

ARTICLE

Genetic association analysis of 13 nuclear-encoded mitochondrial candidate genes with type II diabetes mellitus: the DAMAGE study

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Mitochondria play an important role in many processes, like glucose metabolism, fatty acid oxidation and ATP synthesis. In this study, we aimed to identify association of common polymorphisms in nuclear-encoded genes involved in mitochondrial protein synthesis and biogenesis with type II diabetes mellitus (T2DM) using a two-stage design. In the first stage, we analyzed 62 tagging single nucleotide polymorphisms (SNPs) in the Hoorn study ($n = 999$ participants) covering all common variation in 13 biological candidate genes. These 13 candidate genes were selected from four clusters regarded essential for correct mitochondrial protein synthesis and biogenesis: aminoacyl tRNA synthetases, translation initiation factors, tRNA modifying enzymes and mitochondrial DNA transcription and replication. SNPs showing evidence for association with T2DM were measured in second stage genotyping ($n = 10164$ participants). After a meta-analysis, only one SNP in *SIRT4* (rs2522138) remained significant ($P = 0.01$). Extending the second stage with samples from the Danish Steno Study ($n = 1220$ participants) resulted in a common odds ratio (OR) of 0.92 (0.85–1.00), $P = 0.06$. Moreover, in a large meta-analysis of three genome-wide association studies, this SNP was also not associated with T2DM ($P = 0.72$). In conclusion, we did not find evidence for association of common variants in 13 nuclear-encoded mitochondrial proteins with T2DM.

European Journal of Human Genetics (2009) 17, 1056–1062; doi:10.1038/ejhg.2009.4; published online 11 February 2009

Keywords: type II diabetes mellitus; candidate gene; SNP; mitochondria

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Received 22 July 2008; revised 9 January 2009; accepted 14 January 2009; published online 11 February 2009

Introduction

Mutations in genes involved in mitochondrial protein synthesis have been found to associate with a diabetic state. The 3243A>G mutation in the mitochondrial tRNA

(Leu, UUR) gene, which impairs mitochondrial protein synthesis, is strongly diabetogenic, as are some additional mutations in mtDNA-encoded tRNA genes.^{1,2} Recently, we found that an H324Q variant in the nuclear-encoded mitochondrial leucyl tRNA synthetase (*LARS2*) results in an increased type II diabetes mellitus (T2DM) susceptibility.³ It seems that a change in the activity of mitochondrial protein synthesis makes the organism more vulnerable to develop glucose intolerance.

A change in mitochondrial protein synthesis is expected to result in an unbalance in the stoichiometry of the proteins composing the respiratory chain. Some proteins of the respiratory chain are synthesized through the mitochondrial protein synthesis machinery, whereas the others are synthesized in the cytosol and imported into the mitochondrion. Therefore, impaired activity of the mitochondrial protein synthesis machinery is expected to result in an unbalance between the nuclear DNA- and mitochondrial DNA-encoded proteins of the respiratory chain. A similar situation of an unbalance may arise when the rate of mitochondrial biogenesis is altered.

In T2DM, a reduced activity of the respiratory chain is seen, which is also present in the first degree relatives.^{4,5} The biochemical basis of this phenomenon may arise through an unbalanced expression of the various proteins. On the basis of these considerations, we hypothesized that polymorphisms in genes involved in mitochondrial protein synthesis and mitochondrial biogenesis may alter the balanced expression of proteins of the respiratory chain. As a result, these polymorphisms are expected to modify the risk for T2DM. It is estimated that the complete mitochondrial proteome consists of approximately 1500 proteins, which are mostly encoded by the nuclear genome.⁶ Common variations in genes encoded by the mitochondrial genome are not associated with the onset of T2DM.^{7,8} Based on a literature study, we selected 13 candidate genes divided in several clusters. These candidates are all nuclear genes encoding for mitochondrial proteins.

