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Two common genetic variants near nuclear encoded OXPHOS genes are associated with insulin secretion *in vivo*

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Running title SNPs near OXPHOS genes and insulin secretion

Abstract

Context Mitochondrial ATP production is important in the regulation of glucose-stimulated insulin secretion. Genetic factors may modulate the capacity of the β-cells to secrete insulin and thereby contribute to the risk of type 2 diabetes.

Objective The aim of this study was to identify genetic loci in or adjacent to nuclear encoded genes of the oxidative phosphorylation (OXPHOS) pathway that are associated with insulin secretion *in vivo*.

Design and methods To find polymorphisms associated with glucose-stimulated insulin secretion, data from a genome-wide association study (GWAS) of 1467 non-diabetic individuals, the Diabetes Genetic Initiative (DGI), was examined. 413 single nucleotide polymorphisms (SNPs) with a minor allele frequency (MAF) ≥0.05 located in or adjacent to 76 OXPHOS genes were included in the DGI GWAS. A more extensive population based study of 4323 non-diabetics, the PPP-Botnia, was used as a replication cohort. Insulinogenic index during an oral glucose tolerance test (OGTT) was used as a surrogate marker of glucose-stimulated insulin secretion. Multivariate linear regression analyses were used to test genotype-phenotype associations.

Results Two common variants were indentified in the DGI, where the major C-allele of rs606164, adjacent to *NDUFC2* (NADH dehyrogenase (ubiquinone) 1 subunit C2), and the minor G-allele of rs1323070, adjacent to *COX7A2* (cythochrome c oxidase subunit VIIa polypeptide 2), showed nominal associations with decreased glucose-stimulated insulin secretion (p=0.0009 respective p=0.003). These associations were replicated in PPP-Botnia (p=0.002 and p=0.05).

Conclusion Our study shows that genetic variation near genes involved in oxidative phosphorylation may influence glucose-stimulated insulin secretion *in vivo*.

Introduction

Type 2 diabetes is a heterogeneous disease where multiple genes and environmental factors combine to cause the disease. Genetic factors may directly affect the risk of type 2 diabetes by modulating the capacity of the β-cells to secrete insulin and/or by inducing insulin resistance in target tissues. Mitochondrial dysfunction has been suggested to contribute to both impaired insulin secretion and insulin resistance [1, 2]. In pancreatic β-cells, mitochondria play a key role in regulating insulin secretion. Oxidative phosphorylation (OXPHOS) by the respiratory chain, which contains 5 enzyme complexes embedded in the mitochondrial inner membrane, is coupled to conversion of ADP into ATP. Elevated plasma glucose levels will cause a rise in the β-cell ATP/ADP ratio, which is a main trigger for insulin secretion [3]. Mitochondrial dysfunction could thereby lead to impaired insulin secretion from the β-cells and subsequently to an increased risk of type 2 diabetes. The multi-protein complexes in the OXPHOS system contain approximately 90 protein subunits encoded by both the nuclear and mitochondrial genomes [4], where the mitochondrial genome encodes 13 of the subunits present. A previous study from our group has shown the mRNA expression of *PPARGC1A* (peroxisome proliferator-activated receptor gamma coactivator 1 alpha), a master regulator of mitochondrial genes, to be reduced in islets from patients with type 2 diabetes and this reduction correlates with impaired glucose-stimulated insulin secretion [5]. In addition, a common polymorphism (rs8192678) influences *PPARGC1A* mRNA expression and insulin secretion in the human islets. Even though a mutation in mitochondrial DNA has been found in a rare but specific form of diabetes (Maternally Inherited Diabetes and Deafness; MIDD), common variation in mitochondrial DNA has not been associated with metabolic diseases [6, 7] . Mitochondrial genes encoded by the nuclear genome have been implicated in the pathogenesis of diabetes [8, 9]. However, only a small number of studies have reported whether polymorphisms in nuclear encoded genes involved in oxidative phosphorylation influence risk of the disease [5, 10-12], e.g., a polymorphism in *NDUFB6* (rs540467), has been associated with increased risk of type 2 diabetes [12].

Recent genome-wide association studies (GWASs), including the Diabetes Genetic Initiative (DGI), have provided new insights into the nature of the genetic factors involved in the pathogenesis of type

2 diabetes [13]. Since mitochondrial ATP production is important in the regulation of glucose-stimulated insulin secretion, we examined if polymorphisms in or near nuclear encoded genes of the respiratory chain are associated with insulin secretion using data from the DGI GWAS [13].

