

The *GCKR* rs780094 polymorphism is associated with elevated fasting serum triacylglycerol, reduced fasting and OGTT-related insulinaemia, and reduced risk of type 2 diabetes

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Abstract

Aims/hypothesis Recent genome-wide association studies have suggested that a polymorphism in *GCKR*, the gene encoding the glucokinase regulatory protein, is involved in

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triacylglycerol regulation. Our aim was to examine in large-scale studies the common *GCKR* rs780094 polymorphism in relation to metabolic traits (mainly fasting hypertriacylglycerolaemia) and traits related to pancreatic beta cell function.

Methods The polymorphism was genotyped in 16,853 Danes using Taqman allelic discrimination. Association was analysed in case-control studies and quantitative trait analyses. We also analysed the possible interactive effect between the *GCK* -30G>A polymorphism and the *GCKR* rs780094 variant on metabolic traits.

Results The minor *GCKR* A-allele of rs780094 is associated with an increased level of fasting serum triacylglycerol ($p=6\times 10^{-14}$), impaired fasting ($p=0.001$) and OGTT-related insulin release ($p=3\times 10^{-6}$), reduced homeostasis model assessment of insulin resistance ($p=0.0004$), WHO-defined dyslipidaemia ($p=6\times 10^{-9}$) and a modestly decreased risk of type 2 diabetes ($p=0.01$). Significantly increased fasting serum insulin concentrations were demonstrated when analysing the *GCK* -30A and *GCKR* rs780094 G-alleles in an additive model.

Conclusions/interpretation The *GCKR* rs780094 polymorphism, or another variant with which it is in tight linkage disequilibrium, is likely to increase glucokinase regulatory protein activity to induce improved glycaemic regulation at the expense of hypertriacylglycerolaemia as reflected in the present study of 16,853 Danes. We also suggest an additive effect of *GCK* and *GCKR* risk alleles on plasma glucose and serum insulin release.

Keywords Association · Genetics · Genome-wide association · Glucokinase · Triacylglycerol · Type 2 diabetes

Abbreviations

GK	glucokinase
GKRP	glucokinase regulatory protein
GWA	genome-wide association
HOMA-IR	homeostasis model assessment of insulin resistance
MAF	minor allele frequency
SNP	single-nucleotide polymorphism

Introduction

Liver and beta cell glucokinase (GK) plays a key role in the regulation of blood glucose homeostasis by enhancing insulin secretion from the pancreatic beta cells and by regulating glucose utilisation and production in the liver [1]. GK is regulated by the glucokinase regulatory protein (GKRP), a process depending on fructose 6-phosphate and fructose 1-phosphate [2]. The relationship between GK and diabetes mellitus has been demonstrated by the identification of specific rare mutations in *GCK* (the gene encoding GK) as the cause of subsets of maturity-onset diabetes of the young [3] and permanent neonatal diabetes mellitus [4]. Similarly, a common $-30G>A$ promoter polymorphism of *GCK* increases the risk of hyperglycaemia in the general population [5]. The *GCKR* gene encodes GKRP and is localised to chromosome 2p23.2–3 [6], a genomic region previously linked to metabolic traits like fat mass and circulating lipid concentrations [7]. *Gckr*-deficient mice display reduced production of hepatic GK and exhibit impaired postprandial glycaemic control, albeit with no noteworthy loss in insulin secretion or changes in fasting blood glucose concentrations [8, 9]. Adenoviral-mediated hepatic overproduction of GKRP significantly improved fasting and glucose-stimulated glycaemia in mice and resulted in a concomitant increase in insulin sensitivity, decreased leptin concentrations and increased triacylglycerol levels [10]. Previous studies of *GCKR* have attempted to relate frequent genetic variation to metabolic phenotypes; however, results have until recently been either negative or performed in insufficient study samples [11, 12]. In a study of 57 unrelated French obese participants from families with linkage to chromosome 2p21–23, mutation detection resulted in the identification of three rare amino acid substitutions in addition to a known common Pro446Leu substitution and an intronic polymorphism [12]. None of the rare variants showed co-segregation with obesity. Moreover, in a case–control study of 720 obese and 384 lean participants, no difference in allele or genotype frequencies of the Pro446Leu polymorphism was detected [12]. By contrast, a tendency towards increased postprandial plasma insulin concentrations among codon 446 Leu carriers was observed in 520 Danish twins, although this study was of a limited size [11].

