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ARTICLE

Impact of polymorphisms in *WFS1* on prediabetic phenotypes in a population-based sample of middle-aged people with normal and abnormal glucose regulation

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Abstract
Aim/hypothesis Recently, variants in *WFS1* have been shown to be associated with type 2 diabetes. We aimed to examine metabolic risk phenotypes of *WFS1* variants in glucose-tolerant people and in individuals with abnormal glucose regulation.
Methods The type 2 diabetes-associated *WFS1* variant rs734312 (His611Arg) was studied in the population-based Inter99 cohort involving 4,568 glucose-tolerant individuals and 1,471 individuals with treatment-naive abnormal glucose regulation, and in an additional 3,733 treated type 2 diabetes patients.
Results The *WFS1* rs734312 showed a borderline significant association with type 2 diabetes with directions and relative risks consistent with previous reports. In individuals with abnormal glucose regulation, the diabetogenic risk A allele of rs734312 was associated in an allele-dependent manner with a decrease in insulinogenic index (p=0.025) and decreased 30-min serum insulin levels (p=0.047) after an oral glucose load. In glucose-tolerant individuals the same allele was associated with increased fasting serum insulin concentration (p=0.019) and homeostasis model assessment of insulin resistance (HOMA-IR; p=0.026). To study the complex interaction of *WFS1* rs734312 on insulin release

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and insulin resistance we introduced Hotelling's T^2 test. Assuming bivariate normal distribution, we constructed standard error ellipses of the insulinogenic index and HOMA-IR when stratified according to glucose tolerance status around the means of each *WFS1* rs734312 genotype level. The interaction term between individuals with normal glucose tolerance and abnormal glucose regulation on the insulinogenic index and HOMA-IR was significantly associated with the traits (p=0.0017).

Conclusions/interpretation Type 2 diabetes-associated risk alleles of *WFS1* are associated with estimates of a decreased pancreatic beta cell function among middle-aged individuals with abnormal glucose regulation.

Keywords ADDITION · Beta cell · Case–control study · Genetic association · Insulin resistance · Inter99 · Single-nucleotide polymorphism · Type 2 diabetes

Abbreviations

ER	endoplasmic reticulum
HOMA-IR	homeostasis model assessment of insulin resistance
IFG	impaired fasting glycaemia
IGT	impaired glucose tolerance
LD	linkage disequilibrium
SNP	single-nucleotide polymorphism
WFS1	Wolfram syndrome 1 (wolframin)

Introduction

A study by Sandhu et al. reported the results of genotyping 1,536 single-nucleotide polymorphisms (SNPs) in 84 candidate genes regulating pancreatic beta cell development, growth, function or survival [1]. In a pooled analysis involving six UK studies and one study of an Ashkenazi Jewish population including a total of 9,533 type 2 diabetic patients and 11,389 controls the authors showed that four common variants (rs10010131, rs6446482, rs752854 and rs734312) of the gene encoding wolframin 1 (*WFS1*) were associated with type 2 diabetes. Two independent studies have investigated these initial findings [1]. The first study by Franks et al. [2] replicated the nominal association of the four *WFS1* SNPs with type 2 diabetes, whereas the overall conclusion of the second study by Florez et al. [3] was that no association with type 2 diabetes could be observed. However, a thorough phenotype association analysis was performed to elucidate a possible prediabetic phenotype. The study consisted of 3,530 US individuals at high risk of developing type 2 diabetes (on the basis of overweight, increased fasting plasma glucose and impaired glucose tolerance [IGT]) and investigated the impact of *WFS1* SNPs (including rs10010131 and rs734312) in relation to insulin sensitivity and beta cell function. Interestingly, carriers of the type 2 diabetes risk A allele of rs734312 were characterised by an allele-dependent reduction in insulin release and a concomitant increase in insulin sensitivity [3]. Indeed, the notion that *WFS1* is a true type 2 diabetes gene seems to be established, although some further validation of an intermediate prediabetic phenotype causing the observed diabetes association is still needed.

