A Candidate Type 2 Diabetes Polymorphism Near the *HHEX* Locus Affects Acute Glucose-Stimulated Insulin Release in European Populations

Results from the EUGENE2 study

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OBJECTIVE—In recent genome-wide association studies, two single nucleotide polymorphisms (SNPs) near the *HHEX* locus were shown to be more frequent in type 2 diabetic patients than in control subjects. Based on *HHEX*'s function during embryonic development of the ventral pancreas in mice, we investigated whether these SNPs affect β-cell function in humans.

RESEARCH DESIGN AND METHODS—A total of 854 nondiabetic subjects, collected from five European clinical centers, were genotyped for the *HHEX* SNPs rs1111875 and rs7923837 and thoroughly characterized by an oral glucose tolerance test (OGTT). To assess glucose-stimulated insulin release, a subgroup of 758 subjects underwent an intravenous glucose tolerance test (IVGTT).

RESULTS—SNPs rs1111875 and rs7923837 were not associated with anthropometric data (age, weight, height, BMI, body fat, and waist and hip circumference). After adjustment for center, family relationship, sex, age, and BMI, both SNPs were also not associated with glucose and insulin concentrations in the fasting state and during the OGTT or with measures of insulin sensitivity. Furthermore, *HHEX* SNP rs1111875 was not associated with insulin release during the IVGTT. By contrast, the minor A-allele of *HHEX* SNP rs7923837 was significantly associated with higher IVGTT-derived first-phase insulin release before and after appropriate adjustment ($P = 0.013$ and $P = 0.014$, respectively).

CONCLUSIONS—A common genetic variation in the 3′-flanking region of the *HHEX* locus, i.e., SNP rs7923837, is associated with altered glucose-stimulated insulin release. This SNP's major allele represents a risk allele for β-cell dysfunction and, thus, might confer increased susceptibility of β-cells toward adverse environmental factors. ***Diabetes* 57:514–517, 2008**

Type 2 diabetes is the most prevalent metabolic disease of the Western industrialized world. It is generally agreed that type 2 diabetes is caused by environmental factors, such as high-caloric diet and reduced physical activity, and a polygenic background that confers increased susceptibility toward these environmental challenges (1). To identify the genes involved in the pathogenesis of type 2 diabetes, genome-wide association studies in several cohorts based on large-scale single nucleotide polymorphism (SNP) analysis of genomic variation were recently published (2–7). These studies revealed 10 potential type 2 diabetes candidate genes. Three formerly characterized genes with known effects on insulin sensitivity or insulin secretion, respectively, i.e., *PPARG*, *KCNJ11*, and *TCF7L2*, could be confirmed. However, the role of the other seven candidate genes in the development of pre-diabetes phenotypes, such as insulin resistance and β-cell dysfunction, is not well established. Two of the type 2 diabetes candidate SNPs are located in the 3′-flanking region of the *HHEX* locus (2). *HHEX* encodes the transcription factor hematopoietically expressed homeobox protein (HHEX), which is expressed in the embryonic ventral-lateral foregut that gives rise to the ventral pancreas and the liver (8). Knockout of this gene was shown to impair proliferation of endodermal epithelial cells, positioning of ventral foregut endoderm cells relative to the mesoderm, and budding and morphogenesis of the ventral pancreas (8). This genetic manipulation finally provoked lethality during midgestation (8). Due to these developmental defects of the *HHEX* knockout, it was obvious to investigate whether the two reported type 2 diabetes candidate SNPs near the *HHEX* locus (2) affect β-cell function. This was therefore assessed in five nondiabetic European populations thoroughly characterized by an intravenous glucose tolerance test (IVGTT).

RESEARCH DESIGN AND METHODS

A total of 844 nondiabetic subjects were recruited from the five European clinical centers constituting the EUGENE2 consortium, i.e., the Lundberg Laboratory for Diabetes Research (Göteborg, Sweden), the Polyclinic Mater Domini of the University Magna Graecia (Catanzaro, Italy), the Steno Diabetes

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Received for publication 19 June 2007 and accepted in revised form 16 November 2007.