Subjects and Methods

Study populations

The *Diabetes Genetic Initiative* (DGI) is a case-control based study where patients with type 2 diabetes, geographically matched controls and discordant sib-ships were selected from Finland and Sweden [13]. Patients with type 2 diabetes were classified according to WHO (1999) criteria with fasting plasma glucose ≥7.0 mmol/l or a 2 h glucose ≥11.1 mmol/l during an oral glucose tolerance test (OGTT) and 1464 patients were included in the DGI. Non-diabetic subjects were defined as normal glucose tolerant, with fasting plasma glucose <6.1 mmol/l and 2 h glucose <7.8 mmol/l. Population based non-diabetic subjects had no first degree relatives with type 2 diabetes and included 1467 individuals (Table 1) [13]. Only non-diabetic subjects were included in the current study.

Prevalence, Prediction and Prevention of Diabetes (PPP-Botnia) is a population based study in the Botnia region of Western Finland [14]. The participants were aged 18-75 years (mean age 48.4 ± 15.6 years). Diagnosis of type 2 diabetes was confirmed from subject records or on the basis of fasting plasma glucose concentration ≥7.0 mmol/l and/or 2 h glucose ≥11.1 mmol/l. Only non-diabetic subjects (n=4323) were included in the current study, where 612 (14%) had fasting plasma glucose levels between 6.1-6.9 mmol/l (Table 1).

All participants gave written informed consent for the studies and the local ethics committees approved the protocols.

Assays and Measurements

Blood samples for measurements of plasma glucose and serum insulin concentrations were drawn at 0, 30 and 120 minutes of the 75 g OGTT in both DGI and PPP-Botnia.

Plasma glucose was measured in DGI with a glucose oxidase method (Beckman Glucose Analyzer, Beckman Instruments, Fullerton, CA) and in PPP-Botnia with a glucose dehydrogenase method (HemoCue, Ängelholm, Sweden). Three different methods for measurement of insulin concentrations were used in both DGI and PPP-Botnia: radioimmunoassay (Pharmacia, Uppsala, Sweden), enzyme linked immunoassay (DAKO Diagnostics Ltd, Cambridgeshire, UK), and fluoroimmunoassay (Delphia, Perkin-Elmer Finland, Turku, Finland). The Pharmacia and Delfia values were transformed into "Dako" values using validated correction coefficients.

β-cell function and glucose-stimulated insulin secretion was assessed as insulinogenic index during an OGTT ((insulin at 30 min – insulin at 0 min) / (glucose at 30 min – glucose at 0 min))[15]. Insulin resistance estimated by the homeostasis model assessment (HOMA-IR) was calculated as ((glucose at 0 min * insulin at 0 min) / 22.5)). β-cell function was also assessed in PPP-Botnia as disposition index, insulin secretion adjusted for insulin resistance (Insulinogenic index /HOMA-IR).

Identification of SNPs in the DGI GWAS

We aimed at identifying single nucleotide polymorphisms (SNPs) situated in a region 25kb upstream to 25kb downstream of nuclear encoded OXPHOS genes with a minor allele frequency (MAF)≥0.05, showing nominal associations ($p \leq 0.01$) with insulinogenic index in non-diabetic subjects in the DGI GWAS [13]. Identified polymorphisms were ranked based on the *p*-values of genotype-phenotype association. The GeneChip® Human Mapping 500K Array Set (Affymetrix Inc, Santa Clara, CA, USA) used in the DGI GWAS has coverage in the CEU HapMap population ($r^2 \leq 0.8$) of 67% based on single marker test. In the DGI GWAS, 413 SNPs within or near 76 OXPHOS genes fulfilled the MAF criteria (Supplementary Table 1) and 9 of these SNPs were also nominally associated with insulinogenic index ($p \leq 0.01$). The two top hits were selected for follow-up in an independent cohort.

Genotyping

In the DGI GWAS, genotyping was performed using Affymetrix 500K chip array [13]. In PPP-Botnia, rs606164 and rs1323070 were genotyped using allelic discrimination assays on the ABI 7900 platform (C__2983373_10 and C__3073719_10, Applied Biosystems, Foster City, CA). The genotyping success rates were >95.5% and the concordance rate was 100% based on 4.2% duplicate comparisons.