Recent advances in genotyping techniques have facilitated the use of genome-wide association (GWA) studies in thorough investigations of the genetic background underlying common polygenic and multifactorial traits of the metabolic syndrome [13–19]. Such GWA studies have confirmed several known or putative type 2 diabetes genes such as *PPARG*, *KCNJ11* and *TCF7L2* [13, 15, 16, 18, 19], and have provided several new and promising insights represented by variation in *SLC30A8*, *CDKAL1*, *HHEX*, *CDKN2A*, *CDKN2B* and *IGF2BP2* [13, 15, 16, 18, 19]. Some of these novel candidate genes have no or limited known biological function and both validation of trait association and genotype–physiology studies of such novel findings are crucial. Initial GWA studies of type 2 diabetes and related metabolic traits have revealed a strong association of the *GCKR* gene polymorphism rs780094 (minor A-allele) with hypertriacylglycerolaemia [13, 15]. Curiously, the investigators also observed modestly decreased plasma glucose levels, increased insulin sensitivity as estimated by the homeostasis model assessment of insulin resistance (HOMA-IR) and lower risk of type 2 diabetes [13]. Here, we sought to validate the reported associations between the *GCKR* rs780094 polymorphism and hypertriacylglycerolaemia. We further explored the relationship of this variant with extensive metabolic phenotypes in a large study of Danish white participants. Additionally, we tried to identify possible interactive effects between the *GCKR* rs780094 and *GCK* $-30G>A$ polymorphisms.

Methods

Study population The *GCKR* rs780094 polymorphism was genotyped in 16,853 Danes comprising: (1) the population-based Inter99 sample of middle-aged Danes sampled at Research Centre for Prevention and Health ($n=5,897$) [20]; (2) type 2 diabetic patients sampled through the outpatient clinic at Steno Diabetes Center ($n=2035$); (3) a population-based group of middle-aged glucose-tolerant participants recruited by Steno Diabetes Center ($n=504$); and (4) the ADDITION study group sampled through Department of General Practice at University of Aarhus ($n=8,417$) [21]. Detailed descriptions of study populations are available in the Electronic supplementary material (ESM; ESM Table 1).

Study groups 1 and 3 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. Type 2 diabetes, obesity and dyslipidaemia were defined according to WHO [22].

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. Waist circumference was measured in the standing position, midway between the iliac crest and the lower costal margin; hip circumference was measured at its maximum. Blood samples were drawn after a 12 h overnight fast. Plasma glucose was analysed by a glucose oxidase method (Granustest; 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 an insulin kit (AutoDELFIA; Perkin-Elmer/Wallac, Turku, Finland). Serum C-peptide concentrations were measured by a time-resolved fluoroimmunoassay (AutoDELFIA C-peptide kit; Perkin-Elmer/Wallac). Serum triacylglycerol, total cholesterol and HDL-cholesterol were analysed using enzymatic colorimetric methods (GPO-PAP and CHOD-PAP; Roche Molecular Biochemicals, Basel, Switzerland). HOMA-IR was calculated as described in [23]. (BIGTT-S_I) and (BIGTT-AIR) were calculated as described [24].

Genotyping The *GCKR* rs780094 polymorphism was genotyped using Taqman allelic discrimination (KBioscience, Hoddesdon, UK). Discordance between 1,464 random duplicate samples was 0.3% and the genotyping success rate was 98%. All genotype groups obeyed Hardy–Weinberg equilibrium. Genotyping of the *GCK* –30G>A polymorphism (rs1799884) has been previously described [5].

Statistical analysis Fisher's exact test was applied to examine differences in allele frequencies and genotype distributions between affected and unaffected participants. A general linear model was used to test quantitative variables for differences between genotype groups among glucose-tolerant and untreated participants. In order to assess the combined effect of two single-nucleotide polymorphisms (SNPs) in *GCK* and *GCKR*, we used a linear model assuming equal effects of each risk allele at the two loci and compared this with a model lacking the risk allele parameter. However, in order to make such assumptions, we first tested whether the effect size of each allele was equal. All analyses were performed using SPSS version 14.0 (Chicago, IL, USA and RGui version 2.5.0 (<http://www.r-project.org>)). A *p* value of less than 0.05 was considered to be significant.

