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ARTICLE

Genetic association analysis of *LARS2* with type 2 diabetes

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Abstract
Aims/hypothesis LARS2 has been previously identified as a potential type 2 diabetes susceptibility gene through the low-frequency H324Q (rs71645922) variant (minor allele frequency [MAF] 3.0%). However, this association did not achieve genome-wide levels of significance. The aim of this study was to establish the true contribution of this variant and common variants in *LARS2* (MAF>5%) to type 2 diabetes risk.

Methods We combined genome-wide association data (n=10,128) from the DIAGRAM consortium with independent data derived from a tagging single nucleotide polymorphism (SNP) approach in Dutch individuals (n=999) and took forward two SNPs of interest to replication in up to 11,163 Dutch participants (rs17637703 and rs952621). In addition, because inspection of genome-wide association study data identified a cluster of low-frequency variants with evidence of type 2 diabetes association, we attempted

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replication of rs9825041 (a proxy for this group) and the previously identified H324Q variant in up to 35,715 participants of European descent.

Results No association between the common SNPs in *LARS2* and type 2 diabetes was found. Our replication studies for the two low-frequency variants, rs9825041 and H324Q, failed to confirm an association with type 2 diabetes in Dutch, Scandinavian and UK samples (OR 1.03 [95% CI 0.95–1.12], p=0.45, n=31,962 and OR 0.99 [0.90–1.08], p=0.78, n=35,715 respectively).

Conclusions/interpretation In this study, the largest study examining the role of sequence variants in *LARS2* in type 2 diabetes susceptibility, we found no evidence to support previous data indicating a role in type 2 diabetes susceptibility.

Keywords Genetics · *LARS2* · Mitochondria · SNP · Type 2 diabetes

Abbreviations

DK1	Denmark sample 1
FL1	Finland sample 1
FL2	Finland sample 2
GWAS	Genome-wide association study
MAF	Minor allele frequency
NGT	Normal glucose tolerance
NHS	New Hoorn Study
NL1	The Hoorn Study
NL2	Second Dutch sample
NL3	The Breda study
NL4	ERGO Study
SE1	Sweden sample 1
SNP	Single nucleotide polymorphism
UK1	UK sample 1
UK2	UK sample 2
WTCCC	Wellcome Trust Case Control Consortium

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Introduction

Changes in mitochondrial function are observed in patients with type 2 diabetes and their first-degree relatives. Previous studies have indicated that genes involved in oxidative phosphorylation are downregulated in the muscle cells of type 2 diabetes patients [1]. Furthermore, the muscle mitochondria from patients with type 2 diabetes have an impaired bioenergetic capacity [2]. Mitochondria also play an important role in insulin secretion and sensitivity [3, 4]. Previously, our group has shown that a mutation in the mitochondrial DNA-encoded tRNA-Leu (UUR), encoded by the *MT-TL1* gene is associated with maternally inherited diabetes and deafness [5]. In addition, an H324Q (rs71645922) variant in the nuclear encoded mitochondrial *LARS2* gene has shown an association with type 2 diabetes in work previously carried out by our group [6]. The *LARS2* gene encodes for the mitochondrial leucyl tRNA synthetase (EC 6.1.1.4), which catalyses the aminoacylation of both mitochondrial leucyl tRNAs with leucine and is therefore essential for mitochondrial protein synthesis. By analysing the coding region for the *LARS2* gene, we found the H324Q (rs71645922) variant and demonstrated an association with type 2 diabetes susceptibility in a meta-analysis of four independent cohorts from the Netherlands and Denmark (OR 1.40 [95% CI 1.12–1.76], $p=0.004$, $n=7,836$) [6].

In recent years the advent of genome-wide association studies (GWAS) and the accumulation of large data sets capable of detecting associations to levels of genome-wide significance appropriate for such studies ($p<5\times10^{-8}$) have identified close to 20 loci impacting on type 2 diabetes susceptibility. However, low-frequency variants such as H324Q are generally poorly captured by such studies. We set out therefore to re-evaluate the possible contribution of this low-frequency variant to type 2 diabetes susceptibility in appropriately sized samples. We also used a combination of publicly available (DIAGRAM consortium: www.well.ox.ac.uk/DIAGRAM/, accessed 2 May 2008) and newly derived tagging single nucleotide polymorphism (SNP) data to undertake the most comprehensive assessment of the *LARS2* locus yet performed.