WFS1 encodes an 890 amino acid membrane glycoprotein located in the endoplasmic reticulum (ER). It is ubiquitously expressed in many organs including the brain and pancreas, with weak signals detected in liver, skeletal muscle and kidney [4, 5]. Mutations in *WFS1* are known to cause the Wolfram syndrome, which is an autosomal recessive disorder clinically defined by diabetes insipidus, non-autoimmune diabetes mellitus with juvenile onset, optic atrophy and deafness [5, 6]. Disruption of *Wfs1* in mice resulted in progressive glucose intolerance and concomitant insulin deficiency. In this animal model, beta cell death occurred by an accelerated process of apoptosis; similarly, increased levels of markers reflecting ER stress were also demonstrated [7–9]. In line with the outcome of studies in mice, previous and statistically undersized human studies have indicated that variation in *WFS1* may be associated with type 1 diabetes mellitus [10], type 2 diabetes mellitus [11] and a combination of diabetes mellitus and deafness [12, 13]. Moreover, two patients with the Wolfram syndrome were reported to be without insulin-producing beta cells [14].

The aim of the present study was to establish possible metabolic risk phenotypes of the type 2 diabetes-associated A-allele of rs734312 (His611Arg), the G allele of rs6446482 and the G allele of rs10010131 in a large population-based cohort of middle-aged glucose-tolerant individuals as well as in treatment-naive individuals with abnormal glucose regulation. Furthermore, we aimed to confirm the recently reported [1–3] diabetogenic impact of the *WFS1* risk alleles in our Danish case–control study involving 3,844 type 2 diabetes patients and 4,225 glucose-tolerant control participants.

Methods

Study population Three *WFS1* polymorphisms (rs10010131, rs6446482 and rs734312) were genotyped in 9,772 Danes involving: (1) the population-based Inter99 cohort (ClinicalTrial.gov ID no NCT00289237) of middle-aged individuals sampled at Research Centre for Prevention and Health (n=6,039) [15]; (2) type 2 diabetic patients sampled through the outpatient clinic at Steno Diabetes Center (n=2,107); and (3) the screen-detected type 2 diabetes patients from the Danish ADDITION screening cohort (ClinicalTrials.gov NCT00237549) sampled through Department of General Practice at University of Aarhus (n=1,626) [16]. Study

group 1 underwent a standard 75 g OGTT. Informed written consent was obtained from all participants before participation. The study was approved by the Ethical Committee of Copenhagen County and was in accordance with the principles of the Helsinki Declaration. Normal glucose tolerance, impaired fasting glycaemia (IFG), IGT and type 2 diabetes were defined according to WHO [17].

Biochemical and anthropometrical measurements Height and body weight were measured in light indoor clothes and without shoes; BMI was calculated as weight (kg)/(height [m])^2. Blood samples were drawn after a 12 h overnight fast. Plasma glucose was analysed by a glucose oxidase method (Granutest, Merck, Darmstadt, Germany). HbA_{1c} was measured by ion-exchange high-performance liquid chromatography (normal reference range: 4.1–6.4%) and serum insulin, excluding des(31, 32) and intact proinsulin, was measured using a kit (AutoDELFIA insulin kit; Perkin-Elmer, Wallac, Turku, Finland). Insulinogenic $index_{insulin}$ was calculated as (serum insulin at 30 min [pmol/l]−fasting serum insulin [pmol/l])/plasma glucose at 30 min (mmol/l), which is a surrogate estimate of the initial oral glucose-elicited insulin release. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as described previously [18].

Genotyping The *WFS1* polymorphisms (rs10010131, rs6446482 and rs734312) were genotyped using Taqman allelic discrimination (KBioscience, Hoddesdon, UK). Discordance between 941 random duplicate samples was 0.1% and the genotyping success rate was >97% for all SNPs. All genotype groups obeyed Hardy–Weinberg equilibrium.

