A Single-Nucleotide Polymorphism in a Methylatable Foxa2 Binding Site of the G6PC2 Promoter Is Associated With Insulin Secretion In Vivo and Increased Promoter Activity In Vitro

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OBJECTIVE—The G6PC2 gene encoding islet-specific glucose-6-phosphatase related protein (IGRP) has a common promoter variant, rs573225 (–231G/A), located within a Foxa binding site. We tested the cis-regulatory effects of rs573225 on promoter activity and its association with insulin response to oral glucose.

RESEARCH DESIGN AND METHODS—Functional effects of rs573225 were explored in transfected INS-1 and HIT-T15/H9252 cells. A total of 734 young obese subjects of European ancestry were genotyped for rs573225. Insulin and glucose levels were measured in response to oral glucose, and the insulinogenic index (IGI) of insulin secretion was calculated.

RESULTS—In vitro, the G allele showed a higher affinity for binding Foxa2 transcription factor and increased promoter activity. Foxa2 binding is modified if the C adjacent to the G allele is methylated. IGI was associated with rs573225 by linear regression analysis and was 30% greater in AA or AG than in GG obese children. rs573225 was also associated with fasting glucose.

CONCLUSIONS—rs573225 is functional in cis as a methylatable single-nucleotide polymorphism (SNP) of G6PC2 associated with glucose-insulin homeostasis in obese children, likely to explain the results of recent genome-wide association studies in nondiabetic adults. Diabetes 58:489–492, 2009

The flux through glycolysis is the sensed process that makes β-cells glucose responsive. Thus, altered kinetics of glucose-induced insulin secretion could result from a changed intrinsic activity, cellular level, or allosteric regulation of several enzymes including glucokinase, phospho-fructokinase, G6PC2 (glucose-6-phosphatase, catalytic, 2), or others involved in the regulation of cellular glucose-6-phosphate (G6P) content. Whereas glucokinase, a high K_m enzyme, phosphorylates glucose into G6P in β-cells, G6PC2, a low K_m enzyme, catalyzes G6P dephosphorylation (1), which could antagonize insulin secretion (2). In mice, G6PC2 has only ~5% of the activity of liver G6Pase (1), sufficient however for the knockout of G6PC2 to result in a small decrease in glycemia (3). The human G6PC2 gene is located in 2q24 in a region that we found linked to glycemia (4). The G6PC2 promoter is inactive in HepG2 cells but has 150-fold more activity in HIT-T15 β-cells, due to the region located between −306 and +3 (5). This proximal promoter region regulates G6PC2 expression in transiently transfected βTC-3, HIT-T15, and Min6 cells through the binding of Foxa2 and MaA transcription factors (6). We found three single-nucleotide polymorphisms (SNPs) in the G6PC2 promoter in public banks, among which we selected rs573225, a G/A variant located at position −231. Our choice was based on the fact that this variant is common among European populations and belongs to a binding site for Foxa transcription factors (5,7) that carries a potentially methylatable CG motif created by the G allele. The Foxa proteins, previously designated HNF-3, are known to modulate the expression of pancreatic genes involved in glucose homeostasis (8–11).

We performed a functional analysis of rs573225 in β-cell lines and found that this promoter SNP binds Foxa2 with variable affinity and can act as a transcriptional regulator and then explored whether rs573225 genotypes were associated with insulin responses to oral glucose in 734 obese children of European ancestry. Recently, two independent genome-wide association studies revealed that SNPs in linkage disequilibrium with rs573225, but with no known function, were associated with fasting glucose levels in nondiabetic adults (12,13). We thus suspect rs573225 to be the causative variant for the observed associations.

RESEARCH DESIGN AND METHODS

Methylation-specific PCR. Human pancreatic DNAs were obtained from human islets provided by T. Berney; 1 μg was fragmented with XbaI and denatured by incubation in 3 mol/l NaOH for 15 min at 37°C. Bisulfite conversion was carried out in a solution of sodium bisulfite and hydroquinone at 55°C overnight. After desalting, DNAs were desulfonated by incubation in NaOH at 37°C for 15 min, the solution was neutralized with Na-acetate, and DNAs were ethanol precipitated. Pellets were dissolved in distilled H2O, and DNA aliquots were used for PCR amplifications by TaqDNA polymerase (Invitrogen) under the following conditions: 94°C, 5 min; 45 amplification cycles (94°C, 30 s; 50°C, 30 s; 72°C, 30 s); and finally at 72°C, 10 min. The PCR products were purified using QIAquick kit (Qiagen) and used for automated sequencing (DNA Sequencer, Roche).

