Polymorphisms in *AHII* are not associated with type 2 diabetes or related phenotypes in Danes: non-replication of a genome-wide association result

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Received: 15 October 2007 / Accepted: 14 December 2007 / Published online: 29 January 2008
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Abstract

Aims/hypothesis A genome-wide association study recently identified an association between common variants, rs1535435 and rs9494266, in the *AHII* gene and type 2 diabetes. The aim of the present study was to investigate the putative association between these polymorphisms and type 2 diabetes or type 2 diabetes-related metabolic traits in Danish individuals.

Methods The previously associated polymorphisms were genotyped in the population-based Inter99 cohort ($n=6162$), the Danish ADDITION study ($n=8428$), a population-based sample of young healthy participants ($n=377$) and in additional type 2 diabetes ($n=2107$) and glucose-

tolerant participants ($n=483$) using Taqman allelic discrimination. The case-control study involved 4,104 type 2 diabetic patients and 5,050 glucose-tolerant control participants. Type 2 diabetes-related traits were investigated in 17,521 individuals.

Results rs1535435 and rs9494266 were not associated with type 2 diabetes. Odds ratios (OR) were OR_{add} 1.0 (95% C.I. 0.9–1.2; $p_{add}=0.7$) and OR_{add} 1.1 (0.9–1.2; $p_{add}=0.4$), respectively, a finding supported by meta-analyses: OR_{add} 1.0 (0.9–1.1; $p_{add}=0.6$) and OR_{add} 1.0 (0.9–1.1; $p_{add}=0.6$), respectively. Neither rs1535435 nor rs9494266 were consistently associated with any of the tested type 2 diabetes-related metabolic traits.

Conclusions/interpretation Data from large samples of Danish individuals do not support a role for *AHII* rs1535435 nor rs9494266 as major type 2 diabetes variants. This study highlights the importance of independent and well-powered replication studies of the recent genome-wide association scans before a locus is robustly validated as being associated with type 2 diabetes.

Electronic supplementary material The online version of this article (doi:10.1007/s00125-008-0925-z) contains supplementary material, which is available to authorised users.

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Keywords *AHII* · Association · Chromosome 6 · Genetics · Single nucleotide polymorphism · SNP · Type 2 diabetes

Abbreviations

BIGTT	beta cell function, insulin sensitivity, glucose tolerance test
DGI	Diabetes Genetics Initiative
GWA	genome-wide association
IFG	impaired fasting glycaemia
IGT	impaired glucose tolerance
OR	odds ratio
SNP	single nucleotide polymorphism
WTCCC	Wellcome Trust Case Control Collection

Introduction

Type 2 diabetes is a common, multifactorial disease and a rapidly growing health problem affecting both morbidity and mortality. More than 180 million individuals are reported to suffer from type 2 diabetes with an estimated doubling within the next 15 years [1, 2]. The multifactorial nature of diabetes has made the search for diabetes genes difficult, and only a few genes have rigorously been proven to play a role in type 2 diabetes. Recent technological advances have made genome-wide association (GWA) studies feasible [3, 4] and to date 12 GWA studies for type 2 diabetes have been published [5–16]. These studies have confirmed previously known type 2 diabetes genes (*TCF7L2*, *PPARG* and *KCNJ11*), but have also identified a number of new potential type 2 diabetes genes (*CDKAL1*, *CDKN2A/CDKN2B* locus, *IGF2BP2*, *HHEX/KIF11/IDE* locus, *SLC30A8* and *FTO*) [5–10]. Several of the top-ranked markers in these scans are found in unsuspected type 2 diabetes genes and in regions without annotated genes. Due to the massive number of performed analyses false positive results are expected [17], making replication of GWA data by independent researchers in independent cohorts warranted before a locus is convincingly associated with type 2 diabetes. Genome-wide significant association ($p < 2.3 \times 10^{-7}$) with type 2 diabetes was found in one of the recent GWAs for two polymorphisms (rs1535435 and rs9494266) in the *AH11* locus on chromosome 6q23.3 [11], in proximity of regions that have previously shown suggestive linkage to type 2 diabetes, impaired glucose homeostasis, age at onset of type 2 diabetes and both fasting insulin- and lipid concentrations [18–22]. The association with type 2 diabetes was found for the A-alleles of rs1535435 and rs9494266 in 500 type 2 diabetic patients and 497 control persons originating from four different white populations [rs1535435 odds ratio (OR) 2.3 ($p = 1 \times 10^{-5}$) and rs9494266 OR 2.3 ($p = 2 \times 10^{-5}$)]. These results were replicated in a large French sample (previously used for GWA replication purposes [7]) consisting of 2,573 type 2 diabetic patients and 2,776 control participants [OR=1.3 ($p = 2 \times 10^{-4}$) and OR 1.3 ($p = 5 \times 10^{-5}$)], respectively; French samples and previously mentioned GWA study [11] combined: $p = 1 \times 10^{-6}$ and $p = 2.3 \times 10^{-7}$).

