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ScienceDirect
Metabolism Clinical and Experimental 55 (2006) 1155–1158
Metabolism
Clinical and Experimental
www.elsevier.com/locate/metabol

Endothelial nitric oxide synthase G894T (Glu298Asp) polymorphism was predictive of glycemic status in a 5-year prospective study of Chinese subjects with impaired glucose tolerance

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Received 5 January 2006; accepted 28 April 2006

Abstract

Subjects with impaired glucose tolerance (IGT) have a high risk of developing type 2 diabetes mellitus (DM) and its related complications. However, both environmental and genetic factors may influence the progression or regression of hyperglycemia. Polymorphisms of the endothelial nitric oxide synthase (eNOS) gene have been associated with DM in cross-sectional studies, but their predictive values in glycemic progression are not known. We examined the relationship of the eNOS promoter −T786C (−T786C), intron 4 variable tandem repeat (in4a/b), and exon 7 G894T (G894T) polymorphisms, and their haplotypes, with the long-term glycemic outcome in a Chinese cohort with IGT. Two hundred fifty-six Chinese subjects with IGT at baseline participated in a 5-year follow-up study to assess their glycemic outcome. Each individual was genotyped for the above-mentioned polymorphisms. At 5 years, 40.2% of the subjects had reverted to normal glucose tolerance; 39.9% remained in IGT/impaired fasting glucose and 19.9% had developed DM. A significant gene effect of exon 7 G894T polymorphism on glycemic status at 5 years was demonstrated, with carriers of T_{894} being more likely to have persistent hyperglycemia compared with GG subjects ($P = .003$). On stepwise logistic regression analysis, the presence of the T allele remained a significant risk factor for persistent hyperglycemia (odds ratio, 2.72; 95% confidence interval, 1.36-5.99; T+ vs GG; $P = .013$), together with male sex, high body mass index, and high 2-hour glucose at baseline. No significant effect of −T786C or in4a/b polymorphism on fifth-year glycemic status was observed. The eNOS G894T polymorphism appears to be predictive of persistent hyperglycemia in Chinese subjects with IGT.

1. Introduction

Endothelial nitric oxide synthase (eNOS) mediates the vascular action of insulin, enhancing nitric oxide (NO) production, vasodilatation, and, hence, glucose and insulin delivery to skeletal muscles [1]. Nitric oxide also plays a role in hepatic glucose metabolism [2]. Endothelial nitric oxide synthase knockout mice had reduced peripheral and hepatic insulin sensitivity [3,4], whereas insulin-resistant first-degree relatives of patients with type 2 diabetes mellitus (DM) had reduced NO-mediated cyclic guanosine monophosphate production [5]. Among the eNOS gene polymorphisms described [6], the promoter −T786C polymorphism (−T786C), the 27–base pair variable tandem repeat in intron 4 (in4a/b), and the exon 7 G894T polymorphism (G894T) were reported to affect NO levels [7-10]. The association of −T786C with insulin resistance [11], and G894T with DM and the metabolic syndrome [12], was also observed in case-control studies.

Subjects with impaired glucose tolerance (IGT) are at great risk of developing DM. Whether eNOS gene polymorphisms contribute to the progression of IGT has not been previously studied. In this 5-year prospective study, we examined the relationship between the above 3 eNOS gene polymorphisms and glycemic outcome in 256 subjects with

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doi:10.1016/j.metabol.2006.04.012

Table 1
Baseline characteristics of subjects with IGT and persistent hyperglycemia as compared with those who had regressed to normoglycemia

	NGT (n = 103)	IGT/IFG/DM (n = 153)	*P*	GG (n = 203)	G/T+T/T (n = 53)	*P*
Age (y)	48.3 ± 12.0	51.4 ± 12.1	.042	50.5 ± 12.6	49.0 ± 10.2	NS
Sex (male/female)	33/70	73/80	.013	79/124	27/26	NS
BMI (kg/m^2)	24.5 ± 3.40	26.4 ± 3.52	<.001	25.5 ± 3.68	26.0 ± 3.19	NS
Waist-hip ratio	0.85 ± 0.08	0.88 ± 0.07	<.001	0.86 ± 0.08	0.88 ± 0.83	NS
Fasting glucose (mmol/L)	5.20 ± 0.45	5.51 ± 0.54	<.001	5.35 ± 0.52	5.51 ± 0.54	.06
2-h Glucose (mmol/L)	8.59 ± 0.61	9.08 ± 0.83	<.001	8.89 ± 0.78	8.86 ± 0.83	NS
Fasting insulin[a] (mIU/L)	7.21 ± 6.29	7.62 ± 4.41	NS	7.57 ± 5.52	7.03 ± 3.91	NS
QUICKI	0.37 ± 0.04	0.36 ± 0.04	.029	0.36 ± 0.04	0.36 ± 0.04	NS
HDL (mmol/L)	1.29 ± 0.34	1.16 ± 0.29	.001	1.21 ± 0.32	1.21 ± 0.32	NS
Triglyceride[a] (mmol/L)	1.28 ± 0.74	1.51 ± 0.75	.002	1.43 ± 0.79	1.37 ± 0.59	NS
With hypertension (%)	25.2	30.7	NS	28.6	22.6	NS
Year 5 NGT				44.8%	55.2%	.003
Year 5 IFG/IGT/DM				22.6%	77.4%	

HDL indicates high-density lipoprotein; NS = not significant.
[a] Data logarithmically transformed before analysis.