Electrophoretic mobility shift assay. Nuclear extracts were prepared from HepG2 cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). Binding reactions used binding buffer with 1 μl dDNA (Pierce). A total of 4 μg of the protein extracts was used per gel mobility shift sample. Double-stranded oligonucleotides used to detect protein binding on the G6PC2-231 element were end-labeled with biotin (Operon). The double-strand oligonucleotides were “G” probe, 5’-biotin-AATTTCAGCAAAACATGATC.
RESULTS
In vitro. We found that rs573225 alleles modified the binding of Foxa2 to G6PC2 promoter by comparing the binding of nuclear proteins from HepG2 cells to probes carrying the “G” or the “A” allele or to a “GC” probe (Figs. 1 and 2). Electrophoretic mobility shift assay experiments revealed that the G probe showed a specific binding to HepG2 cell nuclear protein complex, whereas the A probe could not bind this complex and the GC probe bound this complex with a lower affinity (Fig. 2A). The presence of Foxa2 proteins in the DNA-protein complex was confirmed by gel supershift assay experiments (Fig. 2B). These observations indicate that rs573225 alleles create a functional variation in a Foxa2 binding motif, with an added effect of the methylation of the cytosine residue adjacent to the G allele of rs573225. When the proximal segment of G6PC2 gene promoter bearing rs573225 and placed upstream of the luciferase gene in the pG3 basic vector was transfected into INS-1 or HIT-T15 cells, the G allele was consistently associated with a higher promoter activity than the A allele (Fig. 2C).

In vivo. Our hypothesis that the candidate rs573225 variant was associated with parameters of insulin-glucose homeostasis was supported by several findings (Table 1). Linear regression analysis found that rs573225 was associated with IGI (P = 0.0191 under a recessive model and P = 0.0102 under an additive model). Indeed, IGI was ~30% greater in these obese children with AA (36.8 ± 38.1 mmol/l) or AG (33.5 ± 24.2 mmol/l) genotypes compared with GG homozygotes (21 ± 16.9 mmol/l). Insulin levels showed no significant differences sensu stricto, but showed a trend for higher insulin levels in obese children of AA or AG genotype (P = 0.058 for Ins0 and P = 0.059 for Ins30). In addition, linear regression analysis found that rs573225 was associated with fasting glucose (P = 0.032 under a recessive model and P = 0.037 under an additive model) with higher glucose levels in obese children with AA or AG alleles.

DISCUSSION
When studied during their dynamic period of fat accumulation, obese children have glucose-insulin homeostasis yet are unaltered by dietary restriction or drugs, thus allowing bona fide genotype-phenotype studies. This population has the additional advantage of having normal fasting glucose levels, which avoids the interference of glucotoxicity with the studied β-cell parameters. Obese children oversecrete insulin in response to mounting insulin resistance; thus, we could explore genotypic effects over a wide range of insulin levels. Being aware that insulin and glucose levels collected during OGTTs are not

Foxa2 BINDING SITE OF THE G6PC2 PROMOTER

FIG. 1. Representative standard sequencing diagram for bisulfite direct sequencing of G6PC2 gene promoter. Human pancreatic DNA provided from a rs573225 heterozygote is shown. Signals of false conversion were not observed. All cytosines in this sequence were not converted to uracil. The G6PC2 gene promoter region was methylated. On the bottom, the Foxa consensus site in humans is shown. (Please see http://dx.doi.org/10.2337/db08-0587 for a high-quality digital representation of this image.)