Owing to the previous association of *LARS2* with T2DM, the first cluster consisted of the mitochondrial aminoacyl-tRNA synthetases *DARS2*, *TARS2* and *IARS2*. *DARS2* and *TARS2* are located on a widely replicated T2DM linkage locus on chromosome 1q.⁹ Furthermore, variants in the tRNA^{Leu} gene were shown to be associated with metabolic disorders.¹⁰

The second cluster consisted of the two mitochondrial translation initiation factors, *MTIF2* and *MTIF3*. These two genes are involved in initiation of the mitochondrial protein synthesis and their dysfunction could lead to altered expression of mitochondrial proteins, and subsequently to oxidative stress and late-onset disease.^{11–13} We have selected them above other important translation factors, like *TUFM*, *TFSM* and *GFMI*, because mutations in the latter have already been associated with severe early-onset disease.¹⁴

The third cluster consisted of *MTO1*, *TRMU* and *GTPBP3*, which encode tRNA modifying enzymes. Variation in these genes could lead to dysfunctional enzymes and subsequently result in impaired tRNA modification and mitochondrial disease.^{15–17}

In our fourth cluster, we included *TFAM* and *PPRC1*, which are involved in transcription and replication of the mitochondrial genome. It has been shown earlier that a low mitochondrial copy number is associated with T2DM, although this is not confirmed in all studies.^{18,19} *TFAM* is one of the main regulators of mitochondrial transcription and replication, and thus of the mitochondrial copy number.^{20,21} *PPRC1* is a *PPARGC1A*-related gene and involved in mitochondrial biogenesis.²²

In addition, we selected three genes, which did not fit in a specific cluster but may affect mitochondrial biogenesis and function. *SIRT3* and *SIRT4*, which are Sir2 homologs, are involved in mitochondrial function and insulin secretion.^{23,24} Moreover, *SIRT3* has been associated with longevity.²⁵ *GPAM* is the final candidate gene, which is involved in triacylglycerol and phospholipid synthesis. *GPAM* knockout mice show hyperinsulinaemia and reduced glucose tolerance.^{26,27} Therefore, we hypothesized that defects in *GPAM* could lead to insulin resistance and subsequently to T2DM.

Dysfunction of these 13 candidate genes potentially affects mitochondrial function and may therefore increase T2DM susceptibility. Therefore, the aim of this study was to analyze the association of common variants in these gene loci with T2DM, using a tagging single nucleotide polymorphism (SNP) approach.

Materials and methods

Single nucleotide polymorphism selection

Tagging SNPs were selected using the HapMap database (www.HAPMAP.org) and Tagger.^{28,29} The threshold for the minor allele frequency (MAF) was set at 0.05. Using Tagger, 62 SNPs were selected, which cover all common variation in the selected genes according to HapMap data (phase 2, April 2007 (Build 36), population CEU, $r^2 > 0.8$). Aggressive tagging with two- and three-marker tests was used. This resulted in four multimarker tests. The remaining tests were all single marker tests. Gene boundaries include 5' and 3'-UTR regions. Details about the SNP selection for the individual genes, including gene boundaries, are summarized in Table 1.

Genotyping and quality control

A two-stage design was used. For first-stage genotyping, the Sequenom platform (Sequenom, San Diego, CA, USA) was used. First, the allelic discrimination plots were visually observed for good clustering. Assays with bad clustering were not further analyzed. SNPs with a success rate below 95% or not obeying Hardy–Weinberg Equilibrium

Table 1 Gene boundaries and number of SNPs in the selected candidate genes

Gene	Chromosome	Gene boundaries for SNP selection	Number of SNPs (successful)	% covered (MAF > 0.05)
TARS2	1	148726544–148746371	4 (4)	100
DARS2	1	172060581–172094305	2 (1)	28
IARS2	1	218334078–218387999	6 (4)	81
MTIF2	2	55317262–55349888	7 (7)	100
MTO1	6	74228209–74267896	5 (5)	100
TFAM	10	59815181–59825901	5 (5)	100
PPRC1	10	103882777–103900078	3 (3)	100
GPAM	10	113899611–113933507	6 (6)	100
SIRT3	11	205031–226361	10 (10)	100
SIRT4	12	119224546–119235427	3 (3)	100
MTIF3	13	26907783–26922711	3 (3)	100
GTPBP3	19	17309379–17314530	4 (4)	100
TRMU	22	45109962–45131900	4 (3)	81

Gene boundaries and coverage according to HapMap data. Gene boundaries according to genome build 36. The widest gene boundaries were used when more isoforms were known.

Table 2 Descriptive statistics of the first and second stage

	First stage		Second stage	
	Controls	Cases	Controls	Cases
Participants (% male)	519 (55)	480 (52)	7620 (44)	2544 (45)
Age – years (SD)	65 (8)	67 (8)	63 (12)	68 (12)
BMI – kg/m ² (SD)	26 (5)	29 (5)	26 (4)	27 (5)
FPG – mmol/l (SD)	5.4 (0.4)	7.7 (2.2)	5.5 (0.5) ^a	8.3 (2.5) ^a

FPG, fasting plasma glucose.