Results

The rs780094 was genotyped in a total of 16,853 Danes and had an overall minor allele frequency (MAF) of 34.7%.

We analysed quantitative metabolic traits reflecting fasting and glucose-stimulated levels of serum insulin, serum C-peptide and plasma glucose (0, 30 and 120 min during an OGTT) as well as fasting serum lipids (triacylglycerol, total cholesterol and HDL-cholesterol) in the population-based Inter99 study sample of 5,780 individuals with normal glucose tolerance (74%), impaired fasting glucose (8%), impaired glucose tolerance (11%) or screen-detected, treatment-naïve type 2 diabetes (6%). The rs780094 minor A-allele was highly associated with reduced fasting ($p=0.001$), 30 min ($p=1.3\times 10^{-7}$) and 120 min ($p=0.03$) post-OGTT insulin release (Table 1). Concomitantly, reduced fasting, 30 min and 120 min serum C-peptide concentrations were demonstrated, as well as increased insulin sensitivity as assessed by HOMA-IR ($p=0.0004$) and BIGTT-S_I ($p=0.01$; Table 1). Accordingly, a significant decrease was observed in the incremental AUC for serum C-peptide and insulin during an OGTT in participants homozygous for the minor A-allele. No considerable relationship was observed with levels of plasma glucose (fasting, 30 min or 120 min post-OGTT) or the incremental AUC for glucose (Table 1). For fasting serum triacylglycerol we observed a major increase in concentrations related to the rs780094 A-allele ($p=6\times 10^{-14}$) and this was accompanied by a minor elevation of total fasting serum total cholesterol ($p=0.03$).

Three case–control studies of rs780094 were performed in relation to type 2 diabetes, BMI-defined obesity and dyslipidaemia. The polymorphism was associated with type 2 diabetes ($p=0.02$ for genotype distribution and $p=0.01$ for MAF; Table 2). In a second case–control study the participants were stratified according to their BMI levels; however, we did not observe any differences in MAF or genotype distribution between individuals with a BMI < 25 kg/m² and those with a BMI > 30 kg/m² (Table 2). Finally, we investigated the impact of the rs780094 genotypes on healthy normolipidaemic individuals as opposed to participants with WHO-defined dyslipidaemia. Highly significant differences were observed in the MAF [33.3% (95% CI 32.4–34.2%) vs 38.3% (36.9–39.8%), $p=6\times 10^{-9}$] and the distribution of genotypes ($p=6\times 10^{-9}$) between the two groups (Table 2).

We investigated the additive effect of *GCKR* rs780094 and *GCK* –30G>A on fasting and post-OGTT concentrations of plasma glucose and serum insulin in the Inter99 study sample, assuming that the rs780094 G-allele and –30A-allele were the risk alleles (Fig. 1). We observed a significantly increased fasting serum insulin concentration in a model including both of the two SNPs ($p=0.0002$ for additivity) when compared with the null model (no SNPs added). The additive model included one additive covariate ($X_{\text{add}}=0, 1, 2, 3, 4$; for each risk allele of the two SNPs). A test for non-linear interaction (epistasis) between the two SNPs was negative.

Table 1 Anthropometric and metabolic characteristics of 5,780 treatment-naïve Danish participants (Inter99) stratified according to *GCKR* rs780094 genotype

