

Association Analysis Indicates That a Variant GATA-Binding Site in the *PIK3CB* Promoter Is a Cis-Acting Expression Quantitative Trait Locus for This Gene and Attenuates Insulin Resistance in Obese Children

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OBJECTIVE—In search of functional polymorphisms associated with the genetics of insulin resistance, we studied a variant in the promoter of *PIK3CB*, the gene coding for the catalytic p110 β subunit of phosphatidylinositol (PI) 3-kinase, a major effector of insulin action.

RESEARCH DESIGN AND METHODS—The rs361072 C/T variant was selected among single nucleotide polymorphisms of the *PIK3CB* region because we suspected that its common C allele (allelic frequency ~50% in Europeans) could create a GATA-binding motif and was genotyped in five cohorts of obese ($n = 1,876$) and two cohorts of nonobese ($n = 1,490$) European children. To estimate insulin resistance in these children, the homeostasis model assessment for insulin resistance (HOMA-IR) index was measured in strict nutritional conditions. GATA-binding and functional effects of rs361072 were explored in transfected cell lines and in lymphocytes from obese children.

RESULTS—The rs361072 C/T variant was associated with HOMA-IR in the obese children cohorts ($1.7 \times 10^{-12} < P < 2 \times 10^{-4}$ for C/C vs. T/T using regression analysis). HOMA-IR averaged 3.3 ± 0.1 in C/C and 4.5 ± 0.2 in T/T obese children ($P = 4.5 \times 10^{-6}$ by ANOVA). C/T patients had intermediate values. As

shown by the interaction between BMI and genotype ($P = 2.1 \times 10^{-9}$), the association of rs361072 with HOMA-IR depended on BMI and was only marginal in nonobese children ($P = 0.04$). At the molecular level, the C allele of rs361072 was found to create a GATA-binding site able to increase transcription of *PIK3CB*.

CONCLUSIONS—We postulate that the C allele of rs361072 is a causal variant capable of attenuating insulin resistance in obese children through increased expression of p110 β . *Diabetes* 57: 494–502, 2008

Overweight and obesity affect 10–20% of children in Westernized countries. Obese adolescents develop variable degrees of insulin resistance (1–3), a multifactorial trait influenced not only by the amount of deposited fat (2) but by ethnic (3) and genetic factors (4–6). Insulin resistance refers to a decreased ability of insulin to exert its normal biological effects, and a hallmark of this abnormality is decreased insulin-stimulated glucose disposal. It is uniformly agreed that the activation of phosphatidylinositol (PI) 3-kinase plays a central role in the regulation of insulin-stimulated glucose transport (7–10). Class IA PI 3-kinases are heterodimers composed of a p110 catalytic subunit and a p85 regulatory subunit. Insulin causes tyrosine phosphorylation of insulin receptor substrate (IRS) proteins, which then associate with PI 3-kinase through the p85 regulatory domain, leading to increased PI 3-kinase activity (11,12) and to activation of Akt2 and protein kinase C- α , resulting in insulin-stimulated GLUT-4 translocation and increased glucose transport (13,14). Free (i.e., non-p110-bound) p85 subunit has been proposed to negatively regulate PI 3-kinase signaling by competition with p85/p110 dimers for recruitment to phosphotyrosine docking sites (15–19), but this view was recently challenged by Geering et al. (20), who found equimolar amounts of p85 and p110 subunits in mammalian cell lines and tissues and no evidence for free subunits, arguing against a role of free p85 in PI 3-kinase signaling. Although the molecular hierarchy of mechanisms leading to insulin resistance is yet incompletely understood, the major contribution of PI 3-kinase subunits is well established in obese humans (8,21) and animal models of obesity (22,23). The obese state is associated with a mild to severe decrease in the muscle content of various signaling proteins, including the insulin receptor IRS-1 and the p85 and p110 subunits of PI

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Received for publication 6 September 2007 and accepted in revised form 25 October 2007.

Published ahead of print at <http://diabetes.diabetesjournals.org> on 31 October 2007. DOI: 10.2337/db07-1273.

