Research Article

Hepatic glucokinase promoter polymorphism is associated with hepatic insulin resistance in Asian Indians.

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Abstract

Background: The role of glucokinase (GCK) in the pathogenesis of maturity-onset diabetes of the young is well established. However, its role in the common form of type 2 diabetes is far from convincing. We investigated the role of the G-to-A polymorphism in the hepatic GCK promoter on insulin sensitivity and beta cell function in 63 normotensive Asian Indians with normal glucose tolerance. As proposed by Matsuda and DeFronzo, hepatic insulin sensitivity (ISI_H) and total body insulin sensitivity (ISI_M) were estimated from the oral glucose tolerance test. Beta cell function was estimated using %B from the Homeostasis Model Assessment and insulingenic index (dI/dG).

Result: We identified 38 GG, 24 GA, and one AA subjects. The AA subject was pooled with the GA subjects during the analysis. No difference was noted in the demographic features between the two genotypic groups (GG vs. GA/AA). Compared to the GG group, the GA/AA group had a lower ISI_H ($p=0.002$), a lower ISI_M ($p=0.009$), a higher %B ($p=0.014$), and a higher dI/dG ($p=0.030$). Multivariate analysis revealed that this polymorphism is an independent determinant for ISI_H ($p=0.019$) and along with age, waist-hip ratio, gender, and diastolic blood pressure accounted for 51.5% of the variation of ISI_H. However, this polymorphism was a weak, but independent determinant for ISI_M ($p=0.089$) and %B ($p=0.083$). Furthermore, it had no independent effect on dI/dG ($p=0.135$).

Conclusions: These data suggest that the G-to-A polymorphism in the hepatic GCK promoter is associated with hepatic insulin resistance in Asian Indians.

Introduction

Glucokinase (GCK) was originally proposed to be a glucose sensor and metabolic signal generator in pancreatic beta cells and hepatocytes [1]. The discoveries of a linkage and subsequent identification of mutated GCK genes [2,3] in families with maturity-onset diabetes of the young (MODY) provide the strongest evidence for a crucial role of GCK in the pathogenesis of MODY [1]. However, the structural mutations (missense, nonsense mutation, or mutations affecting the splicing mechanism) of GCK were only found in less than 1% of patients with type 2 diabetes [4]. Thus, the mutated GCKs do not play a key role in the pathogenesis of most forms of diabetes.

Nonetheless, some studies suggest that defective liver GCK may play a role in the pathogenesis of insulin resistance and type 2 diabetes [5]. In patients with type 2 diabetes who underwent elective cholecystectomy, hepatic GCK activity was decreased by about 50% in obese dia-

betic subjects compared to lean controls and obese controls [5]. Hyperglycemia in animals has been shown to decrease hepatic GCK activity, which can be reversed by treatment with insulin [6]. We previously reported a G-to-A polymorphism at the nucleotide position -258 of the hepatic GCK promoter [7]. It occurred within a fragment that was completely conserved between human and rat [8,9]. The basic motif surrounding this variant was almost identical to a well-studied insulin responsive sequence (IRS) of the phosphoenolpyruvate carboxykinase (PEPCK) gene [10]. Since hepatic GCK is regulated by insulin [9], we hypothesized that this polymorphism could be related to insulin resistance.

Results

We studied the hepatic GCK promoter polymorphism in 63 normotensive Asian Indians with normal glucose tolerance (Table 1 Clinical characteristics of the studied subjects). Since insulin sensitivity is impaired in non-diabetic subjects with essential hypertension [11], only those with normal blood pressure (< 140/90 mmHg) were enrolled into the study. Since impaired glucose tolerance and overt diabetes are associated with insulin resistance and since glucose toxicity could affect beta cell function and insulin sensitivity [12], only those subjects with a fasting plasma glucose concentration less than 6.1 mM, interval plasma glucose concentrations less than 11.1 mM, and a two-hour plasma glucose concentration less than 7.8 mM were enrolled in the study. By eliminating factors that contribute independently to insulin resistance, such as hypertension and abnormal glucose tolerance, any effect regarding genetic influence per se becomes more apparent.

