Brief Genetics Report

Evidence that the Mitochondrial Leucyl tRNA Synthetase (*LARS2*) Gene Represents a Novel Type 2 Diabetes Susceptibility Gene

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Previously, we have shown that a mutation in the mitochondrial DNA–encoded tRNA$^{Leu(UUR)}$ gene is associated with type 2 diabetes. One of the consequences of this mutation is a reduced aminoacylation of tRNA$^{Leu(UUR)}$. In this study, we have examined whether variants in the leucyl tRNA synthetase gene (*LARS2*), involved in aminoacylation of tRNA$^{Leu(UUR)}$, associate with type 2 diabetes. Direct sequencing of *LARS2* cDNA from 25 type 2 diabetic subjects revealed eight single nucleotide polymorphisms. Two of the variants were examined in 7,836 subjects from four independent populations in the Netherlands and Denmark. A −109 g/a variant was not associated with type 2 diabetes. Allele frequencies for the other variant, H324Q, were 3.5% in type 2 diabetic and 2.7% in control subjects, respectively. The common odds ratio across all four studies was 1.40 (95% CI 1.12–1.76), $P = 0.004$. There were no significant differences in clinical variables between carriers and noncarriers. In this study, we provide evidence that the *LARS2* gene may represent a novel type 2 diabetes susceptibility gene. The mechanism by which the H324Q variant enhances type 2 diabetes risk needs to be further established. This is the first report of association between an aminoacyl tRNA synthetase gene and disease. Our results further highlight the important role of mitochondria in glucose homeostasis. *Diabetes* 54:1892–1895, 2005

Previously, we have shown that an A3243G mutation in the mitochondrial DNA–encoded tRNA$^{Leu(UUR)}$ gene is associated with maternally inherited diabetes and deafness (1,2). Carriers of the 3243 mutation have a reduced glucose-stimulated insulin secretion. Biochemical studies indicate that mutant mitochondrial tRNA^{Leucine} is less efficiently aminoacylated in comparison with the wild-type tRNA (2). Furthermore, it has been shown that there is an altered balance between mitochondrial and nuclear-encoded proteins in mutant mitochondria, resulting in mitochondrial dysfunction (2).

In this study, we have examined whether variation in the nuclear-encoded mitochondrial leucyl tRNA synthetase gene (*LARS2*) is associated with type 2 diabetes. The product of the *LARS2* gene is the leucyl tRNA synthetase protein (LeuRS, EC 6.1.1.4), which catalyzes the charging of tRNA$^{Leu(UUR)}$ with leucine, an essential step in protein synthesis. Subjects (n = 7,836) participating in this study were recruited from four independent population samples in the Netherlands and Denmark to allow independent replication of association results.

RESEARCH DESIGN AND METHODS

Subjects participating in the association studies were selected from four independent study populations in the Netherlands and Denmark. The first cohort consisted of 547 participants of the Hoorn study (3), a population-based study in the Netherlands. Subjects were classified as either type 2 diabetic subjects (n = 211, aged 65 ± 8 years, 48% male) or glucose-tolerant control subjects (n = 336, aged 60 ± 7 years, 41% male) based on a fasting oral glucose tolerance test (OGTT) according to World Health Organization criterion. The second Dutch cohort is the ERGO (Rotterdam) study (4). All subjects are aged >55 years, and nondiabetic subjects underwent a fasting OGTT according to World Health Organization criteria. In total, 457 type 2 diabetic subjects (aged 71 ± 9 years, 44% male) and 457 glucose-tolerant control subjects (aged 64 ± 5 years, 46% male) were randomly selected from the total cohort (n = 7,983).

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 normal glucose–tolerant (NGT) subjects sampled at random through public registers at the Steno Diabetes Center and the Research Centre for Prevention and Health (5). In the group of diabetic patients (n = 706, 48% male), the age was 59 ± 10 years and 57 ± 10 years for NGT participants (n = 514, 46% male) (5).

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NGT, normal glucose tolerant; OGTT, oral glucose tolerance test; SNP, single nucleotide polymorphism.