Methods

Study samples The first part of our study aimed to identify common alleles associated with increased type 2 diabetes susceptibility using DIAGRAM consortium data and a tagging SNP approach. For this we genotyped several European samples.

The first sample was from the Hoorn Study (NL1) [7], a Dutch population-based study from the city of Hoorn, in the north-west of the Netherlands, from which we selected 519 participants with normal glucose tolerance (NGT) and 480 type 2 diabetes patients. Glucose tolerance was assessed using a fasting OGTT, according to 1999 WHO criteria [8]. This sample was used to analyse common variation in *LARS2* gene with a tagging SNP approach. Variants in *LARS2* identified from the DIAGRAM meta-analysis and the tagging SNP approach were then taken forward for replication in three Dutch samples.

The second Dutch sample (NL2) included 1,517 controls and 821 type 2 diabetic patients [9, 10]. The 1,517 controls were randomly selected from the New Hoorn Study (NHS), which is an ongoing, population-based study from the city of Hoorn which does not overlap with the original NL1 sample. Of the type 2 diabetes patients, 147 were from the NHS and the remainder ($n=674$) were recruited from the diabetes clinics of the Leiden University Medical Centre and the Vrije Universiteit medical centre, Amsterdam. All participants in this replication sample were Dutch whites. All NGT participants underwent an OGTT and were classified according to WHO criteria [8].

The third replication sample was ascertained from the Breda study (NL3) [11, 12]. This is a case–control study from the city of Breda, in the south of the Netherlands. The 920 controls were from the Dutch blood bank and self-reported a non-diabetic state. The 501 cases had type 2 diabetes diagnosed on the basis of WHO criteria [8].

For the fourth replication sample we selected 5,183 NGT participants and 1,222 type 2 diabetes patients from the population-based ERGO study (NL4) from Rotterdam in the south-west region of the Netherlands [13].

In total 8,139 controls and 3,024 type 2 diabetes patients were included in our replication study in the Netherlands.

The second part of this study was focused on the follow up of two low-frequency variants in *LARS2*, for which we carried out replication in samples from the Netherlands (NL1–NL4) as well as samples from the UK (UK sample 1 [UK1], UK sample 2 [UK2]), Denmark (Denmark sample 1 [DK1]), Finland (Finland sample 1 [FL1], Finland sample 2 [FL2]) and Sweden (Sweden [SE1]).

Our DK1 sample [14] consisted of 514 NGT controls randomly selected from public registers at the Steno Diabetes Center and the Research Centre for Prevention and Health, Copenhagen, Denmark. The 706 type 2 diabetes patients were recruited from the Steno Diabetes Center. NGT participants underwent an OGTT according to WHO criteria [8].

Of the two UK samples, the first (UK1) was the United Kingdom Type 2 Diabetes Genetics Consortium case–control sample, comprising 4,124 type 2 diabetes patients and 5,126 controls ascertained in Tayside, Scotland. Details of the ascertainment scheme and recruitment criteria for this sample have been described elsewhere [15, 16]. The enlarged sample used here represents continuing recruit-

ment to this resource under precisely the same criteria. The second sample, UK2, consisted of 1,853 type 2 diabetes patients ascertained as part of the BDA Warren 2 collection (Exeter, London, Oxford, Norwich and Newcastle) and 10,220 control samples. The latter represent the full British 1,958 Birth Cohort (n=7,133) and the United Kingdom Blood Services Collection of Common Controls (n=3,087), a subset of which featured in the Wellcome Trust Case Control Consortium (WTCCC) genome-wide association scan (both samples were collected throughout the UK) [15, 16].

Finally, we included samples from Finland and Sweden. The FL1 sample was a case–control sample from the Botnia region of Finland, consisting of 353 controls and 402 type 2 diabetes patients. The sample from Sweden, SE1, was from a case–control study from Skara and Malmö, and consisted of 468 controls and 480 type 2 diabetes patients. We also included a set of trios originating from the Botnia region of Finland. This sample, FL2, consisted of 211 probands (multiple diabetic sibs) and 370 parents [17, 18]. All study samples are summarised in Table 1.