EMSA, electromobility shift assay; eQTL, expression quantitative trait locus; eQTN, expression quantitative trait nucleotide; HOMA-IR, homeostasis model assessment of insulin resistance; INSERM, Institut National de la Santé et de la Recherche Médicale; IRS, insulin receptor substrate; PI, phosphatidylinositol; SNP, single nucleotide polymorphism.

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3-kinase, leading to decreased insulin-stimulated activation of PI 3-kinase activity associated with IRS-1 (8,21–23). Even if PI 3-kinase subunit protein amounts were not measured in these observations, we hypothesized that genetic variation in the function or abundance of p85 or p110 subunits could create variations in the activity of PI 3-kinase heterodimers and thus, possibly, individual variation in insulin sensitivity. Searching for potentially functional polymorphisms in PI 3-kinase subunit genes at the single nucleotide polymorphism (SNP) level, we (P.-H.R.) noticed that the common C allele of the –359 C/T SNP (NCBI rs361072) creates a potential GATA-binding motif in the promoter region of *PIK3CB*. Kossila et al. (24) had previously reported the lack of association of this variant with insulin resistance in 295 nonobese Finnish adults, but we thought that the genetic susceptibility to obesity-related insulin resistance could have its own mechanisms, partly different from those implicated in the insulin resistance of lean people. We thus decided to focus our genetic study on the p110 β promoter variant and to test its association with obesity-related insulin resistance. To match the natural history of insulin resistance, we studied this association in juvenile patients during the initial dynamic phase of obesity (25). Functional studies were performed in parallel to explore potential transcriptional effects of rs361072 and how it could affect the PI 3-kinase–Akt pathway.

RESEARCH DESIGN AND METHODS

Studied cohorts

Inclusion criteria were obesity defined as a BMI >95th centile at time of study, a BMI >85th centile before age 6 years, and a monotonic weight curve since birth (25). Studies were approved by Institutional Ethics Committees, and informed consent was obtained from parents and children. No subject had diabetes, all were healthy, and none was taking any medication. Subjects of Obgen, a hospital-based obese children cohort, were recruited (25) whose European origin was ascertained through family history and grandparents' birthplace. The Saint-Vincent obese children cohort was recruited from all of France (26) in which only patients with all grandparents born in continental Europe were analyzed. The Lille obese cohort included 392 white Caucasian children mostly from Northern France (27). The Napoli cohort recruited obese children of strict Italian origin (28). Ile-de-France is a cohort of French children of European origin whose recruitment started in 2006. We also studied two cohorts of European children whose body weight was within 90–110% of ideal body weight for age, whose four grandparents were white Caucasians born in Europe, and who had no family history of diabetes or abnormally high birth weights. The Leangen cohort included 606 nonobese children recruited among families of investigators, colleagues, nurses, and friends from 1986 to 1999 and among patients hospitalized for reasons not known to interfere with insulin or glucose homeostasis (benign surgery, mild short stature, or cryptorchidism) (29). The Growthgen (Pediatric Endocrinology, Saint Vincent) cohort included 884 nonobese children from 1991 to 2007, 693 evaluated for "idiopathic short stature" and 274 selected among patients hospitalized for benign pediatric conditions. We were careful that none of the studied subjects were from the same family or from related families. Children belonging to isolated communities having obvious high levels of consanguinity like French gypsies or Basques were excluded.

Experimental procedures for clinical studies

During the 3 days preceding insulin measurements, children of Obgen, Saint-Vincent, Ile-de-France, Leangen, and Growthgen cohorts were prescribed a standardized diet. Parents or nurses checked diet observance, and children with insufficient intake (<75% of prescribed carbohydrates) were excluded. In Lille and Napoli cohorts, children were asked to eat normally and have no strenuous physical activity in the days preceding the study. Children fasted 12 h overnight, from the end of dinner (8:00 P.M.) to time of sampling (8:00 A.M.).