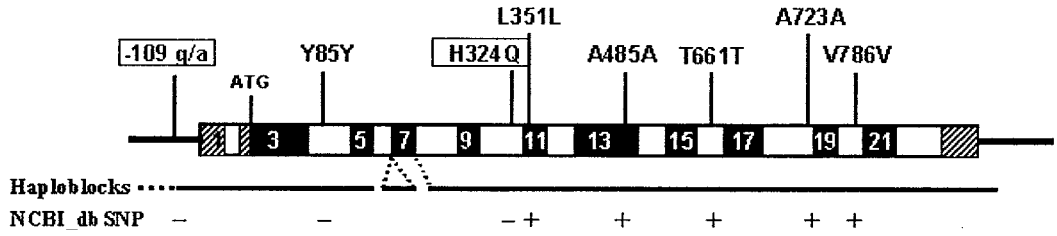

FIG. 1. Locations of SNPs in *LARS2* cDNA. Exon structure is given in black and white (noncoding [parts of] exons are shaded). Those SNPs present in the National Center for Biotechnology Information (NCBI) dbSNP database are indicated below the map. The haploblock structure is depicted below the exon structure based on HAPMAP data. Boxed SNPs are examined for association with type 2 diabetes. Corresponding dbSNP numbers are (if available) NA, NA, NA, rs7610357, rs2128361, rs11549809, rs2170549, and rs267220.

DK2 consisted of unrelated type 2 diabetic patients recruited from the Steno Diabetes Center and the Research Centre for Prevention and Health (n = 654, aged 54 ± 10 years, 42% male) and a group of unrelated NGT subjects sampled from the prospective Inter99 study at the Research Centre for Prevention and Health (n = 4,501, aged 45 ± 8 years, 47% male) (6,7). All control subjects participating in the Danish studies underwent a fasting OGTT according to World Health Organization criteria.

Type 2 diabetic subjects were significantly older in all populations (all $P \leq 0.01$). Questionnaires were used to obtain other relevant information. All participants were Caucasian whites 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.

LARS2 sequencing. The *LARS2* gene is mapped to the chromosome 3p21.3 region and spans ~160 kb. Its 22 exons encode a 903 aa protein (LocuslinkID 23395). Total RNA was isolated from leukocytes and/or pancreas tissue (n = 7) from 25 Dutch type 2 diabetic subjects using standard procedures. First-strand cDNA was made from these RNA samples using a first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA). The *LARS2* gene was amplified in two overlapping segments (primer sequences and assay conditions available on request). The resulting segments encompassing 2,905 bp of the coding region, including 159 bp of the promoter region, were subsequently sequenced on an automated sequencer (Applied Biosystems, Foster City, CA). Haplotypes of the identified single nucleotide polymorphisms (SNPs) (allele frequency, >2.5%) were calculated using the Phase Program (8).

Genotyping. Two SNPs in the coding region of the *LARS2* gene were studied in further detail for association with type 2 diabetes and related quantitative variables like circulating glucose and insulin levels during an OGTT. The first SNP (g/a) was located at position −109 in the promoter area. A 197-bp fragment surrounding the SNP was amplified using a mismatch primer. The PCR fragment was cleaved with the restriction enzyme *MspA1I*, resulting in fragments of 178 and 19 bp in the case of the wild-type sequence and 197 bp in the case of the mutant sequence after separation on a 4% agarose gel.

The second SNP is a coding SNP located in exon 10. The C to A variant changes a histidine at position 324 to glutamine (H324Q). Two different assays were developed for genotyping this SNP. The first method was a restriction fragment–length polymorphism–based method. The genomic region surrounding the SNP was amplified using a mismatch primer introducing an *MspA1I* site when the C allele is present. Primer sequences for both assays are available on request. The second genotyping method for this SNP was based on Taqman SNP genotyping technology (Applied Biosystems, Foster City, CA). Primer and probe design was performed by the manufacturer (sequences available on request), and reactions were done according to the manufacturers' protocol. This method was applied to all samples tested in this study including sufficient replication and control samples. A replication sample of ~1,500 Dutch samples using the restriction fragment–length polymorphism–based method did not identify any mismatches. Furthermore, the accuracy of both measures was further confirmed by direct sequencing of an additional 40 samples.

Overexpression and purification of wild-type and mutant leucyl tRNA synthetase. The wild-type mature enzyme of 864 amino acid residues (Δ39LeuRS) was overexpressed in the BL21-Codonplus(DE3)RIL strain as described (9). A human cDNA clone containing this His-tagged NH_2-terminally truncated LeuRS was obtained from M.P. King (10). The enzyme was purified using nickel affinity chromatography as described (9). Furthermore, we have made a construct with the H324Q polymorphism for production and purification of the mutant enzyme (cloning details available on request).

Aminoacylation and editing reactions. Aminoacylation properties of the wt and H324Q mutant enzymes were determined by measuring [^{3}H]-leucine incorporation according to a previously described method (9), using either total bovine tRNA and/or human mitochondrial RNA as the tRNA source. Furthermore, we have analyzed the mischarging rate of both enzymes using several noncognate [^{3}H]-labeled amino acids including [^{3}H]-isoleucine (11). A priori power calculations have shown that we should be able to detect differences >10–15%.

