The genetics of type 2 diabetes

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Chapter 3

Genetic association analysis of 13 nuclear encoded mitochondrial candidate genes with type 2 diabetes: the DAMAGE study

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European Journal of Human Genetics 2009 Aug 17(8):1056-62

Abstract

Aims/hypothesis

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 2 diabetes using a two stage design.

Methods

In the first stage we analyzed 62 tagging SNPs in the Hoorn study (n = 999 subjects), 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 type 2 diabetes were measured in second stage genotyping (n = 10164 subjects).

Results

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 subjects) resulted in a common 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 type 2 diabetes (p = 0.72).

Conclusion

In conclusion, we did not find evidence for association of common variants in 13 nuclear encoded mitochondrial proteins with type 2 diabetes.

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 2 diabetes 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. Impaired activity of the mitochondrial protein synthesis machinery is therefore 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 type 2 diabetes a reduced activity of the respiratory chain is seen, which is also present in first degree relatives (4;5). The biochemical basis of this phenomenon may arise through an unbalanced expression of the various proteins. Based on 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 type 2 diabetes. 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 type 2 diabetes (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.

Because of previous association of *LARS2* with type 2 diabetes the first cluster consisted of the mitochondrial aminoacyl-tRNA synthetases *DARS2*, *TARS2* and *IARS2*. *DARS2* and *TARS2* are located on a widely replicated type 2 diabetes linkage locus on chromosome 1q (9). Furthermore, variants in the $tRNA^{Ile}$ 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 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*, *TSFM* and *GFM1*, 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 all 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. Previously, it has been shown that a low mitochondrial copy number is associated with type 2 diabetes, 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 3 genes, which did not fit in a specific cluster but may affect mitochondrial biogenesis and function. *SIRT3* and *SIRT4*, which are Sir2 homologues, 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* knock out mice show hyperinsulinemia and reduced glucose tolerance (26;27). Therefore, we hypothesized that defects in *GPAM* could lead to insulin resistance and subsequently type 2 diabetes.

Dysfunction of these 13 candidate genes potentially affects mitochondrial function and may therefore increase type 2 diabetes susceptibility. Therefore, the aim of the present study was to analyze the association of common variants in these gene loci with type 2 diabetes, using a tagging SNP approach.

Materials and methods

SNP 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 2- and 3-marker tests was used. This resulted in 4 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, 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 (HWE) ($p < 0.01$) 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, USA) and evaluated with the same QC guidelines. Duplicate samples (~5%) showed identical genotypes. SNPs showing evidence for association with type 2 diabetes ($p < 0.05$) were selected for second stage genotyping. Taqman SNP genotyping assays were used for this.

Table 1. Gene boundaries and SNPs in the selected candidate genes.

Gene	Chr	Gene boundaries for SNP selection	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 are known.

Study cohorts

The first stage genotyping was performed in the Hoorn study (30). This population based study consists of 519 normal glucose tolerant (NGT) subjects (aged 65 ± 8 years, 55% male) and 480 type 2 diabetes subjects (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 type 2 diabetes subjects. All subjects underwent an OGTT according to WHO criteria (31). In order to increase power we included 674 subjects with type 2 diabetes from the diabetes clinics of the Leiden University Medical Center (LUMC, Leiden) and from the Vrije Universiteit medical center (VUmc, Amsterdam). All subjects were Dutch Caucasians. In total the NHS sample includes 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 consists of 920 healthy controls (according to self report) from the Dutch blood bank (aged 48 ± 13 years, 61% male). The 501 cases (aged 71 ± 10 years, 46% male) are type 2 diabetes patients according to WHO criteria (31).

The third cohort was the ERGO study from Rotterdam (35). This population based study consists of 5183 NGT subjects (aged 69 ± 9 years, 41 % male) and 1222 (73 ± 9 years, 39 % male) type 2 diabetes patients.

In total for the second stage genotyping we used 7620 controls and 2544 type 2 diabetes 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.

Table 2. Descriptive statistics of the first and second stage.

	First stage		Second stage	
	controls	cases	controls	cases
Subjects	519	480	7620	2544
(% male)	(55)	(52)	(44)	(45)
Age - years	65	67	63	68
(std. dev.)	(8)	(8)	(12)	(12)
BMI - kg / m^2	26	29	26	27
(std. dev.)	(5)	(5)	(4)	(5)
FPG - mmol / L	5.4	7.7	5.5	8.3
(std. dev.)	(0.4)	(2.2)	(0.5)[1]	(2.5)[1]

1) available for 2438 controls and 334 cases

First stage consists of subjects from the Hoorn study (30). Second stage is a pooled sample of subjects original from the NHS, Breda studies and ERGO (33-35).

FPG = Fasting Plasma Glucose

Statistical analysis

Differences in genotype distribution and allele frequency were analyzed using a chi-squared 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 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 normal glucose tolerant subjects) 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, 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 came available after completion of our study resulted in the inclusion of two additional SNPs, rs4397793 in *TFAM* and rs2792751 in *GPAM*. Compared to our data both showed a similar effect in at least one of the publically available databases of GWAS (38;39). In total 9 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 ($p_{rec} = 0.002$ and $p_{rec} = 0.07$ resp). However, 2nd 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 second stage data. A meta-analysis of both stages was performed in order to calculate a common OR for the 9 selected SNPs (table 3).