Insulin and glucose measurements

Plasma glucose was determined with a glucose analyzer (Beckman Coulter, Fullerton, CA), and serum insulin was determined by time-resolved fluoroimmunoassay using Wallac Delfia reagents (PerkinElmer SAS, Courtabouff, France). For insulin, the intra- and interassay coefficients of variation at the

level of 10 μ U/ml were 4.1 and 5.0%, respectively; at the level of 27.5 μ U/ml, 3.8 and 5.1%; and at the level of 65 μ U/ml, 3.7 and 4.9%, with a detection limit of 0.5 μ U/ml. Cross-reactivity of C-peptide was 0.01%, and of proinsulin, 0.1%. Insulin measurements were performed in the same laboratory for Obgen, Saint-Vincent, Ile-de-France, Leangen, and Growthgen cohorts and standardized by exchanging samples blindly with Lille and Napoli centers.

Homeostasis model assessment index

The homeostasis model assessment (HOMA-IR) index was calculated as the product of fasting plasma insulin (in microunits per milliliter) and fasting plasma glucose (in millimoles per liter), divided by 22.5 (30). Higher HOMA values indicate higher insulin resistance. This index was the only method that could be applied to estimate insulin resistance in the studied children, including the nonobese children controls, for both ethical and practical reasons. Several studies (1,2,31–34) validated HOMA-IR as a reliable index of insulin resistance in obese children under technical conditions about the insulin assay and experimental circumstances fulfilled in the current study.

Genotyping

SNPs information was extracted from dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>), the International HapMap Project (<http://www.ncbi.nlm.nih.gov/SNP/hapmap.org>), and CNV database. Markers available in the CEU Hapmap dataset (May 2006) within a 400-kb region flanking *PIK3CB* were identified. We calculated pairwise linkage disequilibrium (r^2) between these markers based on the CEU Hapmap genotype data. Then we selected a subset of 11 SNPs with minor allelic frequencies > 0.1 so that all the CEU Hapmap markers with minor allelic frequencies > 0.05 had $r^2 > 0.8$ with at least one of the genotyped markers. Genomic DNA samples were prepared from peripheral blood using PureGene kit (Gentra, Minneapolis, MN) or a standard phenol/chloroform method. The 11 SNPs were genotyped using Taqman Assay-by-Design and analyzed by allelic discrimination using the ABI PRISM 7900HT Sequence Detection System and software (SDSv2.0; Applied Biosystems, CA). For the –359 C/T variant, the PCR reaction was carried out in 50 μ l containing 200 ng genomic DNA, 1 μ mol/l each primer (forward, 5'-CCTGTCAAGTGCTGGTTAACTA-3'; and reverse, 5'-CAATCCATACCACCACTAAAG-3'), 0.2 mmol/l each dNTP, 1.5 mmol/l MgCl₂, and 1.25 units *Taq* polymerase Fast Start (Roche). Allele positivity (presence of a T at position –359) and allele negativity (a C in the same position) were identified through the AATATT sequence recognized by *SspI*. Eight samples previously genotyped were introduced into each 96-well plate as internal controls. We genotyped 3,809 DNA samples, including 288 internal controls and 90 samples that had to be re-run for uncertain results. Sixty-five samples could not be genotyped (1.9%), and successful genotyping was obtained in 3,366 subjects (Table 4). The average error rate was <0.7% (two errors among 288 duplicates). Allele frequencies for all SNPs were in Hardy-Weinberg equilibrium. Haplotypes were estimated using the expectation-maximization algorithm.

Genomic control

The cohorts and genotypic groups were subjected to "genomic control" according to the method described by Pritchard et al. (35). In brief, a set of 374 markers selected based on chromosomal location and heterozygosity that covered the human genome at 10-cM average resolution (ABI Prism Linkage Mapping Sets version 2.5; Applied Biosystems) was genotyped. We found no evidence for population stratification in the studied cohorts considered separately as well as pooled and genotypic subgroups.

Statistical analyses

All values are expressed as means \pm SE. Variables that were not normally distributed (BMI, insulin level, and HOMA-IR) were log-transformed for statistical analysis. However, for clarity of interpretation, results are expressed in tables as untransformed values.