Statistical analyses. Hardy-Weinberg equilibrium was tested in all cohorts before further analysis. Differences in allele frequencies were tested by Fisher's exact tests. Since testing of the homogeneity of the population-specific odds ratios revealed no significant differences, a common odds ratio was calculated using a Mantel-Haenszel test. Differences in clinical variables were tested by linear regression analysis, with adjustment for age, sex, and BMI. Variables were log transformed before analysis, if necessary. The statistical software packages SPSS 10.0 (SPSS, Chicago, IL) and StatXact 6.0 (Cytel, Cambridge, MA) were used.

RESULTS

Sequencing of the *LARS2* cDNA, including 159 bp of the promoter region in 25 type 2 diabetic subjects, revealed eight different SNPs (Fig. 1). We did not find aberrant splicing in any of the samples investigated. Haplotype analysis using four SNPs with allele frequencies ≥2.5% suggests at least six different haplotypes (frequency ≥4%). Two variants (−109 g/a and H324Q) potentially affecting gene function were subsequently investigated in more detail. Double heterozygous subjects were not found, and haplotype and linkage disequilibrium analyses suggest that the two SNPs are present on different haplotypes and haploblocks ($D' = -1$, $r^2 = 0.013$; Fig. 1).

Genotype distributions for both SNPs in the different cohorts were all in Hardy-Weinberg equilibrium. The −109 g/a variant was tested for association in the two Dutch cohorts. Allele frequencies were 31.3 and 32.6% for control (n = 329) and type 2 diabetic (n = 215) subjects in the Hoorn study ($P > 0.5$) and 26.0 and 30.3% for control (n = 263) and type 2 diabetic (n = 200) subjects in the ERGO study ($P \geq 0.2$). Combined analysis of both data sets after testing the homogeneity of the population-based odds ratios revealed no significant difference resulted in a common odds ratio of 1.13 (95% CI 0.93–1.38, $P > 0.2$). Furthermore, we observed no association of this SNP with relevant anthropometric or biochemical variables obtained during OGTT (data not shown). There was also no obvious association with LARS2 mRNA expression levels, as measured during a real-time quantitative PCR experiment on total RNA samples from type 2 diabetic subjects (n = 25 including 8 carriers).

The H324Q variant was examined in all four populations. Allele frequencies for each of the samples are given in Table 1. Significant association between the gene variant and type 2 diabetes was present in the Hoorn cohort (P = 0.04, Table 1). Furthermore, there was a nonsignificant but identical trend toward association in all other cohorts (Table 1). Analysis of the genotype distributions

TABLE 1
Allele frequencies for the H324Q (C→A) SNP in *LARS2* in four different cohorts

Cohort	NGT		Type 2 diabetes		*P*	Odds ratio (95% CI)
	C	A	C	A		
Hoorn study	659	13 (0.019)	404	18 (0.043)	0.04	2.26 (1.10–4.66)
ERGO (Rotterdam) study	889	25 (0.027)	879	35 (0.038)	0.19	1.42 (0.84–2.39)
DK1, Steno case-control study	1,005	23 (0.022)	1,373	39 (0.028)	0.44	1.24 (0.74–2.10)
DK2, Inter99 case-control study	8,746	256 (0.028)	1,259	49 (0.037)	0.08	1.33 (0.97–1.82)
Common odds ratio	—	—	—	—	0.004	1.40 (1.12–1.76)

Data are total number (fraction of total number), unless otherwise indicated. *P* values were calculated using Fisher exact tests.

and adjustment for differences in age and sex yielded very similar results (supplementary Table 1 [available at http://diabetes.diabetesjournals.org]).

A homogeneity test revealed that there was no significant difference between the odds ratios, as obtained in the four different independent cohorts ($P > 0.5$). The common odds ratio across all four studies was 1.40 (95% CI 1.12–1.76, $P = 0.004$), suggesting that this SNP is significantly associated with type 2 diabetes in these European study samples. If we removed the Hoorn cohort, which has the largest odds ratio, from these analyses, the odds ratio was hardly affected (1.33 [95% CI 1.05–1.69, $P = 0.02$]); this was also true if we excluded any of the other cohorts. Linear regression analysis with adjustment for age, sex, and BMI revealed no consistent associations between this SNP and any of the clinical variables tested (Table 2). Furthermore, there was no evidence for an impact on mRNA expression levels (data not shown, 25 individuals tested including 3 carriers). In vitro analysis of aminoacylation and editing properties using purified wild-type and 324Q mutant LeuRS yielded identical results, indicating that in in vitro tests the aminoacylation and editing properties are not significantly affected by the polymorphism (data not shown).