Mutations in *AH11* have previously been shown to cause Joubert syndrome 3 [23], which is an autosomal recessive syndrome characterised by hypoplasia of cerebellar vermis and malformation of the brain stem. Variation in the *AH11* locus has also been associated with schizophrenia in Israeli–Arab families and in an Icelandic population [24–26]. The function of Abelson helper integration site 1 (*AH11*), the protein encoded by *AH11*,

is not known, but it contains multiple SH3 and WD40 domains [27], suggesting that it acts as a scaffolding protein or a docking site [28, 29].

The aim of this study was to validate the recent association with type 2 diabetes ($n=9154$) and explore possible associations with metabolic traits related to type 2 diabetes in Danish individuals ($n=17,521$).

Methods

Participants The two polymorphisms (rs1535435 and rs9494266) were genotyped in 17,521 Danish individuals. Participants from the population-based Inter99 cohort [30] involving 6162 individuals were characterised by an OGTT as having normal glucose tolerance ($n=4567$), impaired fasting glycaemia (IFG; $n=508$), impaired glucose tolerance (IGT; $n=707$) or screen-detected type 2 diabetes ($n=256$) and were investigated for an association between genotype and quantitative metabolic traits; 124 had known type 2 diabetes and were excluded prior to these analyses. An additional 377 individuals, recruited from a population-based sample of young healthy Danish individuals at the Research Centre for Prevention and Health [31] and 8,428 individuals recruited from the ADDITION study sampled through the Department of General Practice at University of Aarhus [32] were enrolled to study quantitative metabolic traits. The case–control study included all unrelated type 2 diabetes patients and healthy glucose-tolerant control individuals from the Inter99 cohort (patients $n=380$, control individuals $n=4,567$) [30], the Danish ADDITION study (patients $n=1,617$; all screen-detected and untreated) [32] and individuals recruited from the outpatient clinic at Steno Diabetes Center (patients $n=2107$, control individuals $n=483$). Detailed description of the participants is given in Electronic supplementary material (ESM; [ESM Supplementary characteristics](#) and ESM Tables 1 and 2). In brief, impaired glucose homeostasis (IFG, IGT and diabetes) was defined according to WHO criteria after a 75 g OGTT [33]. All participants were of Danish nationality and had provided informed written consent before participation. The study was approved by the Ethical Committees of Copenhagen and Aarhus and was in accordance with the principles of the Helsinki Declaration II.

Biochemical and anthropometric measurements Height and body weight were measured in light indoor clothes and without shoes and BMI was calculated as weight (kg) divided by height (m)². Waist circumference was measured in the standing position midway between the iliac crest and the lower costal margin and hip circumference at its maximum. In the Inter99 and Steno Diabetes Center

participants, 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) proinsulin and intact proinsulin, was measured using the AutoDELFIA insulin kit (Perkin-Elmer/Wallac, Turku, Finland). In the 377 healthy participants, serum insulin was measured by ELISA using an insulin kit (Dako Diagnostics, Ely, UK) with overnight incubation. Serum C-peptide concentrations were measured by a time-resolved fluoroimmunoassay (AutoDELFIA C-peptide kit; Perkin-Elmer/Wallac). Serum triacylglycerol and total cholesterol and HDL-cholesterol were analysed using enzymatic colorimetric methods (GPO-PAP and CHOD-PAP; Roche Molecular Biochemicals, Mannheim, Germany). Insulin resistance was estimated according to the homeostasis model assessment index (www.dtu.ox.ac.uk/homa). BIGTT-insulin sensitivity index and BIGTT-acute insulin response, which use information on sex and BMI combined with analysis of plasma glucose and serum insulin levels at time points 0, 30, 120 min to provide indices for insulin sensitivity index (S_I) and acute insulin response (AIR) that highly correlate with indices obtained during an IVGTT, were calculated as described elsewhere [34]. Insulinogenic index_{30 min} was calculated as fasting serum insulin subtracted from plasma insulin_{30 min} (pmol/l) divided by plasma glucose_{30 min} (mmol/l).

Genotyping Genotyping was performed using Taqman allelic discrimination (Kbioscience, Hoddesdon, UK) with a success rate >96.8%. Discordance was <0.1% as judged from re-genotyping of 1202 random duplicate samples.