Statistical analysis Fisher's exact test and logistic regression adjusted for sex, age and BMI were applied to examine differences in allele frequencies and genotype distributions between affected and unaffected participants. General linear statistics was used to test quantitative variables for differences between genotype groups of glucose-tolerant individuals and treatment-naive individuals with abnormal glucose regulation (IFG, IGT and screen-detected type 2 diabetes). The multivariate method, Hotelling T^2 [19], was applied to test the simultaneous effect of rs734312 on two traits of insulin release and insulin sensitivity.

All analyses were performed using RGui version 2.6.0 (http://www.r-project.org). We considered p values below 0.05 after 5,000 permutations using the statistical software BLOSSOM (available from the US Geological Survey; http://www.fort.usgs.gov/products/software/blossom/blossom.asp) to be significant. Since type 2 diabetic patients included in the case–control study were ascertained from three different study populations, we evaluated their possible heterogeneity by comparing allele frequencies of the three *WFS1*variants in type 2 diabetes patients from the different subgroups. No evidence of heterogeneity was observed using Pearson's χ^2 test for count data.

Table 1 Association studies of rs10010131 and rs734312 of *WFS1* in 3,844 type 2 diabetic patients and 4,225 glucose-tolerant control participants

	rs10010131 (genotype risk allele G)			rs734312 (genotype risk allele A)		
	GG	GA	AA	AA	GA	GG
Genotype distribution, *n* (%)						
Glucose tolerance	1,373 (33)	2,105 (50)	747 (18)	1,094 (27)	2,054 (50)	960 (23)
Type 2 diabetes	1,326 (34)	1,878 (49)	640 (17)	1,098 (28)	1,920 (50)	823 (22)
Allele frequency model[a]						
OR (95% CI)	0.94 (0.88–1.00)			0.93 (0.87–0.99)		
p value	0.053			0.024		
Genotype frequency model[a]						
p value	0.13			0.072		
Additive[b]						
OR (95% CI)	0.95 (0.86–1.05)			0.91 (0.83–1.00)		
p value	0.32			0.056		
Dominant[b]						
OR (95% CI)	0.90 (0.78–1.03)			0.85 (0.73–0.99)		
p value	0.13			0.040		
Recessive[b]						
OR (95% CI)	1.01 (0.84–1.20)			0.92 (0.78–1.08)		
p value	0.95			0.30		

Data are number of participants with each genotype (% of each group), risk allele frequencies in per cent (95% CI) and OR (95% CI)
[a] Differences in allele frequencies and genotype distribution not adjusted for age, sex or BMI were calculated using Fisher's exact test
[b] Logistic regression applying an additive, dominant or recessive model adjusted for age, sex and BMI

To investigate whether the effect of the alleles differed between individuals with different glucose tolerance status, we included an interaction term between glucose tolerance status and the genotype of interest in the linear model besides the main effects:

$$y_{\text{trait}} = \mu + \alpha X_{\text{add}} + \beta X_{\text{gts}} + \gamma X_{\text{add}} X_{\text{gts}} + \delta X_{\text{sex}} + \in X_{\text{age}} + \zeta X_{\text{BMI}}$$

In this model, we assumed an additive effect for the genotypes (i.e. X_{add} coded as the number of minor alleles) and treated the glucose tolerance status (X_{gts}) as a binary vector indicating whether individuals belonged to the group of glucose-tolerant individuals (coded as 0) or abnormal glucose regulation (coded as 1). The same procedure was also performed for a bivariate distribution of two traits

Table 2 Anthropometric and metabolic characteristics of 5,384 treatment-naive Danish individuals from the Inter99 cohort stratified according to *WFS1* rs734312 genotype and glucose tolerance status

