Short Communication

The *FOXC2* -512C>T variant is associated with hypertriglyceridaemia and increased serum C-peptide in Danish Caucasian glucose-tolerant subjects

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Abstract

Aims/hypothesis. The transcription factor *FOXC2* plays a key role in adipocyte differentiation and the *FOXC2* gene is a candidate gene for Type 2 diabetes, obesity and dyslipidaemia. We investigated whether the *FOXC2* -512C>T promoter variant is associated with Type 2 diabetes or its intermediary phenotypes in glucose tolerant subjects.

Methods. The variant was genotyped using PCR-RFLP in 705 unrelated Type 2 diabetic patients, 505 unrelated glucose-tolerant control subjects and 219 glucose-tolerant offspring of Type 2 diabetic probands.

Results. The frequency of the T-allele was 58% (95% CI 56–61%) and 59% (56–62%) among the Type 2 diabetic patients and the unrelated glucose-tolerant

control subjects, respectively ($p=0.6$). Among the glucose-tolerant subjects, the T-allele carriers had higher fasting serum triglyceride ($p=0.03$), fasting serum C-peptide concentrations ($p=0.009$) and insulinogenic index ($p=0.04$). Furthermore, in glucose-tolerant women, the waist-to-hip ratio was significantly higher in carriers of the T-allele.

Conclusion/interpretation. Our data suggest that the *FOXC2* -512C>T variant is not associated with Type 2 diabetes. However, among glucose-tolerant subjects the variant is associated with hypertriglyceridaemia and increased fasting serum C-peptide. [Diabetologia (2003) 46:1576–1580]

Keywords *FOXC2*, genetics, single nucleotide polymorphism, Type 2 diabetes, hypertriglyceridaemia, association study.

The forkhead box C2 (*FOXC2*) is a member of the Fox transcription factor family and is expressed in white and brown adipose tissue [1]. Recently, the understanding of the molecular basis of adipose tissue

development was improved with the cloning and characterization of several genes involved in adipocyte differentiation and metabolism. Several classes of transcription and nuclear factors, such as CCAAT-enhancer binding proteins α , β and γ , peroxisome proliferator-activated receptor- γ , and sterol responsive element binding protein 1c, are implicated in the control of adipocyte differentiation and *FOXC2* activates these genes [2, 3].

Transgenic mice over-expressing *FOXC2* in adipose tissue were leaner and had reduced concentrations of serum triglyceride, plasma glucose and insulin and were more insulin sensitive on standard and high-fat diets than their wild-type littermates [4].

In a recent study *FOXC2* mRNA levels were increased in visceral fat compared with subcutaneous fat from human obese subjects and there was a correla-

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Abbreviations: *FOXC2*, Forkhead box C2; UTR, untranslated region; OHA, oral hypoglycaemic agents; Si, insulin sensitivity index; AIR, acute serum insulin response; Sg, glucose effectiveness; HOMA, homeostasis model assessment; IR, insulin resistance.

tion between whole-body insulin sensitivity and *FOXC2* mRNA levels in visceral fat and skeletal muscle [5]. The -512 C>T variant in the 5' untranslated region (UTR) of *FOXC2* was identified and the T-allele of this variant was associated with enhanced insulin sensitivity and lower plasma triglyceride concentrations in women [5].

These data suggest that *FOXC2* is a candidate gene for obesity, Type 2 diabetes and dyslipidaemia. To test this hypothesis, we studied whether the *FOXC*-512C>T variant was associated with diabetes or in glucose-tolerant subjects with alterations in insulin secretion, insulin sensitivity, dyslipidaemia, and estimates of obesity. Furthermore, the analysis was stratified according to sex as previous reports have observed sex-specific effects.