First stage consists of participants from the Hoorn study.³⁰ Second stage is a pooled sample of participants originally from the NHS, Breda and ERGO studies.^{33–35}

^aAvailable for 2438 controls and 334 cases.

($P < 0.01$) were excluded from analysis. SNPs, which did not fit in the Sequenom assay or failed quality control (QC), were genotyped with Taqman SNP genotyping assays (Applied Biosystems, Foster City, CA, USA) and evaluated with the same QC guidelines. Duplicate samples (~5%) showed identical genotypes. SNPs showing evidence for association with T2DM ($P < 0.05$) were selected for second-stage genotyping. Taqman SNP genotyping assays were used for this.

Study cohorts

The first-stage genotyping was performed in the Hoorn study.³⁰ This population-based study consisted of 519 normal glucose tolerant (NGT) participants (aged 65 ± 8 years, 55% male) and 480 T2DM participants (aged 67 ± 8 years, 52% male). Glucose tolerance was tested using a fasting oral glucose tolerance test (OGTT), according to World Health Organization (WHO) criteria.³¹

For the second-stage genotyping, three cohorts from the Netherlands were pooled. The first cohort was the New Hoorn Study (NHS), which is an ongoing, second non-overlapping population-based study in Hoorn.³² From this study, we randomly selected 1517 NGT and 147 T2DM participants. All participants underwent an OGTT

according to WHO criteria.³¹ To increase power, we included 674 participants with T2DM from the diabetes clinics of the Leiden University Medical Center (LUMC, Leiden, The Netherlands) and from the Vrije Universiteit medical center (VUmc, Amsterdam, The Netherlands). All participants were Dutch caucasians. In total, the NHS sample included 1517 controls (aged 53 ± 7 years, 44% male) and 821 cases (aged 61 ± 11 years, 50% male).

The second cohort was the Breda study.^{33,34} This case–control study consisted of 920 healthy controls (according to self report) from the Dutch blood bank (aged 48 ± 13 years, 61% male). 501 cases (aged 71 ± 10 years, 46% male) were T2DM patients according to WHO criteria.³¹

The third cohort was the ERGO study from Rotterdam.³⁵ This population-based study consisted of 5183 NGT participants (aged 69 ± 9 years, 41% male) and 1222 (73 ± 9 years, 39% male) T2DM patients.

In total, for the second-stage genotyping, we used 7620 controls and 2544 T2DM cases. The characteristics of both stages are summarized in Table 2 (Characteristics of the independent cohorts are summarized in Supplementary Table S1.). For additional replication of our strongest signal, we extended the second stage with samples from the Danish Steno study.³⁶ This case–control study

consisted of 514 NGT controls (aged 57 ± 10 , 46% male) randomly selected from public registers at the Steno Diabetes Center and the Research Center for Prevention and Health. The 706 cases (aged 59 ± 10 years, 48% male) were recruited from the Steno Diabetes Center.

Statistical analysis

Differences in genotype distribution and allele frequency were analyzed using a χ^2 test. An additive model was the best fit for most of the SNPs. rs1049432 (*TFAM*) and rs4917960 (*PPRC1*) might better fit a recessive model, and were therefore also analyzed with this model. A common allelic odds ratio (OR) covering both stages was calculated in a meta-analysis using a Mantel–Haenszel test. Homogeneity of ORs between the different cohorts was tested using a Tarone's test. Results from OGTT (only NGT participants) were analyzed with univariate analysis of variance, using additive, dominant and recessive models, and correction for age, BMI and gender, as possible confounders. Power calculations were performed using Quanto.³⁷ Power to detect modest effects in the first stage was limited (80% power to detect, OR 1.45, MAF=0.1). Therefore, we did not correct for multiple testing but rather performed extensive replication of all signals from the first stage with $P < 0.05$. All statistics were calculated using SPSS 14.0 (SPSS Inc., Chicago, IL, USA).

Results

Of the 62 SNPs tested in the first-stage genotyping, 58 SNPs passed QC guidelines. Of these SNPs, we selected the top seven SNPs for second-stage genotyping ($P < 0.05$). Data from genome-wide association studies (GWAS), which became available after completion of our study, resulted in the inclusion of two additional SNPs, rs4397793 in *TFAM* and rs2792751 in *GPAM*. Compared with our data, both showed a similar effect in at least one of the publicly available databases of GWAS.^{38,39} In total, nine SNPs were selected for genotyping in the second stage (Table 3).