Characteristic	A/A	A/G	G/G	p_{add}	p_{int}
Normal glucose tolerance					
n (men/women)	1,091 (495/596)	2,050 (962/1088)	958 (445/513)		
Age (years)	45±8	45±8	45±8		
BMI (kg/m^2)	25.5±4.0	25.5±4.1	25.6±4.1	0.56	
Serum insulin (pmol/l)					
Fasting	39±24	38±23	37±23	0.019	
30 min post-OGTT	290±190	291±178	278±156	0.085	
120 min post-OGTT	174±145	169±126	166±130	0.12	
Plasma glucose (mmol/l)					
Fasting	5.3±0.4	5.3±0.4	5.3±0.4	0.77	
30-min post-OGTT	8.1±1.6	8.2±1.5	8.2±1.5	0.78	
120-min post-OGTT	5.5±1.1	5.5±1.1	5.5±1.1	0.53	
Serum C-peptide (pmol/l)					
Fasting	545±208	537±209	534±220	0.030	
30-min post-OGTT	1,984±668	1,988±710	1,936±666	0.039	
120-min post-OGTT	2,072±804	2,064±794	2,025±796	0.12	
HOMA-IR	9.2±5.8	8.9±5.7	8.7±5.5	0.026	
Insulinogenic index$_{\text{Insulin}}$ (pmol/mmol)	31.2±20.9	31.4±19.9	30.0±18.1	0.075	
Abnormal glucose tolerance[a]					
n (men/women)	359 (224/135)	642 (385/257)	284 (166/118)		
Age (years)	50±8	49±7	49±8		
BMI (kg/m^2)	28.3±4.9	28.3±5.3	28.6±5.2	0.49	0.83
Serum insulin (pmol/l)					
Fasting	54±33	58±37	55±33	1.0	0.28
30 min post-OGTT	291±181	311±224	333±214	0.047	0.0088
120 min post-OGTT	370±304	364±320	390±354	1.0	0.18
Plasma glucose (mmol/l)					
Fasting	6.3±1.35	6.2±1.0	6.2±1.2	0.38	0.27
30 min post-OGTT	10.2±1.9	10.3±1.9	10.2±1.7	0.60	0.35
120 min post-OGTT	8.5±2.9	8.4±2.9	8.2±2.3	0.36	0.70
Serum C-peptide (pmol/l)					
Fasting	756±331	772±348	768±340	0.97	0.56
30 min post-OGTT	2,039±762	2,059±823	2,147±751	0.17	0.021
120 min post-OGTT	3,130±1201	3,054±1223	3,159±1238	0.84	0.19
HOMA-IR	15.3±10.1	16.2±11.6	15.3±9.9	0.81	0.34
Insulinogenic index$_{\text{Insulin}}$ (pmol/mmol)	23.5±16.0	25.1±19.5	27.5±20.5	0.025	0.0025

Data are means±SD. Values of serum insulin and values derived from serum insulin variables were logarithmically transformed before statistical analysis. All analyses were made using an additive model.

Sex and glucose tolerance status were considered as discrete factors and age, genotype and BMI as continuous covariates.

Calculated *p* values were adjusted for age, sex and BMI (where appropriate): the *p* values for an interaction (1 *df*) between glucose tolerance status and genotype, as described in the Methods, are denoted p_{int}.

The insulinogenic index was calculated as fasting serum insulin (pmol/l) subtracted from 30 min post-OGTT serum insulin (pmol/l) and divided by 30 min post-OGTT plasma glucose (mmol/l).

HOMA-IR was calculated as previously described [18]

[a] IFG, IGT, screen-detected diabetes

where the significance of the interaction was assessed by the multivariate method Hotelling T^2 test.

Results

We estimated the linkage disequilibrium (LD) pattern assessed by r^2 of the three *WFS1* SNPs (rs10010131, rs6446482 and rs734312) (Electronic supplementary material [ESM] Fig. 1). As the LD between rs10010131 and rs6446482 was high (r^2>0.95), only rs734312 and rs10010131 were analysed in the present paper. The minor allele frequency in the population-based Inter99 cohort was 42% for rs10010131 and 48% for rs734312.