Subjects and methods

Subjects. An association study was carried out in 705 unrelated Type 2 diabetic patients and 505 unrelated glucose-tolerant control subjects. Studies of relevant intermediary phenotypes were undertaken in the latter group and in 219 glucose-tolerant offspring of Type 2 diabetic probands from 61 families. The diabetic patients were recruited from the outpatient clinic at Steno Diabetes Center. The glucose-tolerant subjects were recruited from the Danish Central Population Register and from the Research Centre for Prevention and Health. The glucose-tolerant offspring were recruited from the Danish family resource bank at the Department of Human Genetics, University of Copenhagen and the outpatient clinic at Steno Diabetes Center. The average age of the diabetic subjects was 59.2 ± 10.4 (mean $\pm$ SD) years, their BMI was 29.2 ± 5.1 kg/m² and their HbA_{1c} $8.1 \pm 1.7\%$ (upper normal value 6.4%). They were treated with diet only (35%), oral hypoglycaemic agents (OHA) (52%) and insulin in combination with OHA (12%). In the glucose-tolerant subjects the age was 56.5 ± 10.2 years and BMI 25.8 ± 3.7 kg/m². In the glucose-tolerant offspring the age was 39.4 ± 8.6 years and BMI 25.6 ± 4.4 kg/m². All glucose-tolerant subjects were examined with an OGTT. Diabetes was diagnosed according to the 1999 World Health Organization criteria [6]. In addition, all glucose-tolerant offspring were examined by a 33-time point tolbutamide-modified frequently sampled IVGTT for the measurement of insulin sensitivity index (Si), acute serum insulin response (AIR), glucose effectiveness (Sg), and disposition index as described [7]. All participants were Danish Caucasian by self-report. Informed written consent was obtained from all participants. The study was approved by the Ethics Committee of Copenhagen and was in accordance with the principles of the Declaration of Helsinki II.

Genetic analysis. The genotyping of the 5'UTR polymorphism of *FOXC2* was done on genomic DNA extracted from human leukocytes using PCR-RFLP. The sequences of the primers were 5'-GTCTTAGAGCCGACGGATCCCTG-3' (forward) and 5'-GATTGGGAAAAGTTT CAAAG-3' (reverse). The reverse primer contained one nucleotide mismatch (underlined). PCR amplification was carried out using 5% DMSO, 0.65 units AmpliTaq Gold polymerase (Perkin Elmer, Foster City, Calif., USA) and 3 mmol/l MgCl₂ with annealing at 53°C for 30 s. The PCR products were digested by *Dpn* II (New England Biolabs, Beverly, Mass., USA) at 37°C overnight and separat-

ed by electrophoresis on a 4% ethidium bromide-stained agarose gel.

Statistical analysis. Fisher's exact test was applied to examine differences in allele frequencies and genotype distributions between diabetic patients and glucose-tolerant control subjects. A variance component model was used to test variables (or transformed variables) for comparisons among genotype groups, fixed factors: genotype and sex; covariates: age and BMI; and random effect: familial coherence. All phenotype analyses were done with PROC MIXED of the SAS/STAT system (version 8.2, SAS Institute). A *p* value of less than 0.05 was considered statistically significant.

Homeostasis model assessment for insulin resistance (HOMA-IR) was calculated from OGTT data as [fasting serum insulin (pmol/l) $\times$ fasting plasma glucose (mmol/l)]/22.5. The insulinogenic index was calculated as [serum insulin at 30 min (pmol/l) - fasting serum insulin (pmol/l)]/plasma glucose at 30 min (mmol/l). The minimal model was used to provide quantitative estimates of Si and Sg. AIR (0–8 min) was calculated as the incremental AUC for insulin from 0 to 8 min. The disposition index was calculated as the product of Si and AIR.

Results

The frequencies of the *FOXC2* -512C>T variant were 58% (95% CI: 56–61%) and 59% (56–62%) among the Type 2 diabetic patients and the control subjects, respectively (*p*=0.6) (Table 1). The genotype distribution was similar in diabetic patients and control subjects (*p*=0.6). All genotype distributions were in Hardy-Weinberg equilibrium.

We tested whether the *FOXC*-512C>T variant influences clinical relevant phenotypes in 724 glucose-tolerant subjects, comprising 505 unrelated glucose-tolerant subjects and 219 glucose-tolerant offspring of diabetic probands. Fasting serum triglyceride and C-peptide concentrations, the insulinogenic index, and the AUC for serum C-peptide (0–120 min during a 75 g OGTT) were significantly higher in subjects with the T-allele and fasting serum insulin; HOMA-IR and the AUC for serum insulin tended to be higher in subjects with the T-allele than in those without (Table 2).