Statistical analyses

Linear regression analyses were performed to test genotype-phenotype associations assuming additive genetic models in both DGI and PPP-Botnia. Phenotype values were logarithmically transformed to fit a normal distribution in both cohorts before analyses.

In DGI, Z scores of insulinogenic index were prepared separately by gender and recruiting region (Botnia, Skara, Malmo or Helsinki) and regressed against genotype adjusted for age, log BMI and type of insulin measurement. Unrelated individuals and siblings were included in this analysis. To correct for inflation caused by inclusion of related individuals, the genomic control inflation factor based on the median test statistic was estimated, and *p*-values based on the test statistic adjusted by this factor are reported [13].

In PPP-Botnia the log-transformed phenotype values were regressed against genotype adjusted for age, sex and BMI.

All analyses were carried out in non-diabetic individuals. Results are presented as median (inter-quartile range) or beta coefficient (s.e.m). Statistical analyses were performed using SPSS version 17 for Windows (SPSS, Chicago, IL, USA) or using STATA/SE 10.0 (STATA Corp LP, College Station, Texas, USA).

Results

Identification of genetic loci associated with insulin secretion

To find genetic loci associated with insulin secretion in or adjacent to nuclear encoded genes of the respiratory chain, we examined data from the DGI GWAS [13] (Table 1). Nine SNPs representing six genes were identified based on a MAF≥0.05, nominal associations to insulinogenic index with $p \leq 0.01$ and they are located in regions of 25 kb up- or downstream of OXHPOS genes (Table 2). Region plots of the six identified OXPHOS genes are presented together with –log10 *p*-values of all SNPs within these regions from the DGI GWAS as well as Haploview presentations of the LD-structure based on HapMap data (Supplementary Figure 1 A-F). Based on the lowest *p*-values, two SNPs were selected for follow-up: rs606164, 12kb upstream of *NDUFC2*, and rs1323070, 24kb downstream of *COX7A2*. The major C-allele of rs606164 and the minor G-allele of rs1323070 are nominally associated with decreased glucose-stimulated insulin secretion during an OGTT (insulinogenic index) in non-diabetic subjects of DGI (rs606164: beta -0.21 ± 0.062, p=0.0009 and rs1323070: beta -0.14 ± 0.046, p=0.003). The common variant rs606164 was not in LD with any of the other two SNPs identified in DGI located adjacent to *NDUFC2* (Table 2 and Supplementary Figure 1A). None of the variants were associated with type 2 diabetes in the case-control based study of DGI GWAS or insulin resistance (HOMA-IR) in non-diabetic subjects of DGI [13] (Supplementary table 1).

Insulin secretion in vivo

We next investigated whether the *NDUFC2* variant, rs606164, and *COX7A2* variant, rs1323070, were associated with insulin secretion in a more extensive replication cohort, the PPP-Botnia Study [14] (Table 1). In line with the findings in DGI, C-allele carriers of rs606164 showed decreased insulinogenic index in the PPP-Botnia study (beta -0.070 ± 0.022, p=0.002) (Table 3). Moreover, G-allele carriers of rs1323070 showed a nominal association with insulinogenic index in PPP-Botnia (beta -0.040 ± 0.021, p=0.05) (Table 3). Disposition index is an additional assessment of β-cell function that considers the insulin resistance-secretion relationship. When analysing disposition-index, C-allele carriers of rs606164 and G-allele carriers of rs1323070 also showed a decreased

insulin secretion adjusted for insulin resistance in PPP-Botnia (rs606164: beta -0.066 ± 0.023, p=0.007; rs1323070: beta -0.040 ± 0.019, p=0.03). Neither rs606164 nor rs1323070 were associated with HOMA-IR in PPP-Botnia (p=0.79 and p=0.57, respectively).

Discussion

In this study, we have demonstrated that two common polymorphisms, rs606164 adjacent to *NDUFC2* and rs1323070 adjacent to *COX7A2,* are associated with insulin secretion *in vivo*. Insulin secretion was measured as insulinogenic index at 30 minutes, a well-known measure of early-phase insulin secretion during an OGTT [15, 16].