Published ahead of print at http://diabetes.diabetesjournals.org on 25 November 2007. DOI: 10.2337/db07-1254.

Additional information for this article can be found in an online appendix at http://dx.doi.org/10.2337/db07-1254.

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HOMA-IR, homeostasis model assessment of insulin resistance; OGTT, oral glucose tolerance test; IVGTT, intravenous glucose tolerance test; SNP, single nucleotide polymorphism.

TABLE 1
Baseline characteristics of the nondiabetic study population

n	844
Age (years)	40.1 ± 10.4
Male/female sex (%)	43/57
BMI (kg/m^2)	26.6 ± 4.9
Waist-to-hip ratio	0.87 ± 0.09
NGT (*n*)	691
IFG (*n*)	23
IGT (*n*)	110
IFG and IGT (*n*)	20

IFG, impaired fasting glucose; IGT, impaired glucose tolerance; NGT, normal glucose tolerance; WHR, waist-to-hip ratio.

Center (Copenhagen, Denmark), the Kuopio University Hospital (Kuopio, Finland), and the Tübingen University Hospital (Tübingen, Germany). The baseline characteristics of the study population are presented in Table 1. All participants underwent standard medical history and physical examination, assessment of smoking status, alcohol consumption habits and activity, routine blood tests, and an oral glucose tolerance test (OGTT). A subgroup of 758 subjects was further characterized by an IVGTT. In addition, hyperinsulinemic-euglycemic clamps were performed in four centers ($n = 575$). The participants gave informed written consent, and the protocols were approved by the local ethical committees.

Genotyping of the study population. For genotyping, DNA was isolated from whole blood using commercial DNA isolation kits. SNPs were genotyped using TaqMan allelic discrimination assays (Applied Biosystems, Foster City, CA). Primer sequences are available upon request from the authors. TaqMan genotyping reaction was amplified on a GeneAmp PCR system 2700 and fluorescence detected using an ABI Prism 7000 sequence detector (Applied Biosystems). The overall genotyping success rate was 99.7%, and rescreening of 3.3% of subjects gave 100% identical results.

Body composition and body fat distribution. Body composition was measured by bioelectrical impedance as the percentage of body fat. BMI was calculated as weight divided by the square of height in meters. Moreover, waist and hip circumferences were measured.

OGTT, IVGTT, and hyperinsulinemic-euglycemic clamp. After an overnight fast, all subjects underwent a 75-g OGTT, and venous blood samples were obtained at 0, 30, 60, 90, and 120 min for the determination of plasma glucose and insulin. On a separate occasion, an IVGTT was performed in overnight-fasted subjects, as described by the Botnia protocol (9). After baseline samples had been collected, a 0.3-g/kg body weight glucose dose of a 20% glucose solution was given at time 0. Blood samples for the measurement of plasma glucose and insulin were obtained at 2, 4, 6, 8, 10, 20, 30, 40, 50, and 60 min. Hyperinsulinemic-euglycemic clamp was performed starting at 60 min after the IVGTT glucose bolus. To this end, subjects received a primed infusion of short-acting human insulin (40 mU/m^2 per min) for 120 min. Variable infusion of 20% glucose was started to clamp the plasma glucose concentration at 5 mmol/l. Blood samples for the measurement of plasma glucose were obtained at 5-min intervals. Plasma insulin levels were measured at baseline and at the steady state of the clamp. Plasma glucose and insulin were determined by standard laboratory methods.