Characteristics	GG	GA	AA	p_a value	p_d value	P_r value
<i>n</i> (men/women)	2,461 (1,209/1,252)	2,617 (1,320/1,297)	702 (349/353)			
Age (years)	46±8	46±8	46±8			
BMI (kg/m ²)	26.2±4.5	26.2±4.5	26.2±4.6	0.9	0.9	0.7
Fasting serum lipids (mmol/l)						
Triacylglycerol	1.27±1.50	1.32±0.98	1.59±1.90	6×10^{-14}	1×10^{-8}	6×10^{-12}
Total cholesterol	5.5±1.0	5.6±1.1	5.6±1.1	0.03	0.03	0.3
HDL-cholesterol	1.4±0.4	1.4±0.4	1.4±0.4	0.08	0.1	0.2
Plasma glucose (mmol/l)						
Fasting	5.6±0.8	5.5±0.8	5.5±0.9	0.008	0.02	0.07
30 min post-OGTT	8.7±1.8	8.7±1.9	8.6±2.0	0.1	0.3	0.2
120 min post-OGTT	6.2±2.0	6.2±2.1	6.3±2.4	0.2	0.4	0.1
AUC (mmol/l×min)	219±133	221±136	225±139	0.3	0.5	0.3
Serum insulin (pmol/l)						
Fasting	35 (24–52)	34 (23–50)	32 (23–49)	0.001	0.003	0.03
30 min post-OGTT	256 (181–369)	238 (174–342)	232 (167–333)	1×10^{-7}	4×10^{-7}	0.001
120 min post-OGTT	158 (98–261)	155 (96–249)	154 (90–242)	0.03	0.1	0.06
AUC (pmol/l×min)	19,400 (13,640–28,940)	18,160 (12,800–26,140)	18,120 (12,140–26,240)	3×10^{-6}	2×10^{-5}	0.002
Serum C-peptide (pmol/l)						
Fasting	602±272	595±276	577±264	0.002	0.02	0.004
30 min post-OGTT	2,042±739	1,985±708	1,920±659	1×10^{-5}	0.0001	0.001
120 min post-OGTT	2,317±1,011	2,315±1,021	2,251±1,001	0.09	0.4	0.02
AUC (pmol/l×min)	163,519±58,812	160,476±57,443	155,907±54,927	1×10^{-4}	0.001	0.002
HOMA-IR	8.6 (5.8–13.0)	8.2 (5.5–12.8)	7.8 (5.3–12.3)	0.0004	0.001	0.01
BIGTT-S _i	9.0 (6.0–11.9)	9.3 (6.5–12.2)	9.7 (6.7–12.2)	0.01	0.01	0.2
BIGTT-AIR	1,639 (1,296–2,097)	1,606 (1,261–2,067)	1,664 (1,314–2,051)	0.04	0.02	0.5

Data are means±SD or median (interquartile range)

Values of serum insulin, values derived from insulin variables and values of serum triacylglycerol were logarithmically transformed before statistical analysis. All analyses were made using additive, dominant and recessive models

Calculated *p* values were adjusted for age, sex and BMI (where appropriate); genotype and sex were considered as fixed factors and age and BMI as covariates

AUC, incremental area under the curve

Discussion

In the present large population-based study of treatment-naïve middle-aged people in Inter99 we validated the recent report [13, 15] of a relationship between the minor A-allele of the *GCKR* rs780094 polymorphism and increased fasting serum triacylglycerol concentrations. Further studies of quantitative variables of metabolism showed that carriers of the minor A-allele had a substantially lower serum insulin release and were more insulin-sensitive. In statistically powered case-control studies involving 7,619 middle-aged participants, we demonstrated that the variant conferred an increased risk of WHO-defined dyslipidaemia and a modestly lower risk of type 2 diabetes. We failed to demonstrate any significant association with obesity.

Improved glycaemic regulation at the expense of hypertriacylglycerolaemia and other abnormalities in energy metabolism has been demonstrated in mice and rat liver overproducing either GK or GKR [10, 25]. GKR is

produced in molar excess over GK and the regulation of GK by GKR may be very sensitive to changes in tissue levels of GKR [6]. Thus, we speculate that the *GCKR* rs780094 polymorphism, or another variant with which it is in tight linkage disequilibrium, influences GKR production levels and thereby induces an increased function of GKR. The observed decrease in serum insulin release among *GCKR* rs780094 carriers may lead to a secondary increase in serum triacylglycerol due to increased hepatic fatty acid oxidation, while the *GCK* -30G>A polymorphism influencing beta cell-specific GK production may act to increase hyperglycaemia by elevating the threshold for glucose-stimulated insulin release. In studies of the general middle-aged population the *GCK* -30G>A polymorphism has previously shown a graded relationship with hyperglycaemia [5]. Therefore, we examined the possible two-way epistasis of this variant and the *GCKR* rs780094 polymorphism on plasma glucose and serum insulin concentrations in the Inter99 study sample, but failed to

Table 2 Genotype distribution and MAF for the *GCKR* rs780094 polymorphism in participants stratified according to glucose tolerance status, BMI levels and dyslipidaemia