Their genotypes were determined using a PCR-RFLP assay. We identified 38 GG, 24 GA, and one AA subjects with the allelic frequencies of 79% and 21%, respectively for the G and A allele. The distribution of genotypes was in compliance with the Hardy-Weinberg equilibrium (p=0.537). Since only one AA subject was identified, this subject was pooled with the GA subjects during the analysis. Both genotypic groups (GG vs. GA/AA) had similar demographic features (Table 2 Demographic features and glycemic parameters by genotypes). During an OGTT, the GA/AA group had a lower plasma glucose concentration at 90 minutes than the GG group (p=0.015, Figure 1) and higher plasma insulin concentrations at fasting and 60 minutes than the GG group (p=0.003 and p=0.008, respectively).

Hepatic insulin sensitivity (ISI_H) and whole body insulin sensitivity (ISI_M) were estimated from the OGTT as described by Matsuda and DeFronzo [13]. Beta cell function (%B) was estimated from the HOMA [14] and dI/dG (the ratio of the incremental response in insulin to that of glucose during the first 30 minutes of the OGTT). The

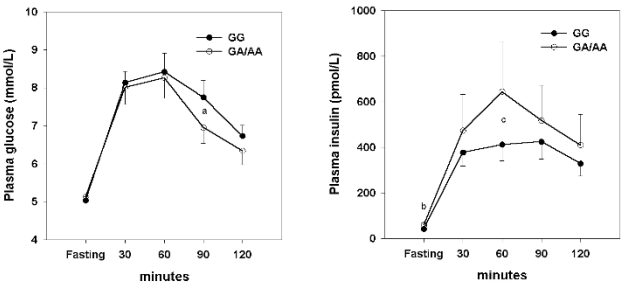

Figure 1
Plasma glucose and insulin concentrations during oral glucose tolerance tests Data present mean (glucose) or geometric mean (insulin) with 95% confidence intervals. [a]p=0.015; [b] p=0.003; [c] p=0.008.

GA/AA group had a lower ISI_H (p=0.002) and ISI_M (p=0.009) than the GG group. This polymorphism accounted for 14.4% and 10.7% of the variations in ISI_H and ISI_M, respectively. In contrast, the GA/AA group had better beta cell function, based on %B and dI/dG, compared to GG group (Table 2). Demographic features and glycemic parameters by genotypes).

Multivariate analysis showed that this polymorphism was an independent determinant for ISI_H (p=0.019) and along with age, waist-hip ratio, gender, and diastolic blood pressure explained 51.5% of the variation in ISI_H (Table 3 Stepwise regression analysis of the estimated indices for insulin sensitivity and beta cell function). However, systolic blood pressure and body mass index had no impact on ISI_H. Since hepatic insulin sensitivity (ISI_H) correlated very well with the whole body insulin sensitivity (ISI_M, $p < 0.0001$, $r^2=0.800$), this polymorphism also had an independent but marginal impact on ISI_M (p=0.089). In contrast to hepatic insulin sensitivity, this polymorphism had less impact on beta cell function (9.5% and 7.5% of the variations in %B and dI/dG, respectively). Multivariate analyses showed that this polymorphism was weakly associated with %B (p=0.083), but not dI/dG (p=0.135).

Discussion

Our data show that the G-to-A polymorphism at the -258 nucletotide position of the hepatic GCK promoter is an independent determinant for ISI_H, but has only marginal impacts on ISI_M and %B, and no impact on dI/dG. Hepatic and whole body insulin sensitivities are well correlated to each other [13] and a better correlation between this polymorphism and ISI_H was observed than with ISI_M. This suggests that the primary impact of this polymorphism is on ISI_H. Since all the subjects were glu-