Calculations. The trapezoidal method was used to calculate the areas under the curve of plasma glucose (in mmol $\cdot$ l^{-1} $\cdot$ min^{-1}) and insulin (in pmol $\cdot$ l^{-1} $\cdot$ min^{-1}). Homeostasis model assessment of insulin resistance (HOMA-IR, in mmol/l $\cdot$ μU/ml) was calculated as $Glc_0 \times Ins_0/22.5$, where Glc_0 is glucose at 0 min and Ins_0 is insulin at 0 min. Clamp-derived insulin sensitivity (*M* value) was calculated as the glucose infusion rate necessary to maintain euglycemia during the last 60 min (steady state) of the clamp (in μmol $\cdot$ kg^{-1} $\cdot$ min^{-1}).

Statistical analyses. Unless otherwise stated, data are given as means ± SD. Hardy-Weinberg equilibrium and differences in genotype distribution between the five centers were tested using χ^2 test. Insulin levels and HOMA-IR were log transformed in order to obtain a normal distribution. Linear mixed model was applied to adjust for confounding factors. For mixed model analysis, we included the center and pedigree (coded as a family number) as random factors, the genotype and sex as fixed factors, and age, BMI, and HOMA-IR as covariates. The SNP genotype was expressed as a nominal value (0, homozygous for the major allele; 1, heterozygous; or 2, homozygous for the minor allele), handled as a covariate, and tested for association with traits in the additive inheritance model. $P < 0.05$ was considered statistically significant. These statistical analyses were performed with SPSS 14.0 (SPSS, Chicago, IL). In the additive model using global *F* test (ANOVA), our study was sufficiently powered (1-β > 0.8) to detect effect sizes with Cohen's $f \geq 0.19$ for SNP rs7923837 and $f \geq 0.17$ for SNP rs1111875 assuming average group sizes of $n = 91$ and $n = 123$, respectively, according to these SNPs' smallest genotype groups. Power calculations were performed using G*power software (available at http://www.psycho.uni-duesseldorf.de/aap/projects/gpower/).

RESULTS

We genotyped the 844 subjects (baseline characteristics given in Table 1) for the two SNPs rs1111875 C/T and rs7923837 G/A in the 3′-flanking region of the *HHEX* gene (chromosome 10). Both SNPs were found to be in Hardy-Weinberg equilibrium ($P = 0.55$ and 0.49, respectively) and displayed minor allele frequencies (MAFs) very similar to those recently reported (2) (MAF 0.39 and 0.34, respectively, Table 2). Analysis of our genotype data revealed that both SNPs were in strong, but not in complete, linkage disequilibrium ($D' = 0.984$, $r^2 = 0.779$). Furthermore, the *HHEX* SNPs rs1111875 and rs7923837 did not show significant differences in genotype distribution between the five centers ($P = 0.09$ and $P = 0.17$, respectively) (supplementary Table 1 [available in the online appendix at http://dx.doi.org/10.2337/db07-1254]). Both SNPs were not associated with anthropometric data such as age, weight, height, BMI, waist and hip circumference, or body fat content (data not shown). Neither SNP rs1111875 nor SNP rs7923837 was associated with fasting glucose and insulin concentrations, glucose levels at 120 min of OGTT, insulin levels at 30 min of OGTT, the areas under the curve of the glucose and insulin responses during the OGTT, or insulin sensitivity before and after adjustment for center, family relationship, sex, age, and BMI (Table 2; data by center presented in supplementary Tables 2–6). Furthermore, *HHEX* SNP rs1111875 was not associated with first-phase (0–10 min) or second-phase (10–60 min) insulin release during the IVGTT (Table 2 and supplementary Tables 2–6). By contrast, *HHEX* SNP rs7923837 was significantly associated with IVGTT-derived first-phase insulin release before as well as after adjustment (Table 2 and supplementary Tables 2–6). Notably, it is the minor A-allele that confers higher insulin responses to an intravenous glucose load. Furthermore, additional adjustment for HOMA-IR and glucose levels at 30 min of OGTT revealed a trend toward an association of *HHEX* SNP rs7923837 with insulin levels at 30 min of the OGTT ($P = 0.066$), supporting the role of this SNP in insulin release. Finally, to assess whether the association of *HHEX* SNP rs7923837 with IVGTT-derived first-phase insulin release is already detectable in healthy, normal glucose-tolerant subjects, we excluded 23 subjects with impaired fasting glucose, 110 subjects with impaired glucose tolerance, and 20 subjects with both impaired fasting glucose and impaired glucose tolerance (Table 1) from statistical analyses. In the remaining normal glucose-tolerant cohort, we no longer detected significant differences between the *HHEX* SNP rs7923837 genotypes after appropriate adjustment ($P = 0.12$ and $P = 0.09$ for first-phase insulin release and for first-phase insulin release over basal, respectively, IVGTT), and this is likely due to the reduced sample size.