In total 25,191 controls and 10,800 type 2 diabetes patients were included for follow up of the low-frequency variants.

All studies were approved by the appropriate medical ethical committees and were in accordance with the principles of the Declaration of Helsinki. All participants provided written, informed consent for this study.

Common SNP selection Common SNPs (minor allele frequency [MAF] >5%) in the *LARS2* locus were selected for follow-up based on data from the DIAGRAM meta-analysis (gene boundaries chr3: 45373001 ... 45698001) [19]. SNPs with a p<0.05 were genotyped in the Dutch replication samples (NL1-NL4). Furthermore, tagging SNPs in *LARS2* were selected for genotyping in the NL1 sample using the HapMap database and Tagger software [20, 21] (selection criteria and SNPs shown in Electronic supplementary material [ESM] Table 1).

Genotyping and quality control SNPs selected for follow-up in our replication samples were genotyped using Taqman SNP genotyping assays (Applied Biosystems, Foster City, CA, USA). Tagging SNPs were genotyped in the NL1 sample using the Sequenom platform (Sequenom, San Diego, CA, USA). Assays showing overlapping clusters, success rates below 95% or not obeying Hardy–Weinberg equilibrium (p<0.05) were excluded from analysis. Duplicate samples (~5%) showed complete concordance.

Statistical analysis Differences in genotype distribution and allele frequencies were analysed using a χ^2 test. Odds ratios were calculated using an additive model, which was the best fit for the data. Homogeneity of ORs between the different samples was calculated with a Tarone's test, after which a common OR was calculated with a Mantel–Haenszel test using a fixed effects model. Results from OGTT (only NGT participants) were analysed with univariate analysis of variance, using additive, recessive and dominant models, and correction for age, BMI and sex as possible confounders. Association was assessed by the transmission disequilibrium test in the Botnia trios. All general statistics were calculated using SPSS 16.0 (SPSS, Chicago, IL, USA). For statistics involving the geographical distribution of the H324Q (rs71645922) variant in the UK population (described below), we used StatXact v 6.0 (Cytel Software, Cambridge, MA, USA).

Power calculations were performed using Quanto [22]. From the DIAGRAM consortium meta-analysis of common variants we selected for replication all common SNPs with a p<0.05. At this alpha, the DIAGRAM consortium meta-analysis had at least 80% power to detect a variant with OR≥1.20 (MAF>0.05) [22]. Combined with our Dutch

Table 1 Description of study samples

Study	Participants, n (% male)		Mean age, years (SD)		Mean BMI , kg/m^2 (SD)	
	Controls	Cases	Controls	Cases	Controls	Cases
NL1	519 (55)	480 (52)	65 (8)	67 (8)	26.4 (4.5)	28.8 (4.6)
NL2	1,517 (44)	821 (50)	53 (7)	61 (11)	25.5 (3.6)	29.0 (4.6)
NL3	920 (61)	501 (46)	48 (13)	71 (10)	n.a.	27.8 (4.1)
NL4	5,183 (41)	1,222 (39)	69 (9)	73 (9)	26.0 (3.9)	27.4 (4.0)
DK1	514 (46)	706 (48)	57 (10)	59 (10)	25.9 (3.8)	29.3 (5.1)
UK1	5,126 (51)	4,124 (55)	60 (13)	66 (6)	26.9 (11.4)	31.2 (13.8)
UK2	10,220 (50)	1,853 (61)	42 (7)	57 (9)	27.2 (6.4)[a]	31.8 (6.7)
FL1	353 (53)	402 (55)	60 (10)	61 (10)	26.1 (3.6)	28.7 (4.5)
FL2	370 (50)[b]	211 (47)[c]	n.a.	40 (9)	28.5 (5.5)	n.a.
SE1	468 (52)	480 (53)	66 (12)	67 (11)	27.5 (4.1)	27.9 (4.1)