	<i>n</i> (men/women)	GG	GA	AA	MAF (95% CI)	OR (95% CI)
Glucose tolerance status						
NGT	4,891 (2,272/2,619)	2,066 (42)	2,234 (46)	591 (12)	34.9 (34.0–35.9)	
Diabetes	3,878 (2,302/1,573)	1,755 (45)	1,681 (43)	442 (12)	33.1 (32.1–34.2)	
p_{GD} value			0.02			0.92 (0.87–0.98)
p_{MAF} value					0.01	
BMI status						
BMI <25 kg/m ²	5,423 (2,270/3,153)	2,358 (44)	2,442 (45)	623 (12)	34.0 (33.1–34.9)	
BMI ≥30 kg/m ²	4,909 (2,535/2,374)	2,161 (44)	2,183 (45)	565 (12)	33.7 (32.8–34.7)	
p_{GD} value			0.8			0.99 (0.93–1.05)
p_{MAF} value					0.7	
Lipid status						
Normolipidaemia	5,415 (2,437/2,978)	2,385 (44)	2,450 (45)	580 (11)	33.3 (32.4–34.2)	
Dyslipidaemia	2,204 (1,378/826)	852 (39)	1,014 (46)	338 (15)	38.3 (36.9–39.8)	
p_{GD} value			6×10^{-9}			1.24 (1.15–1.33)
p_{MAF} value					5×10^{-9}	

Data are number of participants with each genotype (% of each group); frequencies of the minor A-allele (MAF) are in percentages
Dyslipidaemia was defined as serum triacylglycerol ≥1.7 mmol/l or HDL-cholesterol <0.9 mmol/l for men or <1.0 mmol/l for women or current or previous treatment with lipid-lowering drugs

All *p* values were calculated using Fisher's exact test and compare genotype distributions (p_{GD}) and MAFs (p_{MAF})

NGT, normal glucose tolerance; OR, odds ratio

show any evidence of an interaction (data not shown). However, when combining the two risk alleles, we demonstrated a significant effect on fasting serum insulin concentrations in a model including an additive covariate. Even though the two specific gene products may act in different tissues, polymorphisms in *GCK* and *GCKR* possibly combine to further increase the risk of hyperglycaemia compared with the individual risk variants.

Although we observed a large effect of the rs780094 on serum insulin concentrations and a modest effect size on type 2 diabetes compared with persons with normal glucose tolerance, we were somewhat surprised that we were unable to find a significant effect on plasma glucose levels at 30 and 120 min post-OGTT; however, it seems that the effect of

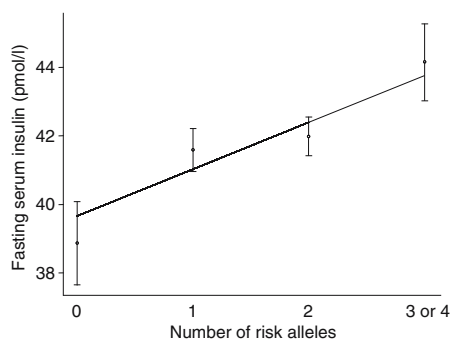


Fig. 1 Additive effect of the *GCKR* rs780094 and *GCK* -30G>A variants on fasting serum insulin. Values are means±SD. Risk alleles are the rs780094 G-allele and -30A-allele. $p=0.0002$ for the two SNPs (estimated)

rs780094 on serum insulin may not be sufficient to influence plasma glucose levels. Moreover, the fact that glucose levels are regulated by various compensatory mechanisms may also explain this observation. In addition, the polymorphism in *GCKR* seems to associate with type 2 diabetes through reduced beta cell function and individuals carrying the risk allele may compensate for this reduction with a healthy life style.

According to the HapMap website (<http://www.hapmap.org/index.html.en>) the *GCKR* rs780094 polymorphism is located in a large haplotype block spanning at least 500 kb and including several other known genes, which indeed questions the proposal that the intronic rs780094 is the causative SNP responsible for the observed metabolic changes. A thorough mutation analysis of the DNA region in question needs to be carried out, e.g. by nucleotide sequencing of selected individuals, in order to identify genetic variation that may explain to a larger extent the inter-individual variation in metabolic variables. In addition, the combined effect (such as an additive, epistatic or haplotypes) of other polymorphisms within the region or the regulatory pathway may contribute significantly to the observed differences in metabolic traits. Also, possible gene–environment interactions need to be considered and investigated [26].

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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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