DISCUSSION

We examined possible associations of two very recently reported type 2 diabetes candidate SNPs near the *HHEX* locus (2) with differences in insulin secretion (and insulin sensitivity) in 844 nondiabetic subjects from five different European clinical centers, 758 of them having undergone an IVGTT. Both SNPs that were found to be in strong, but not complete, linkage disequilibrium did not reveal an

TABLE 2
Associations of *HHEX* SNPs rs1111875 and rs7923837 with metabolic parameters

	HHEX rs1111875 (MAF 0.39)						*HHEX* rs7923837 (MAF 0.34)					
Genotype	CC	CT	TT	P_1	P_2	P_3	GG	GA	AA	P_1	P_2	P_3
n	303	418	123	—	—	—	356	395	91	—	—	—
Fasting glucose (mmol/l)	5.10 ± 0.55	5.09 ± 0.54	5.01 ± 0.44	0.3	0.11	—	5.11 ± 0.56	5.08 ± 0.52	5.01 ± 0.43	0.4	0.3	—
Glucose 120 min OGTT (mmol/l)	6.28 ± 1.58	6.25 ± 1.53	6.20 ± 1.42	1.0	0.8	—	6.27 ± 1.56	6.27 ± 1.54	6.15 ± 1.40	0.9	0.8	—
AUC glucose OGTT (mmol · l^{-1} · min^{-1})	872 ± 175	862 ± 181	863 ± 151	0.7	0.9	—	870 ± 177	863 ± 178	862 ± 152	0.9	0.9	—
Fasting insulin (pmol/l)	48.3 ± 32.5	54.2 ± 73.8	44.4 ± 29.9	0.22	0.06	—	48.1 ± 31.7	54.4 ± 75.9	44.8 ± 27.5	0.5	0.20	—
Insulin 30 min OGTT (pmol/l)	360 ± 225	403 ± 282	385 ± 234	0.11	0.15	—	362 ± 220	407 ± 288	382 ± 240	0.13	0.4	—
AUC insulin OGTT (pmol · l^{-1} · min^{-1})	247,575 ± 154,834	263,378 ± 188,720	246,755 ± 135,307	0.4	0.7	—	244,007 ± 147,702	268,633 ± 194,318	242,962 ± 133,361	0.4	0.7	—
AUC insulin 0–10 min IVGTT (pmol · l^{-1} · min^{-1})*	3,580 ± 2,842	4,013 ± 3,232	3,616 ± 2,359	0.17	0.19	0.23	3,433 ± 2,434	4,173 ± 3,514	3,647 ± 2,209	0.013	0.046	0.014
AUC insulin 0–10 min IVGTT over basal insulin (pmol · l^{-1} · min^{-1})*	3,062 ± 2,502	3,471 ± 2,749	3,198 ± 2,206	0.15	0.20	0.21	2,956 ± 2,282	3,602 ± 2,919	3,220 ± 2,061	0.016	0.049	0.025
AUC insulin 10–60 min IVGTT (pmol · l^{-1} · min^{-1})*	11,164 ± 11,340	11,450 ± 10,699	9,576 ± 7,098	0.3	0.4	1.0	10,559 ± 9,987	11,877 ± 11,649	9,652 ± 6,086	0.19	0.7	0.4
AUC insulin 10–60 min IVGTT over basal insulin (pmol · l^{-1} · min^{-1})*	8,601 ± 9,316	8,737 ± 8,177	7,449 ± 6,222	0.3	0.5	0.8	8,205 ± 8,810	9,006 ± 8,526	7,491 ± 5,196	0.14	0.5	0.22
HOMA-IR ($mmol^{-1}$ · l^{-1} · μU^{-1} · ml^{-1})	11.1 ± 8.2	12.8 ± 20.6	10.0 ± 7.0	0.17	0.042	—	11.1 ± 7.9	12.9 ± 21.1	10.1 ± 6.5	0.4	0.17	—
M (μmol · kg^{-1} · min^{-1})†	42.7 ± 16.5	41.1 ± 16.6	41.0 ± 16.4	0.5	0.6	—	42.8 ± 17.2	41.2 ± 16.5	40.0 ± 14.2	0.4	0.5	—