In the present Danish case–control study, the *WFS1* rs734312 showed a borderline significant association with type 2 diabetes with a direction and a relative risk consistent with previous reports (Table 1).

In order to investigate whether intermediate prediabetic phenotypes are associated with the reported *WFS1* type 2 diabetes risk alleles, we investigated metabolic traits related to type 2 diabetes in 4,568 glucose-tolerant individuals and in 1,471 treatment-naive individuals with abnormal glucose regulation (IFG, IGT and screen-detected type 2 diabetes). In individuals with abnormal glucose regulation, the diabetogenic risk A allele of rs734312 was associated in an allele-dependent manner with a decreased insulinogenic index (p=0.025) and a decreased 30-min serum insulin level (p=0.047) after an oral glucose load, whereas in glucose-tolerant individuals the same allele was associated with increased fasting serum insulin (p=0.019), fasting serum C-peptide (p=0.030) and HOMA-IR (p=0.026; Table 2). Similar results were found for the diabetes risk allele of rs10010131 (ESM Table 1).

We searched results of available web-based genome-wide association studies of *WFS1* SNPs and their potential relationships to traits associated with type 2 diabetes such as fasting serum insulin, 120 min post-OGTT serum insulin and insulinogenic index, as well as estimates of insulin resistance. However, no significant associations with any of the ten SNPs genotyped in *WFS1* were observed [20].

We also examined for interaction between glucose tolerance status and genotype by applying a linear model for quantitative indices of pancreatic beta cell function. In this analysis, we demonstrated a significant interaction of rs734312 with traits related to beta cell function (30-min serum insulin p=0.0088, insulinogenic index p=0.0025), which implies a larger effect size of *WFS1* variants for individuals with abnormal glucose regulation than for glucose tolerant individuals (Table 2, ESM Table 1). In glucose-tolerant people, the effect size on beta cell-related traits such as the insulinogenic index seems to be vague, i.e. 2.2% (95% CI [−4.7, 0.2] reduction per allele, p=0.075), whereas in the group of individuals with abnormal glucose regulation the impact increases considerably, i.e. 6.5% (95% CI [0.8, 12.1] increase per allele, p=0.025). Similar results were observed for 30 min serum insulin and C-peptide after an oral glucose load (ESM Table 2).

The interplay between *WFS1* rs734312 genotype, insulin release and insulin resistance in relation to glucose tolerance status in the Inter99 cohort is shown in Fig. 1. It appears that there is a glucose tolerance interaction affecting the two correlated traits (p=0.0017) and that the largest effect is on insulin release as assessed by the insulinogenic index in A allele carriers with abnormal glucose regulation. With regard to rs10010131, an interaction of glucose tolerance status on the insulinogenic index and HOMA-IR was also found to be statistically significant (p=0.0027, data not shown).

Discussion

In the present study, we assessed the impact of the type 2 diabetes risk alleles of rs734312 and rs10010131 on pre-diabetic phenotypes that may explain the previously reported diabetogenic impact of these variants [1–3]. In subgroups of a population-based cohort of middle-aged people with normal glucose tolerance and treatment-naive abnormal