[a] Based on the British 1958 Birth Cohort (n=7,133) and Panel 2 of the United Kingdom Blood Services Collection of Common Controls (n=1,643)

[b] Parents

[c] Probands

n.a., not available

replication sample, we had at least 80% power to replicate the association of a variant with an OR≥1.09 at the observed MAFs of 0.19 (rs952621) and 0.24 (rs17637703) respectively (α=0.05 or OR≥1.12 at $\alpha=10^{-4}$). Power of the tagging SNP approach in NL1 was limited (80% power to detect a variant with an OR≥1.6 [α=0.05, MAF=0.05] or OR≥1.45 at the observed lowest MAF of 0.10). Therefore we replicated in NL2-NL4 only our strongest signal from the NL1 sample (rs17637703, p=0.07).

While extensive GWAS have indicated that the effect sizes of common variants influencing type 2 diabetes risk are modest, the potential remains for low-frequency variants to have effects on type 2 diabetes risk that are more substantial, which was corroborated by our previous observation regarding the H324Q variant [6]. Power calculations at the start of the project demonstrated that we had at least 99% power to detect an effect size similar to our initial finding for H324Q (rs71645922) (OR 1.4) and at least 80% power to detect an OR of 1.13 (α=0.05) [6]. From the DIAGRAM meta-analysis we used an alpha of 0.05 to select other low-frequency SNPs for replication. At this alpha, the power in DIAGRAM was 80% to detect association for variants with ORs ranging from 1.24 (MAF=0.03) to 1.45 (MAF=0.01 and α=0.05). For replication of the two low-frequency variants (observed MAFs ~0.03 [H324Q, rs71645922] and ~0.05 [rs9825041] respectively), we had in our complete replication sample at least 80% power to detect an OR≥1.13 (25,191 controls and 10,800 type 2 diabetes patients, α=0.05).

Results

Common LARS2 variants in available DIAGRAM GWAS data We analysed the data from the DIAGRAM GWAS meta-analysis [19] for the *LARS2* gene (100% coverage [MAF>5%], according to HapMap phase 2, April 2007, Centre d'Etude du Polymorphisme [Utah residents with northern and western European ancestry] [CEU] population) and observed one common SNP (rs952621, directly typed) showing weak evidence of association with type 2 diabetes (OR 1.11 [95% CI 1.02–1.20], p=0.01 for the T allele). This SNP was also captured in our complementary tagging SNP approach (NL1), with an OR of 1.13 [95% CI 0.89–1.43], p=0.33 for the same allele. However, additional genotyping in the Dutch samples (NL2, NL4) and meta-analysis of all data resulted in a common OR of 1.05 (0.99–1.11, p=0.13, n=19,870). As there was no convincing evidence of association in our samples, this SNP was not analysed further.

No other common SNP in *LARS2* showed evidence of association with type 2 diabetes in the GWAS data. The same was true of the tagging SNP analysis conducted in the NL1 sample (ESM Table 1). In the latter analysis, rs17637703 showed weak evidence of association (OR 1.22 [95% CI 0.99–1.50], p=0.07), but this was not confirmed in the Dutch replication samples (common OR 0.98 [95% CI 0.91–1.06], p=0.62, n=10,087), in line with the DIAGRAM result for this SNP (OR 1.02 [0.94–1.10]).

Low-frequency variants in LARS2 In addition to the common variants, the DIAGRAM meta-analysis also captured fourteen low-frequency SNPs (0.01>MAF<0.05) within the *LARS2* gene, ten of which are in high linkage disequilibrium with each other (r^2>0.95 according to HapMap) (ESM Fig. 1) and showed some evidence for association with type 2 diabetes (ORs 1.17–1.21; p 0.02–0.05). We selected rs9825041 (OR 1.20 [95% CI 1.03–1.39], p=0.02) as a proxy for the group for genotyping in the replication samples, but no association with type 2 diabetes was observed (Table 2). Homogeneity of ORs was tested with a Tarone's test (p=0.67) and we calculated a common OR across all studies of 1.03 (95% CI 0.95–1.12), p=0.45 (8,959 type 2 diabetic patients, 23,003 controls).