n = 844. P values given for unadjusted data (P_1). Additional P values are presented for all data after adjustment for center, family relationship, sex, age, and BMI (P_2) and for IVGTT data after additional adjustment for HOMA-IR (P_3). AUC, area under the curve; MAF, minor allele frequency. M = clamp-derived insulin sensitivity. *subgroup, n = 758; †subgroup, n = 575.

association with insulin resistance. We could not replicate the results of very recent studies showing an association of rs1111875 with acute insulin response (10) and β-cell glucose sensitivity (11), and this is probably due to the limited power of our study to detect smaller effects of this SNP (Cohen's $f < 0.17$) in insulin secretion parameters, which could still be clinically meaningful. However, we observed a clear association of SNP rs7923837 with first-phase insulin release, as derived from IVGTT data. Moreover, the major G-allele, which was formerly identified as a type 2 diabetes risk allele (2,4–6), turned out to represent a risk allele for impaired glucose-stimulated insulin response. This finding not only strengthens the role of the *HHEX* gene in the pathogenesis of impaired glucose tolerance and type 2 diabetes, as suggested by genome-wide association studies (2,4–6), but moreover reveals the underlying mechanisms by which this SNP increases the risk of type 2 diabetes. Thus, knowledge of the *HHEX* genotype could advance the development of appropriate strategies in therapy and prevention of type 2 diabetes, as recently discussed (12). Based on the observations reported from the *HHEX* knockout mouse (8), the association of rs7923837 with differences in glucose-stimulated insulin release could arise from mild alterations in the embryonic organogenesis of the ventral pancreas. This part of the pancreas is also the major site of pancreatic polypeptide production. Therefore, it is possible that the SNP affects this hormone's production during embryogenesis and/or adulthood, provoking the effects on insulin release. These suggestions, however, await further physiological and molecular clarification.

Finally, it is noteworthy that we discovered distribution abnormalities in our insulin data, with heterozygous carriers of both *HHEX* SNPs displaying higher mean insulin levels and higher SD values than the homozygous groups. Further examination revealed that, in four centers, the heterozygous subjects had higher mean insulin levels than the homozygous groups, whereas the heterozygous subjects from the fifth center (Gothenburg) displayed somewhat lower mean insulin levels than the homozygous groups. Even though we have currently no explanation for these abnormalities, the center-specific differences are likely to explain the high SD values for insulin levels in the heterozygous individuals.

In conclusion, a common genetic variation in the 3′-flanking region of the *HHEX* locus, i.e., SNP rs7923837, is associated with altered glucose-stimulated insulin release. This SNP's major allele represents a crucial allele for β-cell dysfunction and, thus, might confer increased susceptibility of β-cells toward adverse environmental factors.

ACKNOWLEDGMENTS

We thank all study participants for their cooperation. The study was supported by the European community's framework program FP6 EUGENE2 (LSHM-CT-2004-512013).

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