DISCUSSION

We provide evidence that a coding SNP in *LARS2* associates with type 2 diabetes in a meta-analysis of four independent study populations from the Netherlands and Denmark. To our knowledge, this is the first report showing association between variation in an (mitochondrial) aminoacyl tRNA synthetase gene and disease. Previously, we have shown that a defect in the mitochondrial $tRNA^{Leu(UUR)}$ gene associates with diabetes and a defect in aminoacylation. Together, this suggests that changes in the biochemical pathways linked to mt-$tRNA^{Leu(UUR)}$ predispose to the development of glucose intolerance.

The risk associated with this gene variant is in the same order of magnitude as shown for the three well-replicated disease-associated genes: KIR6.2, peroxisome proliferator–activated receptor γ, and the Calpain10 haplotypes (odds ratio 1.2–1.4) (12), suggesting again that the contribution of predisposing genes per gene is limited and most likely only a combination of several diabetes risk genes in combination with a detrimental lifestyle results in glucose intolerance. This further implies that only studies with sufficient statistical power will be able to track down and confirm association between such variants and type 2 diabetes.

It is difficult to predict whether this His-to-Gln mutation, present at the surface of the editing domain, affects its function. The editing domain is involved in the repair of mischarged tRNAs, thereby keeping error rates in the synthesis of proteins low. Functional in vitro tests examining aminoacylation and mischarging of the tRNA leucine have not shown major differences. However, additional in vitro and in vivo studies are needed to further elucidate the precise functional impact of this protein variant. Since

TABLE 2
Clinical characteristics of NGT subjects in the different study populations in relation to LARS2 $His^{324}Gln$ polymorphism

	Hoorn study		ERGO study		DK2, Inter99 study	
	HH	HQ + QQ	HH	HQ + QQ	HH	HQ + QQ
n	323	13	432	25	4,248	253
Age (years)	60 ± 7	61 ± 5	64 ± 5	66 ± 5*	45.2 ± 7.8	44.6 ± 7.7
Sex (male/female)	128/195	4/9	238/194	11/14	1,964/2,284	130/123
BMI (kg/m^2)	26.1 ± 3.0	25.7 ± 2.6	25.9 ± 3.2	25.2 ± 4.1	25.5 ± 4.1	25.3 ± 3.7
HbA_{1c} (%)	5.5 ± 0.4	5.2 ± 0.4	5.8 ± 0.5	5.8 ± 0.7	5.8 ± 0.4	5.7 ± 0.4
Fasting glucose (mmol/l)	5.4 ± 0.5	5.1 ± 0.4	5.4 ± 0.4	5.4 ± 0.4	5.3 ± 0.4	5.3 ± 0.4
2-h glucose (mmol/l)	5.2 ± 1.3	5.1 ± 1.3	5.3 ± 1.1	5.4 ± 1.1	5.5 ± 1.1	5.5 ± 1.2
Fasting insulin (pmol/l)	79 ± 36	71 ± 16	69 ± 34	69 ± 32	37 ± 23	39 ± 24
2-h insulin (pmol/l)	NA	NA	60 ± 50	77 ± 56	168 ± 131	164 ± 124
HOMA-IR	19.0 ± 9.8	16.2 ± 3.7	17.0 ± 8.8	16.9 ± 9.0	8.9 ± 5.7	9.2 ± 5.7
Cholesterol (mmol/l)	6.6 ± 1.1	6.4 ± 1.1	6.6 ± 1.0	6.3 ± 1.2	5.4 ± 1.0	5.3 ± 1.0
HDL (mmol/l)	1.33 ± 0.34	1.50 ± 0.65	1.34 ± 0.33	1.31 ± 0.37	1.46 ± 0.40	1.40 ± 0.37†
Triglycerides (mmol/l)	1.41 ± 0.67	1.38 ± 0.58	1.48 ± 0.62	1.70 ± 1.03	1.19 ± 0.95	1.14 ± 0.79
Free fatty acids (mmol/l)	0.58 ± 0.21	0.55 ± 0.17	0.29 ± 0.09	0.34 ± 0.12*	NA	NA

Data are means ± SD. Three QQ homozygotes were detected in the DK2 cohort. *P* values were obtained after general linear regression (SPSS), with age and BMI as covariates and sex and genotype as fixed factors. *$P \leq 0.01$, †$P \leq 0.05$. HOMA-IR, homeostasis model assessment of insulin resistance.

we have studied only two SNPs in detail, it might be that the H324Q variant is merely reflecting linkage disequilibrium with another unknown causative variant in this gene or a nearby-located gene at the same locus. Further, more detailed studies of this gene locus will be needed to replicate and fully examine the nature of the observed association between the H324Q variant and type 2 diabetes.