Table 1: Clinical characteristics of the studied subjects

		n	%	Mean	Standard error	Minimum	Maximum
n		63					
Gender	female	38	60%				
Age	year			41 ± 1		19	68
Body mass index	kg/m²			23.72 ± 0.42		16.93	32.61
Waist hip ratio	cm/cm			0.828 ± 0.010		0.0684	0.969
Systolic blood pressure	mmHg			110 ± 1		88	134
Diastolic blood pressure	mmHg			72 ± 1		58	88
Oral glucose tolerance test							
Fasting plasma glucose	mmol/L			5.07 ± 0.04		4.36	5.77
Plasma glucose at 30 minutes	mmol/L			8.08 ± 0.12		5.00	10.05
Plasma glucose at 60 minutes	mmol/L			8.35 ± 0.18		5.16	10.94
Plasma glucose at 90 minutes	mmol/L			7.43 ± 0.16		4.72	10.60
Plasma glucose at 120 minutes	mmol/L			6.57 ± 0.11		4.39	7.76

Table 2. Demographic features and glycemic parameters by genotypes

			GG Mean (n)	95% CI (%)	GA/AA Mean (n)	95% CI (%)
N			38		25	
Gender		F	24	63%	14	56%
Age		year	42	(39, 45)	40	(37, 43)
Body mass index	[a]	kg/m²	23.13	(22.05, 24.27)	24.09	(22.92, 25.32)
Waist-hip ratio	[a]	cm/cm	0.814	(0.790, 0.839)	0.840	(0.808, 0.873)
Systolic blood pressure		mmHg	108	(105, 112)	111	(107, 116)
Diastolic blood pressure		mmHg	72	(69, 74)	73	(70, 76)
Fasting plasma glucose		mmol/L	5.03	(4.93, 5.13)	5.13	(4.98, 5.28)
Fasting plasma insulin	[a,b]	pmol/L	42	(37, 49)	60	(50, 72)
ISI_H	[a,c]		0.76	(0.66, 0.88)	0.52	(0.43, 0.63)
ISI_M	[a,d]		5.61	(4.87, 6.48)	4.08	(3.33, 5.00)
% B	[a,e]		78	(67, 92)	105	(89, 124)
dI/dG	[a,f]	pmol/mmol	111	(91, 135)	170	(116, 251)

[a] geometric means, (95% CI); [b] p=0.003 for; [c] p=0.002; [d] p=0.009; [e] p=0.014; [f] p=0.030.

cose tolerant, their beta cell function will compensate for the prevailing insulin resistance to maintain plasma glucose concentration wthin a relatively narrow physiological range. The observed differences in %B and dI/dG between the two groups are most likely due to the compensatory increase of beta cell response to the differences in insulin sensitivity. This interpretation is consistent with the nature of this polymorphism, which occurs within the hepatic GCK promoter and not in the beta cell GCK promoter. Therefore, these results indicate that the polymorphism mainly affects hepatic insulin sensitivity.

There are two forms of GCK: liver and islet. Although each tissue has its own exon 1 and promoter, they share common exons 2-10 [8]. The transcript of islet GCK is regulated by glucose [15] while insulin is the key regulator for hepatic GCK transcription [9]. Although substantial work has been accomplished [16], the IRS has not been identified within the hepatic GCK promoter. In contrast, the IRS of PEPCK has been mapped out and studied extensively, which is positively regulated by insulin [10]. This polymorphism (G-to-A substitution) was not only located in a region, which is highly similar to the IRS of PEPCK, but also occurred at the base pair, which was conserved between PEPCK and hepatic GCK and also conserved between human and rat for both PEPCK and hepatic GCK (Figure 2). This suggests that this base pair may be very important in IRS. Transgenic mice with

Table 3. Stepwise regression analysis of the estimated indices for insulin sensitivity and beta cell function