Follow up of the H324Q (rs71645922) variant in LARS2 Finally, we examined the association of the H324Q (rs71645922) variant with type 2 diabetes in our replication samples NL2 to NL4, UK1 and UK2, SE1 and FL1. This variant was not captured by the GWAS and was not captured by any of the SNPs mentioned above (r^2< 0.17). Participants in the Dutch replication samples included in our original study of this variant were excluded from analysis (n=914 from NL4 study). The replication samples did not confirm our previously observed association. A meta-analysis of all available studies including our previous data from the Netherlands (NL1) and Denmark (DK1, DK2) [6] resulted in an overall OR of 0.99 (95% CI 0.90–1.08), p=0.78, n=35,715 participants (10,399 type 2 diabetes patients) (Table 2). In addition, no significant excess of transmission of the risk allele was observed in the Botnia trios (FL2, transmitted/untransmitted=18/14, OR 1.29 [95% CI 0.64–2.59], p=0.48).

To investigate possible heterogeneity between the studies, we performed several analyses. For age stratification we created, based on the age distribution in the Dutch samples, the following age strata: ≤60 years, 61 to 70 years and >70 years. A decreased frequency of the risk allele was observed in type 2 diabetes participants with increasing age in most but not all samples (data not shown, available on request), but this did not reach statistical significance. We also looked at age at diagnosis of type 2 diabetes and allele frequencies in those with early-onset diabetes (≤45 years) and in those with age at diagnosis above 45 years. Although the allele frequency was slightly higher in those with early-onset diabetes, this was not statistically significant, nor was the age at diagnosis in carriers and non-

Table 2 Genotyping results for rs9825041 and H324Q (rs71645922)

Sample	Controls/cases	rs9825041				H324Q (rs71645922)			
		MAF controls (%)	MAF cases (%)	OR (95% CI)	p_{Add}	MAF controls (%)	MAF cases (%)	OR (95% CI)	p_{Add}
NL1	519/480	5.5	7.3	1.35 (0.92–1.98)	0.15	1.9	4.3	2.26 (1.10–4.66)[a]	0.04
NL2	1,517/821	6.0	6.6	1.11 (0.85–1.44)	0.46	3.4	2.7	0.78 (0.49–1.25)	0.18
NL3	920/501	5.7	6.8	1.21 (0.88–1.66)	0.25	3.3	3.0	0.90 (0.57–1.41)	0.74
NL4	5,183/1,222	6.1	5.5	0.89 (0.74–1.08)	0.27	3.1	3.5	1.11 (0.84–1.47)[a]	0.47
DK1	514/706	4.2	4.7	1.12 (0.75–1.68)	0.62	2.2	2.8	1.24 (0.74–2.09)[a]	0.44
DK2	4,501/654	n.m.	n.m.	n.m.	n.m.	2.8	3.7	1.33 (0.97–1.82)[a]	0.08
UK1	5,126/4,124	4.9	4.9	1.00 (0.87–1.14)	1.00	4.8	4.4	0.91 (0.79–1.05)	0.19
UK2	10,220/1,853	4.8	5.1	1.05 (0.88–1.27)	0.57	3.6	3.4	0.94 (0.77–1.15)	0.59
FL1	353/402	n.m.	n.m.	n.m.	n.m.	4.2	4.9	1.18 (0.72–1.93)	0.51
SE1	468/480	n.m.	n.m.	n.m.	n.m.	4.5	4.2	0.94 (0.59–1.48)	0.79
Meta-analysis				1.03 (0.95–1.12)	0.48			0.99 (0.90–1.08)	0.82
DIAGRAM GWAS[b]				1.20 (1.03–1.39)	0.02			NM	NM

[a] Data from previously published results [6]; results from NL4 study are partially from this previous research (n=914)

[b] Meta-analysis of the DIAGRAM consortium GWAS, n=10,128 (4,549 type 2 diabetic patients, 5,579 controls) [19]

n.m., not measured

p_{Add}, p value, additive model

carriers (all $p>0.05$, data not shown). Stratification for sex and BMI (where data available) did not affect the outcome in the Dutch studies (NL1-NL4, data not shown) and was therefore not further investigated.