We analyzed the second-stage results for rs1049432 in *TFAM* and rs4917960 in *PPRC1* first with a recessive model, because that was the best fit for the first-stage data ($P_{\text{rec}} = 0.002$ and $P_{\text{rec}} = 0.07$, respectively). However, second-stage results showed no support for this model; therefore, the additive model was used for further analyses. There was no evidence of heterogeneity of ORs between the independent cohorts; therefore, we pooled all the second-stage data. A meta-analysis of both stages was performed to calculate a common OR for the nine selected SNPs (Table 3). Only the G allele of rs2522138 in *SIRT4* remained significant ($P = 0.01$), but this was mainly caused by first-stage results. To confirm the observed association, we expanded the second stage with the Danish Steno study resulting in a common OR of 0.92 (0.85–1.00), $P = 0.06$.

The C allele of rs4917960 in *PPRC1* was borderline significant after the meta-analysis (OR = 1.06 (1.00–1.13) $P = 0.06$). This association was nominal and mainly caused by first-stage data. The ORs in all independent cohorts are shown in Supplementary Table S2. Correction for age, gender and BMI (when available) did not influence the results.

Finally, we analyzed OGTT data for association of the selected genes with differences in clinical variables like glucose and insulin. Furthermore, association with BMI was assessed when the data were available. No differences were observed using different models (data not shown).

After completion of our study, a meta-analysis of three T2DM GWAS appeared.⁴⁰ The coverage of common variation in the 13 selected genes in the GWAS was between 80 and 100% except *GTPBP3*, which was covered for 50%. We compared our results with the GWAS data, using the OR-based analysis (Table 4). The G allele of rs2522138 in *SIRT4* was not associated with T2DM susceptibility in the GWAS data (OR = 1.02 (0.93–1.11), $P = 0.72$). The C allele of rs4917960 in *PPRC1* showed an OR of 1.04 (0.97–1.12), ($P = 0.23$) in the GWAS data, which is in the same direction as observed in our study (OR = 1.06 (1.00–1.13), $P = 0.06$). Two SNPs located in *IARS2* (the C alleles of rs17007135 and

Table 3 Results of first and second-stage genotyping

rs number	Gene	MAF ^a		First Stage			Second Stage		Meta-analysis	
		Controls	Cases	OR (95% CI)	P_{Add}	OR (95% CI)	P_{Add}	OR (95% CI)	P_{Add}	
rs1937 ^b	<i>TFAM</i>	0.14	0.09	0.66 (0.50–0.88)	0.005	1.00 (0.86–1.17)	0.97	0.91 (0.79–1.04)	0.17	
rs4397793	<i>TFAM</i>	0.41	0.37	0.86 (0.72–1.03)	0.10	1.04 (0.97–1.11)	0.25	1.02 (0.95–1.08)	0.62	
rs1049432	<i>TFAM</i>	0.19	0.14	0.77 (0.61–0.97)	0.03	1.00 (0.91–1.09)	0.95	0.97 (0.89–1.05)	0.39	
rs4917960	<i>PPRC1</i>	0.38	0.42	1.12 (1.00–1.43)	0.05	1.05 (0.98–1.12)	0.21	1.06 (1.00–1.13)	0.06	
rs17129583	<i>GPAM</i>	0.12	0.16	1.32 (1.02–1.70)	0.04	0.92 (0.84–1.02)	0.12	0.97 (0.88–1.06)	0.50	
rs2792751	<i>GPAM</i>	0.26	0.24	0.87 (0.71–1.06)	0.18	1.01 (0.94–1.09)	0.81	0.99 (0.93–1.06)	0.82	
rs535716	<i>SIRT3</i>	0.24	0.19	0.78 (0.63–0.96)	0.02	1.05 (0.97–1.13)	0.25	1.01 (0.94–1.09)	0.78	
rs2522138 ^c	<i>SIRT4</i>	0.18	0.13	0.69 (0.54–0.88)	0.003	0.93 (0.85–1.02)	0.13	0.92 (0.85–1.00)	0.06	
rs17085633	<i>MTIF3</i>	0.44	0.50	1.25 (1.05–1.50)	0.01	1.02 (0.97–1.09)	0.55	1.05 (0.98–1.11)	0.15	

^aMAF in first-stage genotyping (Hoorn study).

^bSecond-stage genotyping only in NHS and Breda study ($n = 3759$).

^cMeta-analysis includes Steno study.