Mitochondrial ATP production by oxidative phosphorylation in the respiratory chain is necessary for glucose-stimulated insulin release by β-cells [17]. Insulin secretion is impaired in pancreatic islets from patients with type 2 diabetes partially due to impaired hyperpolarization of the inner mitochondrial membrane and a failure to respond with a rise in ATP levels [18], but the number of identified polymorphisms in genes of the oxidative phosphorylation process associated with type 2 diabetes and its risk factors is limited.

Recent GWAS have focused on identifying genes associated with type 2 diabetes and today more than 35 common variants have been identified that affect the risk of the disease [13, 19-28] . Although many of these variants seem to affect the β-cell function and insulin secretion [29, 30], only a few GWAS have included measurements of glucose-stimulated insulin secretion [31]. Due to the importance of OXPHOS in the regulation of insulin secretion, we examined if common variants near nuclear encoded genes of the respiratory chain affect glucose-stimulated insulin secretion using data from a GWAS, the DGI. The identification of variants was based on one trait and for variants in genes of one specific molecular pathway. Among variants near genes of the respiratory chain, rs606164 and rs1323070 showed the strongest associations with insulinogenic index in DGI. Although these associations were not genome-wide significant in the DGI, one should keep in mind that the study was underpowered for these associations, as they could only reliably be assessed for non-diabetic individuals. We therefore used a lower threshold for selection of SNPs for replication in an independent study, PPP-

Botnia. Our inclusion criteria was to identify SNPs near nuclear encoded OXPHOS genes, which are associated with insulinogenic index with $p \leq 0.01$ and a MAF$\geq$0.05. Nine variants fulfilled the criteria. To confirm the association of the two top hits, we replicated the results in a more extensive population based cohort, the PPP-Botnia study from Western Finland [14]. In accordance with DGI, C-allele carriers of rs606164 showed decreased insulin secretion and there was a nominal association between rs1323070 and insulin secretion in PPP-Botnia. This effect was maintained when adjusting for insulin resistance by using disposition index. The inclusion of individuals with impaired fasting glucose (IFG) in PPP-Botnia did not affect our findings since an association between rs606164 and insulinogenic index as well as a trend towards association for rs1323070 were observed even when individuals with IFG were excluded.

The common variant rs606164 is located 12kb upstream of the *NDUFC2* gene, a position where genetic variation may influence gene transcription. *NDUFC2* encodes NADH dehyrogenase (ubiquinone) 1 subunit C2, which is located in complex I of the electron transport chain. Complex I is the largest component of the OXPHOS system and entry point of electrons from NADH into the electron transport chain, thereby playing a key role in regulating oxidative phosphorylation. *NDUFC2* and its flanking genes thyroid hormone responsive (*THRSP)* and asparagine-linked glycosylation 8 (*ALG8*) appears to be conserved among chickens, humans, mice and rats. Since rs606164 are in LD with SNPs located in both *THRSP* and *ALG8*, we can not exclude that the effect on insulin secretion is mediated by these genes. However, based on the function of these genes, they are not likely to be involved in the regulation of insulin secretion, and we therefore suggest that the found association between rs606164 and insulinogenic index is mediated via *NDUFC2*.

The position of rs1323070 is approximately 24 kb downstream of *COX7A2*. *COX7A2* encodes cytochrome c oxidase VIIa polypeptide 2, which is located in complex IV of the electron transport chain. Complex IV catalyses the electron transfers from cytochrome c to the reduction of oxygen to water. Although, the location of rs1323070 is downstream of *COX7A2* and not in LD with any SNP within this OXPHOS gene, it may affect the expression of *COX7A2*. It is also possible that rs1323070

influences a gene upstream of the SNP or a so far unknown regulatory region that mediates the effect on insulin secretion. Taken together, common polymorphisms in or near genes involved in oxidative phosphorylation may be of importance in insulin secretion and/or the pathogenesis of type 2 diabetes.

In conclusion, we have identified two polymorphisms associated with glucose-stimulated insulin secretion *in vivo*. These polymorphisms, rs606164 and rs1323070, are located adjacent to nuclear encoded genes involved in oxidative phosphorylation. This is an exploratory follow-up study of a published GWAS, DGI, where we have selected SNPs nominal associated with insulin secretion for replication in an independent study. It remains to be shown whether the findings can be replicated in even larger studies and influence risk of type 2 diabetes.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Table 1 Clinical characteristics of non-diabetic participants in the Diabetes Genetics Initiative (DGI) and the Prevalence, Prediction and Prevention of Diabetes (PPP-Botnia).