We also analysed the data after stratifying the subjects according to sex. In glucose-tolerant men, the insulinogenic index and the AUC for serum C-peptide were higher and the AUC for serum insulin tended to be higher in the T-allele carriers (Table 2). In glucose-tolerant women, WHR, fasting plasma glucose, and fasting serum C-peptide concentrations were significantly higher and HOMA-IR tended to be higher in subjects with the T-allele than in those without (Table 2).

In glucose-tolerant offspring, no differences in Si, AIR, or disposition index were observed. However, Sg was lower in female T-allele carriers (CC: 2.26 ± 0.49 ; CT: 1.90 ± 0.50 , TT: $1.95 \pm 0.53 \times 10^{-2}$ /min; *p*=0.03).

Table 1. Allele frequencies and distribution of genotype groups of the *FOXC2* -512C>T variant among Type 2 diabetic patients and glucose-tolerant control subjects^a

<i>n</i>	All			Men			Women		
	Diabetic patients	Control subjects	<i>p</i>	Diabetic patients	Control subjects	<i>p</i>	Diabetic patients	Control subjects	<i>p</i>
	705	505		440	240		265	265	
CC	129 (18)	82 (16)	0.6	77 (18)	44 (18)	0.9	52 (20)	38 (14)	0.3
CT	332 (47)	247 (49)		207 (47)	115 (48)		125 (47)	132 (50)	
TT	244 (35)	176 (35)		156 (35)	81 (34)		88 (33)	95(36)	
T-allele frequency (%) (95% CI)	58.2 (55.6–60.7)	59.3 (56.3–62.3)	0.6	59.0 (55.7–62.2)	57.7 (53.3–62.1)	0.7	56.8 (52.6–61.0)	60.8 (56.6–64.9)	0.2

^a Data in parentheses are percentages of genotype distributions

Discussion

The allele and genotype frequencies of the *FOXC2* -512C>T variant did not differ significantly between Type 2 diabetic patients and glucose-tolerant control subjects. Our findings show that the *FOXC2* -512C>T substitution is not a major cause of Type 2 diabetes in the Danish Caucasian population. This is consistent with previous reports in Japanese subjects [8] and Pima Indian subjects [9].

However, in glucose-tolerant subjects fasting serum triglyceride concentrations were higher among the T-allele carriers, suggesting that the T-allele might be associated with fasting hypertriglyceridaemia. A recent study in families with familial combined hyperlipidaemia and low serum HDL-cholesterol concentrations also showed an association of the -512C>T variant with serum triglyceride concentrations [10]. In Pima Indians fasting plasma triglyceride concentrations were lower in women carrying the T-allele and tended to be higher in men carrying the T-allele [9]. In glucose-tolerant women values of WHR and HOMA-IR were higher among the T-allele carriers, which could indicate that the T-allele is associated with visceral adipose depots, which cause insulin resistance. Both our study and the previous reports from Sweden [5] and the Pima Indians [9] show sex-specific effects. However, the mechanism is not known. Furthermore, fasting serum C-peptide concentrations, AUC for serum insulin, AUC for serum C-peptide and the insulinogenic index were higher in subjects with the T-allele. Considering the phenotype of transgenic mice [4], our findings are in agreement with the view that the expression of *FOXC2* mRNA from the subjects with T-allele is lower. Contrary to these findings, a Swedish sib pair study in non-diabetic siblings showed that the T-allele of the *FOXC2* -512C>T variant was associated with enhanced insulin sensitivity and lower plasma triglyceride concentrations in women. The same authors showed that *FOXC2* mRNA levels in adipose tissue and skeletal muscle correlated negatively with serum insulin concentrations and insulin sensitivity [5]. In the Swedish study *FOXC2* mRNA levels were investigated only for obese subjects and the authors showed that *FOXC2* mRNA is increased in visceral fat among T-allele carriers. In contrast to the findings in visceral fat they found a non-significant increased gene expression of *FOXC2* among C-allele carriers in muscle tissue [5].