Dependent Variable	Covariate entered	Covariate removed	r^2	p
ISI_H	GCK polymorphism		0.144	0.002
ISI_H			0.515	
	Age			< 0.001
	Waist-hip ratio			< 0.001
	Gender			0.003
	GCK polymorphism			0.019
	Diastolic blood pressure			0.052
		Systolic blood pressure		0.316
		Body mass index		0.597
ISI_M	GCK polymorphism		0.107	0.009
ISI_M			0.414	
	Age			< 0.001
	Waist-hip ratio			0.008
	Gender			0.029
	Systolic blood pressure			0.085
	GCK polymorphism			0.089
		Body mass index		0.360
		Diastolic blood pressure		0.574
%B	GCK polymorphism		0.095	0.014
%B			0.437	
	Age			< 0.001
	Gender			0.003
	Systolic blood pressure			0.026
	Waist-hip ratio			0.035
	GCK polymorphism			0.083
		Body mass index		0.189
		Diastolic blood pressure		0.433
dI/dG	GCK polymorphism		0.075	0.030
dI/dG			0.180	
	Age			0.005
	Waist-hip ratio			0.011
	Gender			0.100
		GCK polymorphism		0.135
		Diastolic blood pressure		0.720
		Body mass index		0.913
		Systolic blood pressure		0.954

overexpressed PEPCK developed hyperinsulinemia [17]. Increased GCK gene copies in mice leads to increased hepatic glucose metabolism and, consequently, a lower plasma glucose concentration [18]. In addition, overexpression of human hepatic GCK in mice liver also results in decreased glucose concentration and reduced body weight [19]. Furthermore, mice that lack GCK only in the liver are only mildly hyperglycemic but display pronounced defects in both glycogen synthesis and glucose turnover rate during a hyperglycemic clamp [20]. Therefore, it is tempting to speculate that reduced expression of hepatic GCK could lead to hepatic insulin resistance as we observed in this study (seen in a lower ISI_H for the GA/AA subjects). Initially, glucose homeostasis is maintained by the compensatory hyperinsulinemia (as observed from the higher plasma insulin concentration for

the GA/AA subjects in this study) through an increase in insulin secretion by the pancreatic beta cells, which was also observed in this study (a higher %B and dI/dG for the GA/AA subjects). However, the cause-effect relationship between this polymorphism and insulin resistance remains to be elucidated.

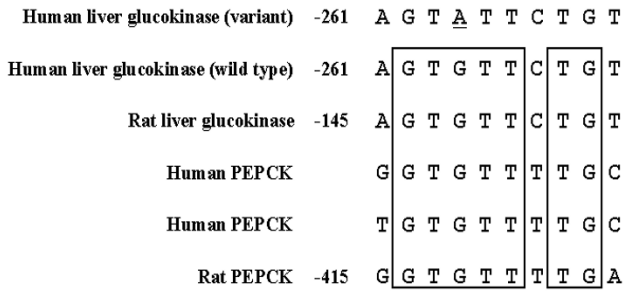

Figure 2
Similarity of putative insulin regulatory sequence with known insulin regulatory sequence of rat phosphoenolpyruvate carboxykinase (PEPCK) All the sequences shown are from the coding strand of the respective gene promoter. The 5' end positions are expressed relative to the transcription start site (GenBank accession: M90298 for human liver glucokinase; M24943 for rat liver glucokinase; K03243 for rat PEPCK). Human PEPCK has two copies of insulin regulatory sequences in its promoter and the transcription start site has not been mapped yet (Chiu KC, unpublished data). The underlined A in the variant human liver glucokinase is the position where the G-to-A substitution occurs. The blocked area is conserved between human and rat and also between PEPCK and glucokinase.

In conclusion, we demonstrated that the G-to-A polymorphism at the -258 nucleotide position of hepatic GCK promoter is associated with hepatic insulin resistance in normotensive and glucose tolerant subjects. To our knowledge, this is the first study, which attempts to dissect genetic influence among hepatic and whole body insulin sensitivity and beta cell function. However, to understand the molecular basis of insulin resistance of this polymorphism requires further studies.