General linear regression models were created to estimate effects of BMI and genotype adjusted for age, sex, and puberty level assessed by the Tanner score. Multivariate analyses were adjusted on sex and pubertal stage. An interaction between BMI and genotype was introduced in the model and was significant. This means that genotype effect on HOMA-IR depends on BMI level. Sensibility analyses were performed to assess the level of BMI for which genotype has a significant effect on HOMA-IR. Moreover, the model was tested in five different cohorts of children to check consistency of results between cohorts. Normality of residuals was systematically verified, and robustness analyses were systematically performed by dropping outliers from analysis. Then, the five different cohorts of obese children were pooled, and a linear mixed-effects model was computed to assess the consistency of the effect of genotype on HOMA-IR adjusted for BMI with an interaction between BMI and genotype, taking into account center effect as a random effect. In all cohorts, the level of the cutoff of BMI for which there was a significant effect genotype on HOMA-IR was assessed by sensibility analysis. This cutoff value was around 31 in all cohorts. All *P* values were two-sided, and a *P* value <0.05 was considered significant. Data analyses used the R statistical software.

TABLE 1
Application of the general linear regression model to the five cohorts of obese children

Factors	Obgen obese children (n = 358)		Saint Vincent obese children (n = 397)		Lille obese children (n = 350)		Napoli obese children (n = 607)		Ile-de-France obese children (n = 164)		Pooled five cohorts (n = 1,876)	
	Regression coefficients*	P value	Regression coefficients*	P value	Regression coefficients*	P value	Regression coefficients*	P value	Regression coefficients*	P value	Regression coefficients*	P value†
BMI	0.07 ± 0.03	5 × 10 ⁻⁴	0.13 ± 0.04	2 × 10 ⁻⁴	0.17 ± 0.06	5 × 10 ⁻³	0.17 ± 0.05	8 × 10 ⁻⁴	0.09 ± 0.05	0.01	0.10 ± 0.03	1.2 × 10 ⁻¹⁴
p110β	2.15 ± 0.38	3.3 × 10 ⁴	2.55 ± 0.35	1.7 × 10 ⁻¹²	1.30 ± 0.48	7 × 10 ³	1.54 ± 0.35	1 × 10 ⁻⁵	1.46 ± 0.38	2 × 10 ⁻⁴	2.03 ± 0.16	3.2 × 10 ⁻¹⁵
	C/T	9 × 10 ⁻⁴	1.47 ± 0.33	1.0 × 10 ⁻⁵	0.38 ± 0.44	0.15	0.70 ± 0.30	2 × 10 ⁻³	1.13 ± 0.35	1.5 × 10 ⁻³	1.12 ± 0.14	3.7 × 10 ⁻⁸
	C/C	0	0	0	0	0	0	0	0	0	0	0.0000
p110β × BMI	0.29 ± 0.05	6 × 10 ⁶	0.38 ± 0.05	1.9 × 10 ⁻¹²	0.19 ± 0.08	0.01	0.25 ± 0.07	6 × 10 ⁻¹	0.14 ± 0.07	0.048	0.30 ± 0.30	2.1 × 10 ⁻⁸
	C/T	8 × 10 ⁴	0.17 ± 0.04	2 × 10 ⁻⁴	0.10 ± 0.07	0.2	0.07 ± 0.06	0.2	0.12 ± 0.06	0.035	0.17 ± 0.02	2 × 10 ⁻⁶
	C/C	0	0	0	0	0	0	0	0	0	0	0.0000

Data are means ± SE. Estimates of regression coefficients were calculated for a BMI of 35 kg/m². This modeling shows the expected influence of BMI and the consistent influence of the -359 genotype on insulin resistance. It also shows a significant modification of the HOMA-BMI relationship by the -359 genotype. *Estimate of regression coefficient, mean ± SE. †Overall significance for the pooled 5 obese cohorts. ‡Using a mixed-effects model was used to account for potential center effects when pooling the 5 cohorts together. The value of P < 0.0000 is the lowest possible generated by the use of the latter model.