Characteristics	DGI	PPP-Botnia
N (male/female)	1467 (707/760)	4323 (2043/2280)
Age (years)	58.8 ± 10.1	47.6 ± 15.2
BMI (kg/m^2)	26.6 ± 3.7	26.3 ± 4.3
Fasting plasma glucose (mmol/l)	5.3 ± 0.5	5.3 ± 0.6
Glucose 30min (mmol/l)	8.3 ± 1.5	8.3 ± 1.6
Glucose 120 min (mmol/l)	5.6 ± 1.3	5.2 ± 1.6
Fasting insulin (mU/l)	5.2 (4.3)	5.3 (4.2)
Insulin 30 min (mU/l)	50.2 (48.9)	50.4 (38.3)
Insulin 120min (mU/l)	36.2 (29.7)	23.7 (26.2)
Insulinogenic index	14.1 (14.8)	15.9 (16.1)
HOMA-IR	1.46 (1.08)	1.26 (1.04)

Data are expressed as mean ± SD or median (IQR). Insulinogenic index; calculated as ((insulin at 30 min – insulin at 0 min) / (glucose at 30 min – glucose at 0 min)). HOMA-IR; calculated as ((glucose at 0 min * insulin at 0 min)/22.5)).

Table 2 Identified SNPs from DGI GWAS located in a region of ~25kb up- or downstream of OXHPOS genes with an association to insulinogenic index in non-diabetic individuals of DGI with $p \leq 0.01$and MAF$\geq$0.05.

SNP	Chromosome	Nearest OXPHOS gene	Alleles[a] (Major/Minor)	MAF	Beta (s.e.m)	*p*-value
rs606164	11	~12 kB upstream NDUFC2	**C**/G	0.16	-0.21 (0.062)	$9*10^{-4}$
rs1323070	6	~24 kB downstream COX7A2	A/**G**	0.36	-0.14 (0.046)	$3*10^{-3}$
rs10793285	11	~20 kB upstream NDUFC2	**T**/G	0.36	-0.12 (0.044)	$6*10^{-3}$
rs1133322	15	~0.3 kB downstream COX5	A/**G**	0.49	-0.13 (0.045)	$7*10^{-3}$
rs2643338	8	Intron UQCRB	**A**/G	0.47	-0.12 (0.045)	$1*10^{-2}$
rs7827095	8	~3 kB downstream UQCRB	**T**/C	0.47	-0.12 (0.045)	$1*10^{-2}$
rs10734905	12	Intron ATP6OA2	**G**/T	0.32	-0.13 (0.049)	$1*10^{-2}$
rs1264913	1	~15 kB upstream ATP5F1	A/**G**	0.11	-0.18 (0.071)	$1*10^{-2}$
rs2845556	11	~20 kB upstream NDUFC2	**C**/T	0.49	-0.12 (0.46)	$1*10^{-2}$

In Diabetes Genetic Initiative (DGI) *p*-values are based on linear regression to test association between genotype and insulinogenic index z-score with the covariates gender, recruiting region, age, BMI and type of insulin measurement. A genomic control inflation factor was used to adjust for related individuals. MAF, minor-allele frequency.

[a]Allele denoted in bold associated with decreased insulinogenic index.

Table 3 Effects of rs606164 and rs1323070 on insulinogenic index in non-diabetic individuals of the PPP-Botnia study.

Study	SNP	Phenotype	Genotypes (Genotype Frequency)			Beta	s.e.m	*p*-value
PPP-Botnia (n=4201)	rs606164		GG (0.024)	CG (0.285)	CC (0.691)			
		Insulinogenic index	19.08 (14.54)	16.42 (15.92)	15.63 (16.13)	-0.070	0.022	0.002
PPP-Botnia (n=4140)	rs1323070		AA (0.432)	AG (0.441)	GG (0.127)			
		Insulinogenic index	16.17 (16.13)	15.90 (15.81)	15.25 (15.17)	-0.040	0.021	0.05

Data are expressed as median (IQR). Beta coefficiants (s.e.m) are from linear regression analyses adjusted for age, sex and BMI based on an additive model.