CAACTGTG-3'; “A” probe, 5'-biotin-AATTCTCAAACACATGATCCACT GTT-3'; and “GC” probe, 5'-biotin-AATTCTCAAGGCT-3'. Samples were subjected to electrophoretic separation on a 6% nondenaturing polyacrylamide gel. The gel was transferred to a Nylon membrane (Pierce), which was cross-linked using a UV-Light cross linker instrument (Bio-Rad) and incubated with Nucleic Acid Detection buffer (Pierce). Signals were visualized using Chemiluminescent Substrate.
entirely replicable in the same obese child (16), we mini-
mimized stress and diet variations in controlled in-hospital
conditions. These strict criteria do not allow the study of
the thousands of subjects necessary to well-powered
association studies. We therefore consider our results as
tentative and needing further replication. Already, two
genome-wide association studies have reported the asso-
ciation of \( G6PC2 \) variants and fasting glycemia. In 9,353
normoglycemic subjects, Bouatia-Naji et al. (13) found an
association between fasting glycemia and rs560887. The
magnitude of the genotypic effect on glycemia was only
0.09 mmol/l, explaining why thousands of subjects were
necessary to reach significance. Chen et al. (12) found a
significant association between rs563694 and fasting glu-
cose in 5,088 nondiabetic individuals from Finland and
Sardinia and a \( P \) value of \( 5.7 \times 10^{-7} \) for rs573225. Because
our rs573225 is in almost complete linkage disequilibrium
\( (r^2 = 0.96) \) and with rs563694 \( (r^2 = 0.95) \) (according to the HapMap CEU reference panel), it is
likely that these three SNPs represent the same associa-
tion signal. Bouatia-Naji et al. (13) found an association
with the HOMA-\( \beta \) index of insulin secretion derived from

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TABLE 1
Clinical and biological characteristics of insulin secretion in
our young obese Caucasian cohort, according to rs573225
variant

|             | G/G | G/A | A/A |
|-------------|-----|-----|-----|
| \( n \)     | 60  | 293 | 381 |
| Age (years) | 12.4 ± 2.8 | 11.8 ± 3 | 11.7 ± 2.8 |
| BMI (kg/m²) | 30.7 ± 5.6 | 29.7 ± 5.3 | 29.6 ± 5.5 |
| Fasting glucose (mmol/l) | 4.41 ± 0.51* | 4.53 ± 0.53 | 4.56 ± 0.57 |
| Glycemia at 30 min (mmol/l) | 7.47 ± 1.22 | 7.67 ± 1.23 | 7.53 ± 1.3 |
| Fasting insulin (µU/ml) | 15 ± 7.7 | 15.7 ± 8.7 | 15.9 ± 9.9 |
| Insulin at 30 min (µU/ml) | 87 ± 52 | 109 ± 77 | 106 ± 85 |
| IGI | 21 ± 16.9† | 33.5 ± 24.2 | 36.8 ± 38.1 |
| HOMA-\( \beta \) | 68.5 ± 34.1 | 69.6 ± 38.8 | 69.9 ± 41.4 |

Data are means ± SD. \( P \) values under a recessive model: *\( P = 0.032 \)
and \( P = 0.037 \) under an additive model, †\( P = 0.0191 \) under a
recessive model, and \( P = 0.0102 \) under an additive model.
fasting insulin and glucose values, whereas our study found only an association of rs573225 with IGI, an index derived from OGTT responses, not with HOMA-β. IGI is an index of insulin secretion that is based on the first-phase response to oral glucose and possibly more sensitive to acute changes in β-cell G6P level modulated by G6PC2 activity than is HOMA-β. We suspect that these apparent differences between results in large genome-wide association studies and our candidate variant study may be due to differences in sample size and nature of proxies used to estimate insulin secretion.

Our functional analysis of the rs573225 variant in β-cell lines found that the G allele was able to bind Foxa2 factor more efficiently than the A allele and was associated with a higher G6PC2 promoter activity. Previous studies have found that several mutated transcription factor sites located in G6PC2 promoter could modify its expression (6,17). Studies reviewed in Martin et al. (6) support a positive role for Foxa2 in G6PC2 expression and a negative impact on insulin secretion in vitro (18–20). G6PC2 appears to negatively affect stimulus-secretion coupling (3). Based on these studies and our own observations, we postulate that the higher affinity of Foxa factor for the G allelic form of the binding promoter motif could result in 1) a higher expression of G6PC2, 2) a diminution of β-cell G6P content, and 3) a diminution of insulin secretion in GG carriers. The cytosine adjacent to the G allele creates a CG/C/Gm epi-allele that showed variable Foxa2 binding capacity, which could possibly add allele-specific epigenetic effects to the Foxa2-dependent transcriptional activity of the promoter. Rs573225 can thus be called an epiSNP.

In summary, the present study reports that the G6PC2 promoter carries an rs573225 variant that modulates Foxa2 binding and promoter activity and could influence insulin secretion consistently with the known role of G6PC2 in β-cell physiology.

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