In conclusion, the *FOXC2* -512C>T variant is not associated with Type 2 diabetes in Caucasian subjects. However, the T-allele of the *FOXC2* -512C>T variant is associated with fasting higher serum triglyceride concentrations and increased fasting serum C-peptide concentrations in Danish glucose-tolerant subjects and might therefore be a gene variant contributing to components of the metabolic syndrome.

Table 2. Clinical and biochemical characteristics of glucose-tolerant subjects according to the *FOXC2* -512C>T genotype^a

<i>n</i>	All	<i>p</i>			<i>p^b</i>			<i>p</i>	<i>p^b</i>			<i>p</i>	<i>p^b</i>		
		CT	TT	240	CC	CT	TT		Men	CT	TT			Women	CC
	CC 119	365	240		59	168	111		60	197	129				
Age (years)	52±12	51±12	51±13		54±12	53±13	52±14		50±12	50±12	51±12				
BMI (kg/m ²)	25.5±3.7	25.9±4.1	25.6±3.9	0.4	0.3	25.8±2.8	25.9±3.6	26.5±3.2	0.3	0.3	25.1±4.3	25.9±4.5	24.9±4.3	0.1	0.4
WHR	0.86±0.10	0.87±0.10	0.88±0.10	0.2	0.1	0.95±0.07	0.94±0.07	0.94±0.06	0.5	0.2	0.78±0.05	0.82±0.09	0.82±0.10	0.04	0.01
% body fat	30.6±8.8	31.6±8.5	30.3±8.4	0.4	0.7	26.0±5.2	26.0±6.9	26.5±6.1	0.8	0.9	35.1±9.2	36.3±6.7	33.6±8.7	0.1	0.7
F.s-total cholesterol (mmol/l)	5.6±1.1	5.7±1.2	5.7±1.1	0.3	0.1	5.5±1.0	5.6±1.0	5.7±1.0	0.4	0.2	5.6±1.1	5.7±1.3	5.7±1.2	0.8	0.8
F.s-HDL cholesterol (mmol/l)	1.5±0.5	1.4±0.4	1.4±0.4	0.6	0.4	1.3±0.4	1.3±0.4	1.2±0.4	0.5	0.7	1.6±0.5	1.5±0.4	1.6±0.4	0.7	0.5
F.s-triglyceride (mmol/l)	1.1±0.5	1.3±0.7	1.2±0.7	0.09	0.03	1.2±0.6	1.3±0.9	1.4±0.9	0.3	0.1	1.1±0.4	1.2±0.6	1.1±0.5	0.4	0.2
F.p-glucose (mmol/l)	5.1±0.4	5.1±0.4	5.1±0.4	0.7	0.5	5.2±0.4	5.2±0.4	5.2±0.4	0.4	0.3	4.9±0.4	5.0±0.5	5.0±0.4	0.09	0.04
F.s-insulin (pmol/l)	35±16	39±21	41±25	0.1	0.06	36±15	39±23	44±27	0.3	0.5	34±17	39±19	38±22	0.3	0.1
F.s-C-peptide (pmol/l)	491±173	545±192	542±200	0.03	0.009	516±174	558±224	578±218	0.4	0.2	468±170	535±160	511±178	0.07	0.03
HOMA-IR	7.9±3.8	9.0±5.1	9.3±5.9	0.1	0.06	8.4±3.9	9.2±5.8	10.2±6.6	0.5	0.6	7.5±3.7	8.9±4.5	8.6±5.2	0.2	0.06
AUC-insulin (pmol/l)	22799±14865	24289±13437	25179±15747	0.1	0.05	20809±11180	24478±14013	26888±17690	0.1	0.06	24689±17560	24124±12948	23702±13753	0.8	0.5
AUC-C-peptide (pmol/l)	137343±55389	148580±52795	149073±46510	0.09	0.03	131795±49888	149254±54916	148221±47657	0.09	0.03	142614±60097	147993±51014	149803±45682	0.7	0.5
Insulinogenic index (pmol/l)	28.7±22.7	30.3±17.8	31.1±18.9	0.1	0.04	25.7±16.1	29.8±17.7	31.9±20.6	0.09	0.03	31.5±27.5	30.7±17.9	30.4±17.5	0.8	0.7

^a Data are means ± SD. F, fasting. *s*, serum. *p*, plasma^b *p* value when analysing CC against CT+TT

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