Statistical analyses Fisher's exact test and logistic regression analyses were used to analyse differences in allele and genotype frequencies between patients and control persons; analyses were adjusted for age, BMI and sex. A general linear model was used to test quantitative variables for genotype differences between type 2 diabetes patients and healthy control participants, adjusting for age, sex, BMI and diabetes status (0 vs 1 variable) in analyses of the complete cohort. Meta-analyses of published data for the putative association between rs1535435 and rs9494266 and type 2 diabetes were performed using a Mantel–Haenszel test to estimate the combined OR of these studies. Power was calculated using the Genetic Power Calculator (<http://pngu.mgh.harvard.edu/~purcell/gpc/>) [35] where type 2 diabetes prevalence was estimated to 10% and with a relative risk of 1.15 (as described in the replication sample in the original article) [11]. The statistical analyses were performed using

RGui version 2.5.0 (available at www.r-project.org). The p values were not adjusted for multiple testing.

Results

The rs1535435 and rs9494266 were genotyped in 17,521 Danish individuals and the potential association with type 2 diabetes was evaluated in 4,104 patients and 5,050 glucose-tolerant control individuals who had previously been used to replicate the associations between *HHEX*, *CDKN2A/B*, *IGF2BP2*, *GCKR* and type 2 diabetes [36]. Allele frequencies were in accordance with previously published results and in Hardy–Weinberg equilibrium ($p > 0.05$). Also, D' and r^2 values ($D' = 0.99$; $r^2 = 0.97$) were in the same range as previously reported. Power to reject the null hypothesis was estimated to ~85% assuming an additive model, risk allele frequency of 8.5% and a relative risk of 1.15 per allele.

The previously reported association between rs1535435 and rs9494266 and type 2 diabetes was not confirmed in our case–control study, which generated the following ORs: OR_{add} 1.0 (95% CI 0.89–1.2; $p_{add} = 0.7$) and OR_{add} 1.1 (95% CI 0.90–1.2; $p_{add} = 0.4$), respectively (Table 1). Meta-analyses for rs1535435 and rs9494266 combining data from the present study with data from the Diabetes Genetics Initiative (DGI) [5] and from the Wellcome Trust Case Control Consortium (WTCCC) [10] GWA studies did not support an association with type 2 diabetes: OR_{add} 0.97 (95% CI 0.89–1.06; $p = 0.6$) and OR_{add} 0.98 (95% CI 0.91–1.06; $p = 0.6$), respectively (Fig. 1a,b).

Furthermore, rs1535435 and rs9494266 were investigated for an association with type 2 diabetes-related quantitative traits in the population-based Inter99 sample involving 6,038 treatment-naïve middle-aged individuals (ESM Table 2). The rs1535435 A-allele was nominally associated with increased serum insulin and C-peptide concentrations at 120 min ($p_{add} = 0.01$ and $p_{add} = 0.004$, respectively). Increased AUC for serum insulin, plasma glucose and C-peptide concentrations was also observed ($p_{add} < 0.05$, $p_{add} = 0.04$ and $p_{add} = 0.03$, respectively). The rs9494266 A-allele was nominally associated with increased serum insulin and C-peptide concentrations at 120 min ($p_{add} = 0.01$ and $p_{add} = 0.004$, respectively). Increased AUC for plasma glucose was observed for the rs9494266 A-allele ($p_{add} = 0.01$).

Registered data on plasma glucose (ADDITION, capillary blood glucose), BMI, total cholesterol and HDL-cholesterol were available for 17,521 participants (from Inter99, ADDITION and Steno study samples). Participants homozygous for the rs1535435 and rs9494266 A-alleles

Table 1 Association study of type 2 diabetes in 4,104 type 2 diabetes patients and 5,050 glucose-tolerant control participants

SNP	Allele (minor/major)	MAF (%)	Genotype	Genotype distribution		Allele association OR (95% CI) ^a	Additive model OR (95% CI) ^b	Dominant model OR (95% CI) ^b	Recessive model OR (95% CI) ^b
				NGT, n (%)	T2D, n (%)				
rs1535435	A/G	8.4	GG	4,019 (84)	3,228 (84)	1.0 (0.9–1.1)	1.0 (0.9–1.2)	1.0 (0.9–1.2)	0.9 (0.4–1.9)
			GA	731 (15)	594 (15)	<i>p</i> =0.8	<i>p</i> =0.7	<i>p</i> =0.6	<i>p</i> =0.8
			AA	40 (1)	25 (1)				
rs9494266	A/G	8.5	GG	3,971 (84)	3,222 (84)	(0.9–1.1)	1.1 (0.9–1.2)	1.1 (0.9–1.3)	1.2 (0.6–2.6)
			GA	729 (15)	598 (15)	<i>p</i> =1.0	<i>p</i> =0.4	<i>p</i> =0.5	<i>p</i> =0.6
			AA	35 (1)	26 (1)				

MAF, minor allele frequency; NGT, normal glucose tolerance; T2D, type 2 diabetes

^aNot adjusted for age, BMI or sex^bAdjusted for age, BMI and sex

had reduced BMI compared with carriers of the G-allele [27.8 ± 5.0 vs 26.7 ± 4.4 kg/m² ($p_{\text{rec}}=0.03$) and 27.8 ± 5.0 vs 26.9 ± 4.5 kg/m² ($p_{\text{rec}} < 0.05$), respectively] (ESM Table 2). Analyses were adjusted for age, BMI, sex and diabetes status, since the complete cohort also includes individuals with type 2 diabetes.

