37 ± 23
2-h post-OGTT insulin (pmol/l)	NA	NA	NA	NA	168 ± 130	170 ± 134
HOMA-IR	19.4 ± 9.7	17.9 ± 6.9	12.3 ± 6.4	12.9 ± 6.2	8.9 ± 5.7	8.8 ± 5.5
Fasting serum total cholesterol (mmol/l)	6.7 ± 1.1	6.5 ± 1.0	5.9 ± 1.0	5.6 ± 1.0	5.4 ± 1.0	5.5 ± 1.1
Fasting serum HDL cholesterol (mmol/l)	1.32 ± 0.35	1.34 ± 0.37	1.51 ± 0.40	1.46 ± 0.45	1.46 ± 0.40	1.44 ± 0.40
Fasting serum LDL cholesterol (mmol/l)	4.71 ± 1.11	4.48 ± 0.97	3.84 ± 0.86	3.50 ± 0.88	NA	NA
Fasting serum triglycerides (mmol/l)	1.43 ± 0.62	1.42 ± 0.69	1.26 ± 0.56	1.48 ± 0.91	1.17 ± 0.94	1.22 ± 0.90

Data are means ± SD. *P* values were obtained after general linear regression (SPSS) with age and BMI as covariates and sex and genotype as fixed factors. All $P > 0.05$. HOMA-IR, homeostasis model assessment of insulin resistance; NA, not applicable; OGTT, oral glucose tolerance test.

ent between normoglycemic carriers and noncarriers (Table 2).

We determined SCHAD enzyme activity in mitochondrial fractions isolated from fibroblasts of 5 carriers and 10 noncarriers of the P86L variant. Enzyme activities were, however, not significantly different (0.64 ± 0.14 vs. 0.60 ± 0.07 units/UCS, $P > 0.5$). SCHAD enzyme activities in total fibroblast lysates showed a similar nonsignificant difference. Furthermore, there were no detectable differences in SCHAD protein and mRNA levels between carriers and noncarriers (data not shown). Together, this suggests that the P86L variant has no effect on enzyme levels or function, although small differences cannot be fully excluded.

According to the Hapmap Phase I CEPH data, *HADHSC* is present on one single haploblock spanning the whole locus (online appendix Fig. 1). The four common haplotypes (>1%) in *HADHSC* can be tagged by four tagging SNPs, which were typed in subsets of the HS1 ($n = 630$) and DK1 ($n = 520$) studies. We also examined the performance of our tagging SNPs in the recently available HapMap Phase II CEPH (January 2006) dataset. Our tagging SNPs captured almost all of the SNPs identified in the *HADHSC* region from the Phase II dataset, capturing 48 of 55 common SNPs with a mean r^2 of 0.991 and 100% of alleles with $r^2 > 0.8$.

Haplotype frequency distributions are very similar in both populations. In a joint analysis of all haplotypes in relation to disease status, there was no significant difference between case and control subjects in both cohorts. There were also no significant differences in the frequency of the four most common haplotypes (all $P > 0.1$), although we observed similar trends in both populations, especially with the 0-0-0-1 haplotype (Table 3). Our combined case-control sample provides at least 80% power to detect an effect with an OR ≥1.35 (assuming $f = 0.33$ [0-0-0-1 haplotype] and $\alpha = 0.05$).

To examine the possibility that the 0-0-0-1 haplotype is associated with altered enzyme function and a minor effect on type 2 diabetes susceptibility, we determined SCHAD activity in mitochondrial fractions from fibroblasts of four homozygous carriers of this haplotype and four homozygous carriers of the common 0-0-0-0 haplotype. Enzyme activity was not detectably different between the two haplotypes (0.57 ± 0.13 vs. 0.62 ± 0.11 for 0-0-0-0 haplotypes, $P > 0.5$). There was also no evidence for differences in expression levels between carriers and noncarriers (data not shown).

DISCUSSION

In this study, which is the first ever to examine the relationship between variations in *HADHSC* and type 2 diabetes, we have found no evidence for an association between variants at this gene locus and susceptibility to develop type 2 diabetes.

Based on the above-described results, the fact that we found no evidence for alterations in circulating glucose, insulin, or lipid levels in NGT carriers of these gene

TABLE 3
Frequency of *HADHSC* haplotypes in two different populations

	Hoorn Study 1		DK1	
Haplotype	NGT	Type 2 diabetes	NGT	Type 2 diabetes
0-0-0-0	0.54	0.54	0.56	0.55
0-0-0-1	0.31	0.34	0.30	0.33
1-1-1-0	0.09	0.07	0.07	0.06
0-1-0-0	0.06	0.05	0.04	0.04
Rare	<0.01	<0.01	0.03	0.02
*n**	840	420	404	636

* Total number of haplotypes in each group. SNP order: rs4521419-rs4590125-rs4956145-rs141066. Differences in haplotype distribution were tested by overall distribution and by 2 × 2 Fisher's exact tests (all $P > 0.1$).

variants, and given that the data show that the variants investigated in this study have no detectable effect on expression levels or enzyme function, we conclude that variation in *HADHSC* is unlikely to have a major effect on susceptibility to develop type 2 diabetes in these cohorts.

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