Data issues

Data of the association study will be made available on request so that others can analyze them independently.

Functional studies of rs361072

EMSAs and binding of hGATA-3 and cGATA-2 peptides to the C probe. Following the hypothesis that the C allele creates, on the noncoding DNA strand, a potential binding motif, AGATAT, for GATA transcription factors (Fig. 2A3), we characterized this GATA binding site by electromobility shift assays (EMSAs). Radiolabeled oligonucleotides containing AAATAT (T probe) or the variant AGATAT sequence (C probe) of p110 β promoter (Fig. 2A1) were used to detect the binding of GATA proteins from cell nuclear extracts. The specificity of the binding of the C probe was shown using increasing amounts of cold canonical GATA probe as competitor (Fig. 2A1).

The DNA binding domains of chicken GATA-2 and human GATA-3 were tested by competitive EMSA for binding to a probe derived from the p110β gene promoter containing only the C site, with a canonical GATA site, or an oligonucleotide of random sequence as competitors (Fig. 2A2). Dissociation constants were determined as described previously (36).

Transient transfections of C and T promoters in cell lines. A DNA fragment spanning nucleotides -441 to +23 relative to the transcription initiation start site and carrying a T or a C in position -359 was inserted upstream of the firefly luciferase-coding sequence and transfected in Jurkat, HEK293, and HepG2 cells, and luciferase activities were determined using the dual-luciferase assay (Promega, Madison, WI).

p110β mRNA and protein in TCD4+ cells from obese children. Fresh circulating TCD4+ lymphocytes were purified from venous blood. A fraction of cells were stimulated for 72 h with PHA/IL2 and lysed. Total RNA was extracted, and real-time quantitative RT-PCR was performed using RPLPO transcripts for normalization (37). To establish the individuality of mRNA levels, intra-assay and intra-individual SD-to-mean ratios of measurements in six subjects sampled repeatedly were calculated to be 7.2 and 16%, respectively, whereas the inter-individual SD-to-mean ratio in cohorts was >150%, consistently with transcriptome analysis data in circulating human lymphocytes (38).

For immunoblot protein detection, 5.10⁵ TCD4+ cell extracts were electrophoresed through 8% polyacrylamide/SDS gels and transferred onto nitrocellulose membranes (Hybond ECL; Amersham) incubated with polyclonal antibodies to p110β (Santa Cruz Biotechnology, Santa Cruz, CA), p85 (Upstate, Charlottesville, VA), P-Akt and Akt (Cell Signaling, Danvers, MA), and actin (Sigma, St. Louis, MO). Recombinant p85 and p110β proteins were given to us by B. Vanhaesebroeck (Ludwig Institute for Cancer Research, London, U.K.). The proteins were visualized by the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and monitored in an imaging system (ChemioGenius²; SynGene, Frederick, MD). Band intensity was analyzed using GeneTools (SynGene) and normalized to actin. Measurements were carried out in duplicate and re-analyzed if values discorded by >50%. We checked P-Akt and Akt measurements using another method. Cell lysates separated by SDS-PAGE were transferred to nitrocellulose membranes saturated with Blocking Buffer Odyssey (Li-Cor Biosciences, Lincoln, NE) and incubated overnight with a polyclonal rabbit anti-P-Akt antibody (Cell Signaling, Danvers, MA) and a mouse anti-Akt antibody (R&D System, Minneapolis, MN). Secondary antibodies labeled with IRDye 800w and 680 (Li-Cor Biosciences, Lincoln, NE) were used for visualization. Blotted proteins were detected and quantified with the Odyssey Infrared Imaging System (Li-Cor Biosciences). Scanning of membranes was performed at 680 nm (Akt) and 780 nm (P-Akt) with an Odyssey instrument. The Li-Cor results correlated closely with chemoluminescence (1.0 > r > 0.89). We used chemoluminescence because it showed the best scores for precision and reproducibility and correlated closely with Li-Cor measurements and because the initial set of patients' lymphocytes was studied using chemoluminescence. The SD-to-mean ratio for protein measurements was 7-12% (intra-assay) and 13-22% (intra-individual), indicating that our evaluation of protein levels was reasonably consistent for a given individual.