In conclusion, we report the association of an amino acid polymorphism in the leucyl tRNA synthetase gene and type 2 diabetes in a combined analysis of four independent study populations in the Netherlands and Denmark. To our knowledge, this is the first report showing association between variation in an aminoacyl tRNA synthetase gene and disease, and it further highlights the importance of correct mitochondrial function in glucose homeostasis (1,13–15).

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REFERENCES

1. van den Ouweland JMW, Lemkes HHPJ, Ruitenbeek W, Sandkuyl LA, de Vijlder MF, Struyvenberg PAA, van de Kamp JJP, Maassen JA: Mutation in the mitochondrial $tRNA^{LEU(UUR)}$ gene in a large pedigree with maternally transmitted type II diabetes mellitus and deafness. *Nature Genet* 1:368–371, 1992
2. Maassen JA, 't Hart LM, van Essen E, Heine RJ, Nijpels G, Jahangir Tafrechi RS, Raap AK, Janssen GM, Lemkes HH: Mitochondrial diabetes: molecular mechanisms and clinical presentation. *Diabetes* 53 (Suppl. 1): S103–S109, 2004
3. Mooy JM, Grootenhuis PA, de Vries H, Valkenburg HA, Bouter LM, Kostense PJ, Heine RJ: Prevalence and determinants of glucose intolerance in a Dutch caucasian population: the Hoorn study. *Diabetes Care* 18:1270–1273, 1995
4. Hofman A, Grobbee DE, de Jong PTVM, van de Ouweland FA: Determinants of disease and disability in the elderly: the Rotterdam Elderly Study. *Eur J Epidemiol* 7:403–422, 1991
5. Drivsholm T, Ibsen H, Schroll M, Davidsen M, Borch-Johnsen K: Increasing prevalence of diabetes mellitus and impaired glucose tolerance among 60-year-old Danes. *Diabet Med* 18:126–132, 2001
6. Glumer C, Jorgensen T, Borch-Johnsen K: Prevalences of diabetes and impaired glucose regulation in a Danish population: the Inter99 study. *Diabetes Care* 26:2335–2340, 2003
7. Jorgensen T, Borch-Johnsen K, Thomsen TF, Ibsen H, Glumer C, Pisinger C: A randomized non-pharmacological intervention study for prevention of ischaemic heart disease: baseline results Inter99. *Eur J Cardiovasc Prev Rehabil* 10:377–386, 2003
8. Stephens M, Smith NJ, Donnelly P: A new statistical method for haplotype reconstruction from population data. *Am J Hum Genet* 68:978–989, 2001
9. Sohm B, Frugier M, Brule H, Olszak K, Przykorska A, Florentz C: Towards understanding human mitochondrial leucine aminoacylation identity. *J Mol Biol* 328:995–1010, 2003
10. Park H, Davidson E, King MP: The pathogenic A3243G mutation in human mitochondrial tRNALeu(UUR) decreases the efficiency of aminoacylation. *Biochemistry* 42:958–964, 2003
11. Chen JF, Guo NN, Li T, Wang ED, Wang YL: CP1 domain in Escherichia coli leucyl-tRNA synthetase is crucial for its editing function. *Biochemistry* 39:6726–6731, 2000
12. McCarthy MI: Progress in defining the molecular basis of type 2 diabetes mellitus through susceptibility-gene identification. *Hum Mol Genet* 13:R33–R41, 2004
13. Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstrale M, Laurila E, Houstis N, Daly MJ, Patterson N, Mesirov JP, Golub TR, Tamayo P, Spiegelman B, Lander ES, Hirschhorn JN, Altshuler D, Groop LC: PGC-1 alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nature Genet* 34:267–273, 2003
14. Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, Kashyap S, Miyazaki Y, Kohane I, Costello M, Saccone R, Landaker EJ, Goldfine AB, Mun E, Defronzo R, Finlayson J, Kahn CR, Mandarino LJ: Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: potential role of PGC1 and NRF1. *Proc Natl Acad Sci USA* 100:8466–8471, 2003
15. Petersen KF, Dufour S, Befroy D, Garcia R, Shulman GI: Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. *N Engl J Med* 350:664–671, 2004