Only the G allele of rs2522138 in *SIRT4* remained significant (p = 0.01), but this was mainly caused by stage 1 results. In order to confirm the observed association we expanded the second stage with the Danish Steno study resulting in a common odds ratio 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 stage 1 data. The ORs in all independent cohorts are shown in supplementary table 2. 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).

Table 3. Results of first and second phase genotyping.

Rs-number	Gene	MAF [1]		1st Stage		2nd Stage		Meta-analysis	
		controls	cases	OR 95% CI	P_{Add}	OR 95% CI	P_{Add}	OR 95% CI	P_{Add}
rs1937 [2]	*TFAM*	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	*TFAM*	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	*TFAM*	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	*PPRC1*	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	*GPAM*	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	*GPAM*	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	*SIRT3*	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 [3]	*SIRT4*	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	*MTIF3*	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

1) MAF in first stage genotyping (Hoorn study).
2) Second stage genotyping only in NHS and Breda study (n = 3759).
3) Meta-analysis includes Steno study.

After completion of our study, a meta-analysis of three type 2 diabetes 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 type 2 diabetes 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, 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 rs2289191) showed evidence of nominal association in the GWAS meta-analysis ($p = 6 * 10^{-4}$ 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 type 2 diabetes GWAS meta-analysis.

Table 4. Comparison DIAGRAM 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

T2D: type 2 diabetes

Discussion

We analyzed 58 tagging SNPs in 13 genes involved in mitochondrial function for association with type 2 diabetes 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 subjects 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 minor allele frequency ≥ 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 type 2 diabetes susceptibility. Rare variants are not covered by our approach so 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 type 2 diabetes GWAS (38-40;42;43). Nevertheless, previous studies suggested that they may be involved in type 2 diabetes 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 type 2 diabetes.

In conclusion, we were unable to detect association of common SNPs in 13 nuclear encoded mitochondrial candidate genes with type 2 diabetes or 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.

Supplementary table S1. Descriptive statistics of all individual cohorts.

	Hoorn Study		NHS		Breda Study		ERGO Study		Steno Study	
	controls	cases	controls	cases	controls	cases	controls	cases	controls	cases
Subjects	519	480	1517	821	920	501	5183	1222	514	706
(% male)	(55)	(52)	(44)	(50)	(61)	(46)	(41)	(39)	(46)	(48)
Age	65	67	53	61	48	71	69	73	57	59
(std. dev.)	(8)	(8)	(7)	(11)	(13)	(10)	(9)	(9)	(10)	(10)
BMI	26	29	26	29	n.a.	28	26	27	n.a.	n.a.
(std. dev.)	(5)	(5)	(4) [1]	(5)[1]		(4)	(4)	(4)		
FPG	5.4	7.7	5.3	7.6	n.a.	n.a.	5.7	8.7	n.a.	n.a.
(std. dev.)	(0.4)	(2.2)	(0.4)[2]	(2.1)[2]			(0.6)[3]	(2.6)[3]		

1) Only available for NHS subjects selected from the ongoing study, not from diabetic subjects from the LUMC and VUmc diabetes clinics (n=1664).
2) 1517 controls and 147 cases
3) 920 controls 187 cases
n.a. = not available
FPG = Fasting Plasma Glucose

Supplementary table S2. Results of replicated SNPs for every independent cohort

Rs number	1st stage				2nd stage				Steno Study	
	Hoorn Study		NHS		Breda Study		ERGO Study			
	OR 95% CI	P_{Add}	OR 95% CI	P_{Add}	OR 95% CI	P_{Add}	OR 95% CI	P_{Add}	OR 95% CI	P_{Add}
rs1937	0.66 0.50-0.88	0.005	1.08 0.88-1.33	0.46	0.89 0.70-1.13	0.37	n.a.	n.a.	n.a.	n.a.
rs4397793	0.86 0.72-1.03	0.10	1.10 0.95-1.28	0.20	1.00 0.85-1.17	1.00	1.06 0.97-1.16	0.22	n.a.	n.a.
rs1049432	0.77 0.61-0.97	0.03	1.11 0.91-1.35	0.29	0.93 0.74-1.17	0.56	0.99 0.88-1.12	0.90	n.a.	n.a.
rs4917960	1.12 1.00-1.43	0.05	1.09 0.94-1.26	0.28	1.00 0.86-1.18	0.97	1.03 0.94-1.13	0.51	n.a.	n.a.
rs17129583	1.32 1.02-1.70	0.04	0.75 0.60-0.93	0.01	1.09 0.88-1.36	0.46	0.90 0.79-1.04	0.15	n.a.	n.a.
rs2792751	0.87 0.71-1.06	0.18	1.08 0.92-1.27	0.35	0.94 0.79-1.12	0.50	1.01 0.91-1.11	0.92	n.a.	n.a.
rs535716	0.78 0.63-0.96	0.02	1.05 0.88-1.25	0.59	0.97 0.81-1.16	0.78	1.06 0.96-1.18	0.27	n.a.	n.a.
rs2522138	0.69 0.54-0.88	0.003	0.98 0.82-1.18	0.89	0.84 0.67-1.07	0.15	0.98 0.86-1.10	0.71	1.13 0.91-1.42	0.27
rs17085633	1.25 1.05-1.50	0.01	1.10 0.95-1.28	0.21	0.97 0.83-1.14	0.72	0.99 0.91-1.09	0.91	n.a.	n.a.

n.a. = not available

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