Determination of PI 3-kinase activity in blood cells

Using a previously reported method (39), we were not able to obtain reliable individual measurements of PI 3-kinase enzymatic activity in lymphocytes or platelets that could be used to compare patients. Instead, we used the cellular P-Akt content as a reflection of PI 3-kinase activity.

RESULTS

Association of rs361072 with HOMA-IR in the OB-GEN cohort. The distribution of rs361072 alleles was comparable in obese and nonobese cohorts, indicating that this variant is not a significant marker of the susceptibility to early-onset obesity.

TABLE 2

Characteristics of the 11 common SNPs neighboring the functional -359 C/T variant over 80 kb of the p110β gene region

Single nucleotide polymorphism	Gene	MAF	Significance of association with HOMA (<i>P</i> value)	Physical position	Distance from -359 C/T (bp)	HWE (<i>P</i> value)
rs1679168	FAM62	0.26	0.99	139674890	286352	0.847
rs1618357	FAM62	0.41	0.65	139677133	284109	0.490
rs6439809	Cep70	0.48	0.30	139709731	251511	0.878
rs9855074	Cep70	0.23	0.39	139727626	233616	0.191
rs12636246	FAIM	0.38	0.98	139829284	131958	0.261
rs601032	FAIM	0.23	0.39	139832030	129212	0.823
rs600590*	FAIM	0.49	5.10 ⁻⁵	139832105	129137	1
rs9878820	p110β	0.37	0.27	139868113	93129	0.606
rs693293	p110β	0.11	0.88	139891874	69368	0.673
rs361080*	p110β	0.50	1.3 × 10 ⁻⁶	139900687	60555	0.323
rs361072 (-359 C/T)	p110β	0.49	3.3 × 10⁻⁸	139961242	0	0.99
rs11716652	—	0.11	0.23	140048087	86845	0.144

Bold type, -359 C/T variant. Association of each SNP with HOMA is described using the general linear model. *Two SNPs (rs600590 and rs361080) were found to be in near complete linkage disequilibrium ($D/D_{\max} > 0.985$) with the functional -359 SNP, and thus both showed association with HOMA. MAF, minor allele frequency.

Linear regression analysis found that rs361072 was strongly associated with HOMA-IR in the *Obgen* cohort. For a given BMI, HOMA-IR values were lower in obese children with C/C genotype, intermediate with C/T genotype, and higher with T/T genotype (Table 1). There was a strong interaction between BMI and genotype (Table 1), reflecting the dependence of the genotype effect on BMI. We were thus not surprised to find only a marginal association of rs361072 with HOMA-IR in the nonobese children of the Leangen cohort ($P = 0.04$), supporting that the effect of rs361072 on insulin resistance has a relevant magnitude only in conditions of excess fat accumulation. **Test of neighboring SNPs for association with HOMA-IR.** Among the 11 common SNPs neighboring rs361072, only 2 rs361080 in intron 10 of p110β and rs600590 in intron 5 of *FAIM* were found to be in near complete linkage disequilibrium with rs361072 and thus to

associate with HOMA-IR ($P = 1.3 \times 10^{-6}$ and 5×10^{-5}) (Table 2). We were not able to assign any potential function to these SNPs, which together with rs361072 formed a haplotype block that associated with HOMA-IR to the same extent as rs361072. No other SNP or haplotype showed association with HOMA-IR. We replicated these results in the Saint-Vincent obese children cohort. Following these observations, our study focused exclusively on rs361072 for further genetic and functional analyses.