IGT. These polymorphisms were chosen because of their reported functional significance [7-11] and associations with diabetes or the metabolic syndrome [11,12]. The G894T polymorphism was among the 4 proposed tagging single nucleotide polymorphisms (SNPs) of the eNOS gene in Han Chinese, based on data from the HapMap Project (www.HapMap.org). It did not show high linkage disequilibrium ($r^2 > 0.8$) with any of the other SNPs reported. The −T786C variant was not among the 24 SNPs reported in that database.

2. Subjects and methods

From the population-based Hong Kong Cardiovascular Risk Factors Prevalence Study [13], subjects with IGT, diagnosed on a 75-g oral glucose tolerance test (OGTT), were invited to return for reassessment, including a 75-g OGTT, at 2 and 5 years. Glycemic status was defined using the World Health Organization 1998 criteria. Subjects with impaired fasting glucose (IFG), IGT, or DM at year 5 were collectively defined as having persistent hyperglycemia. Details of methods and results of second-year assessment were reported previously [14,15]. Insulin sensitivity was estimated using the quantitative insulin sensitivity check index (QUICKI), calculated as 1/[log (fasting glucose in mmol/L × 18) + log (fasting insulin in mIU/L)] [16]. All subjects were given similar dietary and exercise advice at baseline, without initiating active medical therapy. Only subjects with consent for genetic analysis were included in this report. The protocol was approved by the local ethics committee.

Genotyping was performed with polymerase chain reaction–restriction fragment length polymorphism using the restriction enzymes *Mbo*I and *Ban*II for G894T [17] and *Hae*III for −T786C. For in4a/b, the 2 common alleles containing 4 (a) or 5 (b) repeats were identified by electrophoresis on 6% polyacrylamide gels after polymerase chain reaction amplification [18]. All primers and reaction conditions are available on request.

2.1. Statistical analyses

Results are expressed as mean and SD. Skewed data were logarithmically transformed before analysis. The association of baseline demographic, biochemical, or genotypic parameters with fifth-year glycemic status was tested using analysis of variance or χ^2 as appropriate. Backward stepwise logistic regression was used to identify independent predictors of fifth-year glycemic status. *P* values of less than <.05 were considered significant.

Linkage disequilibrium was analyzed using GCUtilities (http://www.smd.qmul.ac.uk/statgen/dcurtis/software.htm/). The distribution of genotypes and alleles in association with glycemic status was compared using the χ^2 test and the WHAP program (http://statgen.iop.kcl.ac.uk/), respectively. Haplotypes representing more than 1% of the subject population were analyzed using the WHAP program.

3. Results

Among the 256 subjects with IGT at baseline, 40.2% had reverted to normal glucose tolerance (NGT), 39.9% remained in IGT/IFG, and 19.9% had developed DM at year 5. Subjects with persistent hyperglycemia at year 5 had a more adverse metabolic profile at baseline (Table 1).

Table 2
Relationship between allelic frequencies of the polymorphisms and glycemic status at year 5

Polymorphism	Allele	Year 5 glycemic status			*P*
		NGT (n = 103)	IGT/IFG (n = 102)	DM (n = 51)	
G894T	G	94.2	84.3	86.3	.005
	T	5.8	15.7	13.7	
−T786C	T	87.4	89.1	86.3	.79
	C	12.6	10.9	13.7	
In4a/b	b	87.6	89.6	86.0	.64
	a	12.4	10.4	14.0	

The results are percentages within each glycemic group.

The genotype distributions in the subjects with IGT were 79.3% GG, 18.7% G/T, and 2.0% TT for G894T; 76.5% TT, 22.7% T/C, and 0.8% CC for −T786C; and 77.0% bb, 22.2% a/b, and 0.8% aa for in4a/b, each following Hardy-Weinberg equilibrium. −T786C and in4a/b were in tight linkage disequilibrium with each other (r = 0.977, D' 1.0, P < .0001), but not with exon 7 G894T (r = 0.115). Homozygotes for the rare allele in each polymorphism were grouped with the respective heterozygous groups for analysis.

For each polymorphism, there was no significant difference in baseline characteristics between the respective genotypes. Results on G894T are shown in Table 1.

Significant gene effect on fifth-year glycemic status of those with IGT was observed only with G894T, with T+ having significantly greater risk of persistent hyperglycemia compared with GG subjects (P = .003) (Table 1). Similar findings were observed when allelic frequencies were analyzed (Table 2). Weight change over 5 years was comparable between the 2 groups (T+ vs GG: 0.18 ± 3.36 vs −0.7 ± 3.85 kg; P = .66). On stepwise logistic regression analysis (Table 3), presence of the eNOS T_{894} variant was an independent predictor of persistent hyperglycemia at 5 years (odds ratio, 2.721; 95% confidence interval, 1.236-5.992; T+ vs GG, P = .013), together with male sex (P = .037), body mass index (BMI) (P = .006), and 2-hour post-OGTT glucose (2hG) (P < .001) at baseline, in a model that initially also included age, high-density lipoprotein, and QUICKI. Similar significance for G894T (P = .018) was obtained if fasting glucose replaced 2hG in the model.