Discussion

The *AHII* locus is located on chromosome 6q23.3, which has shown suggestive linkage to type 2 diabetes and related metabolic traits [18–22]. In a recent GWA study for type 2 diabetes, two single-nucleotide polymorphism (SNPs;

Fig. 1 Meta-analysis for **a** rs9494266 and **b** rs1535435. Values are ORs with 95% CI. For each study, the vertical tick represents the OR for the given genotype and the horizontal line represents its 95% CI. Imputed data for rs1535435 was available from the WTCCC [10], but

not from the DGI [5] study. Numbers within parentheses represents the number of type 2 diabetes patients and glucose-tolerant control participants in the individual studies

rs1535435 and rs9494266) in the *AHII* locus were associated with type 2 diabetes and were internally replicated with significant p values in an independent large cohort [11]. This is the only one of 12 recent GWAs for type 2 diabetes that has shown GWA ($p < 2.3 \times 10^{-7}$) at this locus. Based on the strong association data and data describing suggestive linkage to type 2 diabetes and related metabolic traits adjacent (centromeric) to this locus, we decided to investigate the putative association with type 2 diabetes and its related quantitative metabolic traits for the recently described polymorphisms associated with type 2 diabetes in the *AHII* locus in a large independent sample of well-characterised Danish individuals.

We were not able to confirm the previously reported association with type 2 diabetes for rs1535435 and rs9494266, either in our case–control sample comprising >9,000 individuals ($p=0.7$ and $p=0.4$), or in a meta-analysis comprising >16,300 individuals drawn from our study and from the publicly available DGI and WTCCC GWAs [5, 10] ($p=0.6$ and $p=0.6$, respectively). Neither were we able to show significant association with investigated type 2 diabetes-related quantitative metabolic traits in the large population-based Inter99 cohort ($n=6038$) or when analysing all participants ($n=17521$, recruited from the Inter99 cohort, the ADDITION and Steno study samples).

Our results are in contrast to those from Salonen et al. [11], who showed that rs1535435 and rs9494266 in the *AHII* locus were associated with type 2 diabetes. The discrepancy between the results in that study and ours might suggest that the results in the original study were due to chance. That initial report showed surprisingly high OR (2.3), possibly representing a ‘jackpot’ effect due to heterogeneity of the GWA samples (derived from four different white populations), differences in study design and the low power in the sample used in the genome-wide approach. It should be appreciated that $p < 2.3 \times 10^{-7}$ used in the original study to declare genome-wide significance only represents borderline significance in the context of GWAs [37]. Other explanations for the discrepant results could be that the two polymorphisms are in high linkage disequilibrium with a causative variant in the original publication investigating Finns, Ashkenazi Jews and UK, German and French individuals; but this was not the case in our study, which investigated Danes. In this context, it is important to note that we limited our search to just these two SNPs and could therefore have missed a potential causative variant in the *AHII* locus that is not in linkage disequilibrium with these two SNPs in our population. Despite the strong internal replication in a large well-powered sample of different ethnic origin (French), no association with type 2 diabetes was seen for rs9494266 (or imputed data for rs1535435) in the recent DGI and WTCCC GWA studies

[5, 10], which cover ~77% ($r^2 > 0.8$) of the common genetic variation at this locus. Internal replication efforts are often biased (errors in phenotyping or genotyping and population stratification) and might be an explanation for the results in the original publication, indicating the need for independent validation. In this respect, our study is an independent statistically well-powered association study in >17,000 individuals, looking for a possible role of two previously genome-wide associated SNPs in the *AHII* locus in the development of type 2 diabetes.

In conclusion, we have shown that the two previously associated SNPs (rs1535435 and rs9494266) in *AHII* locus are not associated with type 2 diabetes or related quantitative metabolic traits.

Acknowledgements The authors wish to thank A. Forman, I.-L. Wantzin and M. Stendal for technical assistance and G. Lademann for secretarial support. The study was supported by grants from the European Union (EUGENE2, grant no. LSHM-CT-2004–512013), the FOOD Study Group/the Danish Ministry of Food, Agriculture and Fisheries and the Ministry of Family and Consumer Affairs (grant no. 2101–05–0044).

Duality of interest K. Borch-Johnsen holds stock in Novo Nordisk and has received lecture fees from pharmaceutical companies. The remaining authors declare that there is no duality of interest associated with this manuscript.

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