Association of rs361072 variant with HOMA-IR in replication cohorts. Strict nutritional and fasting conditions are required to allow a reliable HOMA-IR measurement that restrain the number of children cohorts allowing reliable genetic studies of insulin resistance. We were able to investigate the effect of rs361072 in four additional independent replication cohorts of obese children (26–28) of strict European ancestry: The association of rs361072

TABLE 3

Main characteristics of rs361072 genotypic groups in the five pooled cohorts of obese children and the two pooled cohorts of nonobese children

	Total obese children* ($n = 1,876$)			<i>P</i> value
	TT	CT	CC	
<i>n</i>	468	930	478	
Percent female	56	56	54	
Age (years)	11.9 ± 0.1	12.0 ± 0.1	12.1 ± 0.1	0.68
BMI (kg/m ²)	32.3 ± 0.3	31.9 ± 0.2	32.2 ± 0.3	0.52
Fasting insulin (μU/ml)	20.9 ± 0.7	18.5 ± 0.4	16.1 ± 0.4	1.6 × 10 ⁻⁵
Fasting glucose (mg/dl)	86 ± 0.5	84 ± 0.3	84 ± 0.4	0.02
HOMA-IR	4.5 ± 0.2	3.9 ± 0.1	3.3 ± 0.1	4.5 × 10 ⁻⁶
	Nonobese children† ($n = 1,490$)			
	TT	CT	CC	<i>P</i> value
<i>n</i>	355	746	389	
Percent female	61	62	66	
Age (years)	12.1 ± 0.1	11.8 ± 0.1	11.7 ± 0.1	0.21
BMI (kg/m ²)	18.2 ± 0.1	18.3 ± 0.1	18.1 ± 0.1	0.58
Fasting insulin (μU/ml)	9.2 ± 0.2	8.8 ± 0.1	8.7 ± 0.1	0.04
Fasting glucose (mg/dl)	84 ± 0.2	83 ± 0.2	83 ± 0.2	0.62
HOMA-IR	2.0 ± 0.04	1.9 ± 0.02	1.9 ± 0.03	0.04

Data are means ± SE. *P* values refer to analysis of variance between genotype groups after log transformation of insulin, glucose, and HOMA-IR. For clarity, real values of these parameters are displayed. *Pooled from Obgen, Saint Vincent, Lille, Napoli, and Ile-de-France cohorts. †Pooled from Leangen and Growthgen cohorts. To convert values for insulin to picomoles per liter, multiply by 6; to convert values for glucose to millimoles per liter, multiply by 0.055.