Table 4 Comparison of the results of the T2DM GWAS meta-analysis and our replication SNPs

rs number	Gene	Meta-analysis GWAS		Meta-analysis of our study	
		OR (95% CI)	P-value	OR (95% CI)	P-value
rs1937	TFAM	1.02 (0.91–1.14)	0.76	0.91 (0.79–1.04)	0.17
rs4397793	TFAM	1.03 (0.96–1.09)	0.43	1.02 (0.95–1.08)	0.62
rs1049432	TFAM	1.02 (0.94–1.12)	0.69	0.97 (0.89–1.05)	0.39
rs4917960	PPRC1	1.04 (0.97–1.12)	0.23	1.06 (1.00–1.13)	0.06
rs17129583	GPAM	1.00 (0.92–1.09)	0.99	0.97 (0.88–1.06)	0.50
rs2792751	GPAM	1.04 (0.98–1.11)	0.22	0.99 (0.93–1.06)	0.82
rs535716	SIRT3	1.03 (0.96–1.11)	0.42	1.01 (0.94–1.09)	0.78
rs2522138	SIRT4	1.02 (0.93–1.11)	0.72	0.92 (0.85–1.00)	0.06
rs17085633	MTIF3	1.00 (0.94–1.06)	0.89	1.05 (0.98–1.11)	0.15

Meta-analysis of three GWAS from the DIAGRAM consortium, $n = 10128$ (4549 cases and 5579 controls).⁴⁰ ORs are fixed-effect results.

rs2289191) showed evidence of nominal association in the GWAS meta-analysis ($P = 6 \times 10^{-4}$ and $P = 0.003$, respectively). Unfortunately, the assays for these two SNPs failed in our study. None of the other SNPs in our 13 genes showed evidence of association in the T2DM GWAS meta-analysis.

Discussion

We analyzed 58 tagging SNPs in 13 genes involved in mitochondrial function for association with T2DM susceptibility using a two-stage design. After meta-analysis of the data of both stages, only rs2522138 in *SIRT4* remained borderline significant. However, after extension of the second stage with samples from the Steno study, this association did not remain significant.

For several gene variants measured in our study, there is substantial heterogeneity of ORs between the first and second stage, but not within each stage (Table 3 and Supplementary Table S2). Heterogeneity between the independent cohorts might be caused by the differences in the age, gender or selection criteria used, and might obscure true association (Supplementary Table S1). However, we were unable to identify the reasons for this heterogeneity in our study. Differences in LD between the measured and causal SNP are also an unlikely cause, because the study participants in our cohorts are from similar ancestry.⁴¹ In the context of testing multiple SNPs, our nominal significant results in *SIRT4* and *PPRC1* should be interpreted as consistent with statistical noise.

One of the limitations of our study is that it is statistically underpowered to detect SNPs with a very small impact or low allele frequency. Although power in the first stage alone was low, we had at least a power of 80% at an observed $MAF \geq 0.13$ to detect ORs comparable to those recently reported in GWAS (1.12–1.37) when first and second stage are combined.⁴⁰ Our negative results for these 13 genes are confirmed by the DIAGRAM GWAS meta-analysis, which has a much higher power to detect also

more modest effects.^{38–40,42} Taken together, we can exclude that common variants in the selected genes have a major impact on T2DM susceptibility. Rare variants are not covered by our approach; therefore, we cannot exclude a role for such variants. Deep sequencing and analysis of novel rare variants in large well-phenotyped cohorts should provide more insight into these important issues.

Other nuclear-encoded mitochondrial genes are also not among the top hits of reported T2DM GWAS.^{38–40,42,43} Nevertheless, earlier studies suggested that they may be involved in T2DM susceptibility.^{3,44–47} Further combined and detailed analysis of the available GWAS datasets, and analysis of copy number and so-called low-frequency intermediate-penetrance variants are necessary to fully explore the role of nuclear-encoded mitochondrial genes in the pathogenesis of T2DM.

In conclusion, we were unable to detect association of common SNPs in 13 nuclear-encoded mitochondrial candidate genes with T2DM or with related continuous traits.

Acknowledgements

This study was supported by the Netherlands Organization for Scientific Research, ZonMW RIDE program and the Dutch Diabetes Research Foundation. The Danish study was supported by grants from the Lundbeck Foundation Centre of Applied Medical Genomics for Personalized Disease Prediction, Prevention and Care (LUCAMP), the Danish Health Research Council, and The European Union (EUGENE2, Grant no. LSHM-CT-2004-512013). The authors would like to acknowledge the participants of all study cohorts for their cooperation.

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