Materials and methods

Studied subjects:

The study was approved by the Institutional Review Board and written informed consent was obtained at the entry of the study from each participant. We confirm that the study has complied with the recommendations of the Declaration of Helsinki. Asian Indians who resided in the metropolitan Los Angeles area were recruited from local Indian temples. Only normotensive subjects, who were not taking any medications, were included. Glucose tolerance was determined by an oral glucose tolerance test (OGTT) after an overnight fast. The subjects were biologically unrelated. They were instructed to fast overnight and not to take any medication before the study. Two baseline blood samples were obtained at -10 and -5 minutes before an oral glucose challenge with 75 gm glucose. Four additional blood samples were obtained at 30, 60, 90, and 120 minutes. Blood pressure was measured three times with a mercury sphygmomanometer while the subject was in the seated position. The mean of the last two measurements was used in the analysis.

Hepatic and whole body insulin sensitivity were estimated from the OGTT according to Matsuda and DeFronzo [13]. They were calculated from the following formulae: [405 / (fasting plasma concentration in μU/mL X fasting insulin concentration in mg/dL)], which was modified from the Homeostasis Model Assessment (HOMA) [14], for hepatic insulin sensitivity (ISI_H) and [10,000 / (fasting plasma glucose concentration in mg/dL X fasting insulin concentration in μU/mL X mean plasma glucose concentration in mg/dL X mean plasma insulin concentration in μU/mL)^0.5] for whole body insulin sensitivity (ISI_M). We also estimated beta cell function using %B [20 X fasting plasma insulin concentration in μU/mL / (fasting plasma glucose concentration in mmol/L - 3.5)] from the HOMA [14] and dI/dG [(plasma insulin concentration at 30 minutes - fasting plasma insulin concentration in μU/mL) / (plasma glucose concentration at 30 minutes - fasting plasma glucose concentration in mmol/L)].

Laboratory methods:

Genomic DNA was extracted from the peripheral lymphocytes as described previously [21]. A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay was developed for the 174-base pair fragment containing nucleotide -411 to -238 of the liver GCK promoter [8]. Since the substitution occurs within a region that is not cut by any known restriction enzyme, we created a *de novo* restriction site by placing the reverse primer close to the site of variation and replacing one of the nucleotides in the reverse primer. By substituting T with A at nucleotide -256 within the reverse primer, a *de novo ACC I* restriction site was created when the molecular variation of G-to-A substitution was present. The standard PCR reaction was a 10-μl reaction mixture containing 0.1 μg of genomic DNA, 1 pmole of each primer, 0.2 mM of dNTP, 2 mM of $MgCl_2$, 1X PCR buffer, and 0.25 U of Thermal stable Taq polymerase. The PCR was performed with an initial denaturation at 94°C for 5 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds, extension at 72°C for 30 seconds, and then a final extension at 72°C for 10 minutes. The forward primer was CAGACCCTGGATTGTATGAAATG and the reverse primer was GGCTGCCTTGGCCACAGTA. The restriction digestion

was carried out in a 10 μl reaction containing 2.5 μl of PCR reaction and 0.1 U of *Acc I* in the buffer supplied by the vender (Promega Inc., Madison, WI, USA) at 37°C for 3 hours. The reaction was resolved on a 8% acrylamide gel which was scored under a UV illuminator after staining with ethidium bromide. The wild type (G at nucleotide -258) was not cut by *Acc I* and was isolated as a larger fragment (173 bp), while the variant (A at nucleotide -258) was cut by *Acc I* to produce a smaller fragment (154 bp).

Statistical analysis:

Variables with skewed distributions were logarithmically transformed before analysis. They were body mass index, waist-hip ratio, insulin concentrations, %S, ISI_M, %B, and dI/dG. Data were presented as means (or geometric means when appropriate) with 95% confidence intervals, unless otherwise specified. Two-sided t-tests or chi-square tests were used to evaluate the differences between the two groups. To examine the influence of multiple variables on either insulin sensitivity or beta cell function, multivariate analysis was performed with a backward stepwise option. The probability to enter or to remove was set at 0.10. A nominal P value of less than 0.05 was considered significant. SYSTAT 8.0 for Windows from SPSS, Inc. (Chicago, Illinois) was used for the statistical analyses.

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