The prevalence of hypertension, defined as 130/85 or more or on regular antihypertensive treatment, was 28.6% in GG and 22.6% in T+ at baseline (Table 1; P = .3) and was 49% in both groups at 5 years. Among the 183 subjects with no hypertension at baseline, 16 (39%) of 41 subjects with T+ had developed hypertension by year 5, compared with 43 (30%) of 142 for those with GG (P = .29).

In contrast, both −T786C and in4a/b polymorphisms showed no gene effect on the fifth-year glycemic status of subjects with IGT at baseline. As expected, haplotypes formed from combinations of the 3 polymorphisms did not result in stronger associations with fifth-year glycemic status as compared with G894T alone (results not shown).

Table 3
Final model of a stepwise backward logistic regression analysis, illustrating the independent baseline predictors of persistent hyperglycemia at year 5

Parameter	Odds ratio	95% Confidence interval	P
Sex (male)	1.876	1.037-3.394	.037
Baseline BMI (kg/m^2)	1.127	1.035-1.227	.006
Baseline 2hrG (mmol/L)	2.452	1.624-3.703	<.001
eNOS 894T+	2.721	1.236-5.992	.013

The analysis initially also included age, high-density lipoprotein cholesterol, and QUICKI. 2hrG indicates plasma glucose at 2 hours after a 75-g oral glucose load.

4. Discussion

Subjects with IGT have an increased risk of developing DM and its related complications. We have demonstrated in this prospective study that the eNOS exon 7 G894T polymorphism plays a role in determining the glycemic outcome after 5 years, in a cohort of Chinese subjects with IGT, as do baseline 2hG, sex, and BMI. As the Chinese in Hong Kong predominantly originated from Southern China, our finding may reflect only the significance of this genetic factor in Southern Chinese. It is interesting to note that, in the HapMap project, the −T786C SNP was not reported in 45 Han Chinese in Beijing. Whether this reflects a difference between Southern and Northern Chinese is uncertain as the project was not intended to provide a comprehensive database for any given gene.

Monti et al [12] previously reported an association of G894T with DM in a cross-sectional study of white patients without macrovascular disease and with insulin resistance in control subjects. Although an earlier study in whites found no difference in the allelic frequency of G894T between controls and patients with DM and macrovascular disease [19], higher levels of plasma very low-density lipoprotein–containing lipoproteins were found in male patients homozygous for this variant, which could suggest the presence of increased insulin resistance that is known to contribute to both reduced clearance and increased production of these lipoproteins in DM [20]. Taken together with our data, the findings of the above studies [12,19] would support a deleterious effect of the G894T variant on insulin resistance, accounting for its association with more adverse glycemic outcome in this prospective study of subjects with IGT. We did not observe any association of this eNOS polymorphism with the prevalence or incidence of hypertension, in contrast to reported findings in a study of African Americans and whites [21]. Whether this represents a true ethnic difference remains to be confirmed in studies involving a larger number of subjects.

It has been reported that the replacement of glutamate by aspartate at amino acid position 298 in the G894T polymorphism renders the eNOS protein more susceptible to intracellular cleavage [22]. In computer analysis, G894T induces a conformational change of the protein from a helix into a tight turn [17], which may affect its function, interaction with other chaperone proteins, or responsiveness to insulin or other activators [23]. The T variant has been reported in some studies, but not others [11,24,25], to result in impaired NO production in transfected cells in culture [26], decreased eNOS activity in human placental tissues [9], reduced NO levels [10] or activity [12], and may thus contribute to insulin resistance.

We did not find any association of −T786C or in4a/b with the fifth-year glycemic status, similar to a previous cross-sectional study [11], although the authors observed a lower insulin-mediated glucose uptake during euglycemic hyperinsulinemic clamp in −T786C carriers. The functional

effects of −T786C [11,25] and in4a/b [8,9,27] remain uncertain, with different studies yielding conflicting results.

Our study cohort is relatively small and any gene effect may be influenced by type I error. However, the risk associated with the G894T polymorphism is probably genuine, as well-known risk factors including BMI and 2hG were also predictive of fifth-year glycemic status in this cohort and our spontaneous reversion rate to NGT was comparable to that reported in other studies [28]. Future systematic analysis or meta-analysis of studies on eNOS genotype and glycemia would help to confirm these findings.

In conclusion, it appears that the presence of the eNOS exon7 G894T polymorphism predisposes to an increased risk of persistent hyperglycemia, especially when challenged by risk factors, such as obesity.

Acknowledgment

This study was supported by funding from the Committee on Research and Conference Grants of the University of Hong Kong, the Hong Kong Research Grant Council (HKU7290/97M), and the Health Care & Promotion Fund (no. 212907) of the Hong Kong Government.

We thank Ms Rachel Wong for laboratory assistance.

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