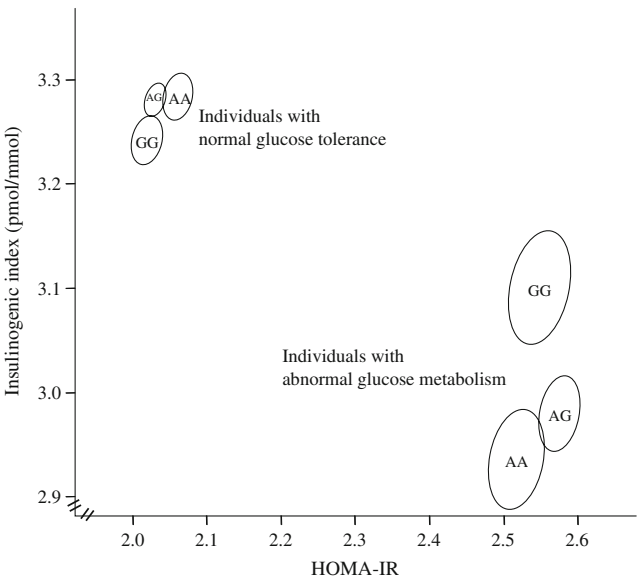

Fig. 1 Standard error of the mean of the insulinogenic index and HOMA-IR stratified according to glucose tolerance status and rs734312 genotype in middle-aged individuals from the Danish Inter99 cohort. Assuming bivariate normal distribution, we constructed standard error ellipses around the means of each genotype level. The interaction term between individuals with normal glucose tolerance (AA: 980, AG: 1898, GG: 874) and abnormal glucose regulation (AA: 355, AG: 632, GG: 286) on the insulinogenic index and HOMA-IR was significantly associated with the traits (p=0.0017) when applying Hotelling T^2

glucose regulation we found that the effect size of *WFS1* diabetes risk alleles differed depending on glucose-tolerance status. In the individuals with abnormal glucose regulation, defined as IFG, IGT or screen-detected and untreated type 2 diabetes, the reported *WFS1* diabetes risk alleles were significantly associated with impaired insulin release as assessed by insulinogenic index (6.5% per allele), whereas the same alleles in glucose-tolerant individuals were associated with increased fasting serum insulin and HOMA-IR. To date, two studies [2, 3] have investigated the association between *WFS1* variants and type 2 diabetes suggested by Sandhu et al. [1]; however, only one study has considered the underlying prediabetic phenotype [3]. The latter study involved 3,530 US individuals of various ethnicities, all at high risk of developing type 2 diabetes. The authors of that report demonstrated an increased post-oral glucose load insulin release as estimated by the insulinogenic index and a decreased insulin sensitivity index for the reported protective G-allele of rs734312. Thus, these results [3] on insulin release are in line with our findings in Danish middle-aged participants with untreated abnormal glucose regulation. However, due to the modest effect size of the *WFS1* variants, both reported results should be considered as preliminary findings and obviously they will need to be replicated in independent and statistically powered study samples.

Taken together, the known pathophysiology of the Wolfram syndrome and the numerous studies of the comparable gene in mice suggest that the Wolfram syndrome 1 (wolframin) protein (WFS1) primarily mediates its action at the pancreatic beta cell level. Specifically, it has been localised to the ER, indicating a role in membrane trafficking, secretion and regulation of ER calcium homeostasis, i.e. biological functions that cause ER stress if disturbed. These mechanisms have also been examined in *Wfs1*-deficient mice that exhibited increased phosphorylation of RNA-dependent protein kinase-like ER kinase, chaperone gene expression and active X-box binding protein 1 levels, all of which are factors pointing to an enhanced ER stress response.

In line with the known function of WFS1, the diabetes risk allele-dependent decrease in post-oral glucose load insulin release observed in individuals with abnormal glucose regulation and not in glucose-tolerant individuals may tentatively be explained by the fact that other diabetogenic factors are operating in the group of people with abnormal glucose regulation. Thus, individuals with abnormal glucose regulation may have several stressors of their pancreatic beta cell function, including insulin resistance, proinflammation, glucotoxicity and lipotoxicity, all of which may interact with susceptibility variants in *WFS1* and hereby predispose to ER stress-mediated apoptosis.

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Duality of interest K. Borch-Johnsen and O. Pedersen hold stock in Novo Nordisk and have received lecture fees from pharmaceutical companies. All other authors declare that there is no duality of interest associated with this manuscript.

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