H324Q (rs71645922) shows marked variation in MAF between the various European-descent samples examined (control MAF ranges from 1.9 to 4.8%). In the two large UK control samples, for example, there was a highly significant ($p=5\times10^{-7}$, using an exact implementation of the Cochran–Armitage trend test) difference in allele frequencies between UK1 (recruited exclusively in Scotland) and UK2 (recruited throughout the UK), which made us consider the possibility that this variant showed variation in allele frequency along the south–north cline as previously described in the WTCCC study and others [16, 23–26]. To test this, we used information on the region of ascertainment that was available from the UK 1958 Birth Cohort and UK Blood Service, and analysed genotype frequencies based on subdivisions of the UK into four major regions, namely (1) Scotland, (2) northern England, (3) Midlands and (4) southern England (see ESM Fig. 2). We found some evidence (ESM Fig. 2) for a north–south gradient across the UK (MAF 4.66%, 3.41%, 3.31% and 3.28% from north to south respectively, with $p=0.038$, calculated using the Jonckheere–Terpstra Test [StatXact]). No such MAF gradient was observed in other European samples (ESM Fig. 3)

Discussion

We found no evidence of common SNPs in *LARS2* being associated with type 2 diabetes in our samples. We therefore conclude that it is unlikely that common SNPs in *LARS2* are associated with type 2 diabetes susceptibility.

Several low-frequency SNPs, all in high linkage disequilibrium with each other ($r^2>0.95$), showed nominal evidence of association with type 2 diabetes in the DIAGRAM meta-analysis. However, we have been unable to confirm this association in our large replication samples from the Netherlands, Denmark and the UK (n=31,962). We therefore conclude that the nominal p values observed in the GWAS are most likely to be consistent with statistical noise.

The previously observed association of the H324Q (rs71645922) variant in *LARS2* with type 2 diabetes was not confirmed in our replication samples (Table 2). Power calculations at the start of the project demonstrated that we had at least 99% power to detect an effect size similar to our initial finding for H324Q (rs71645922) (OR 1.4) and at least 80% power to detect an OR of 1.13. Before excluding the previous association as false, we considered the possibility of heterogeneity, but found no evidence that age, age at diagnosis, BMI and sex were responsible. Another possibility, raised by the evidence for clinal variation in H324Q MAF across the UK, is that the previous

association in Dutch and Danish participants reflected the effects of hidden population structure. However, it seems unlikely that population stratification effects were responsible for the original reports of H324Q (rs71645922) associations, as the cases and controls in that study were recruited from the same relatively narrow geographic regions within the Netherlands and Denmark [6]. Also in our additional Dutch replication cohorts we could not detect a MAF gradient across the country. However, there are differences in MAF between different countries (ESM Figs 2 and 3). Migration patterns in the UK appear to reflect an increase in the MAF of H324Q (rs71645922) and therefore this may be a potential migration marker. However, this needs to be demonstrated in other populations. Since stratification for BMI and sex did not affect our result, we can exclude the possibility that these variables confounded our observation. The reason for the discrepancy between our first and current study is likely to be chance.

Three other low-frequency non-synonymous SNPs are present in the *LARS2* locus: K727N (rs36054230), E831D (rs9827689) and E868K (rs34965084). However, according to the dbSNP database and our own sequencing efforts [6], these SNPs are only identified in the African population and not polymorphic in the European population. Therefore, these additional non-synonymous variants were not analysed in this study. As our study did not include a thorough resequencing of the complete *LARS2* locus, we cannot fully exclude that other, as yet unknown low-frequency variants are present and associated with type 2 diabetes. Results from the 1,000 genomes project (www.1000genomes.org) should facilitate a thorough investigation of low-frequency SNPs in *LARS2* in the future.

In conclusion, our findings do not support the hypothesis that common variants in *LARS2* are major type 2 diabetes susceptibility factors. We have also conducted one of the largest (up to 35,715 participants) replication studies for two low-frequency variants for type 2 diabetes susceptibility. These variants also do not play a significant role in type 2 diabetes susceptibility. We therefore conclude that currently known genetic variation in *LARS2* does not play an important role in type 2 diabetes susceptibility.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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