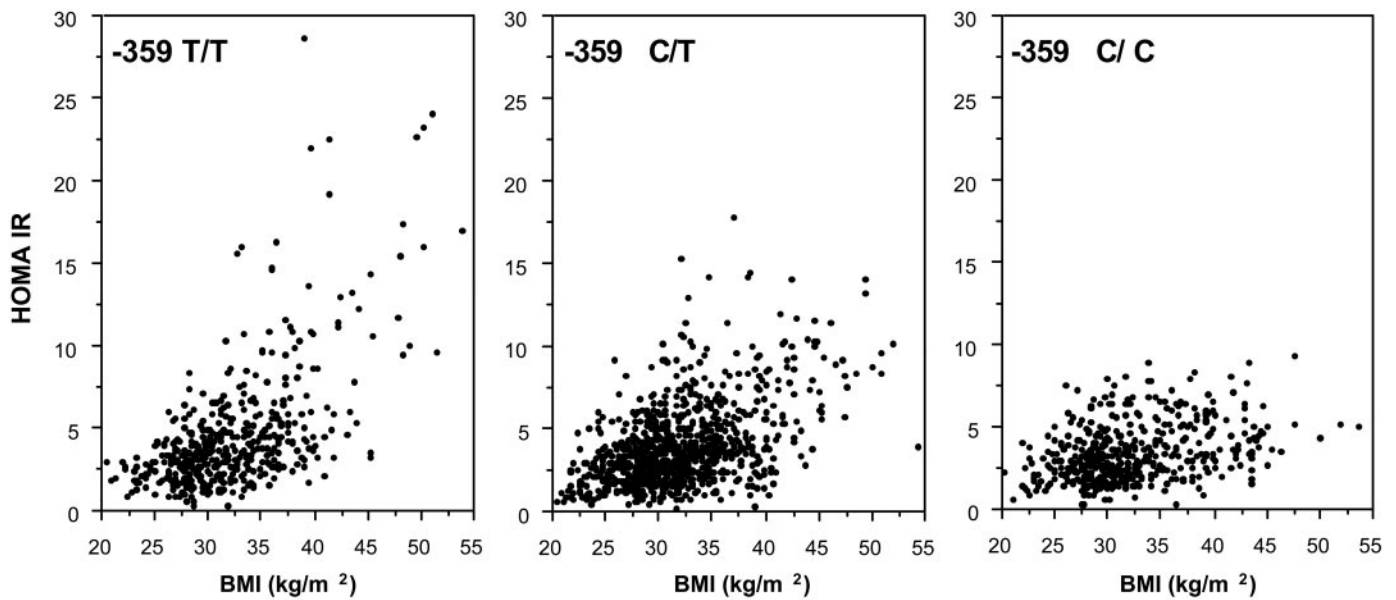


FIG. 1. The -359 SNP influences the relationship between HOMA index and BMI in the obese children pooled from the five cohorts. The regression between HOMA index and BMI is described by the equations $y = 0.40x - 8.4$ ($r = 0.60$) in the T/T obese children, $y = 0.26x - 4.3$ ($r = 0.53$) in the C/T heterozygote patients, and $y = 0.11x - 0.2$ ($r = 0.36$) in C/C.

with HOMA-IR was significant in all four cohorts (Table 1). A linear mixed-effects model found no cohort effect on the results, allowing pooling of the five obese cohorts for a global presentation in Tables 1 (linear regression analysis) and 3 (ANOVA) and in Fig. 1. HOMA-IR showed consistent differences across the rs361072 genotypic groups, with C/C obese children having lower HOMA-IR values (Table 3). The effect of BMI on HOMA-IR was different by genotype (Fig. 1), as reflected by the results for interaction estimates (Table 1). The greatly attenuated insulin resistance-BMI relationship in C/C (Fig. 1) and the intermediate HOMA-IR values in C/T obese children support an additive effect of the C allele. Fasting glucose was slightly higher in T/T patients (Table 3).

Again, we found no significant effect of rs361072 in another cohort of 884 nonobese European children (Growthgen, mean age 11.5 years, BMI <19.2 kg/m²). However, pooling the two nonobese cohorts allowed observance of a marginally significant ($P = 0.04$) difference for HOMA-IR between the genotypic groups (Table 3). Age, puberty, and sex had no significant effect on HOMA-IR variation in obese children, whereas age, and thus puberty, showed a major effect in the nonobese Leangen ($P = 4.3 \times 10^{-7}$) and Growthgen cohorts ($P = 7.9 \times 10^{-10}$).

The prevalence of the C allele of rs361072 in the studied cohorts was 0.48–0.52 (Table 4), with 0.24–0.27 C/C

homozygotes compared with 0.13 in Finnish studies (24). The prevalence of C was 0.35 and 0.41 in Burkina Faso ($n = 319$) and Cameroon ($n = 291$) and only 0.035 in Japanese ($n = 128$), concordant with HapMap estimations. **Functional in vitro and in vivo studies of rs361072.** We found that the C allele creates a potential GATA binding motif, AGATAT. The AGATAT probe corresponding to the C allele showed a specific binding to a Jurkat cell nuclear protein complex similar to the one obtained with the canonical GATA sequence, whereas the AAATAT probe (T allele) could not bind this complex (Fig. 2A1). These results were replicated with HepG2 extracts. Competitive EMSAs showed that both cGATA-2 and hGATA-3 DNA binding domain peptides bound with high affinity to the C probe and with low affinity to the T promoter (Fig. 2A2).

The in vitro transcriptional activity of the C promoter was increased versus the T promoter. Transfected into Jurkat, HEK293, or HepG2 cells, the C promoter fragment induced a 45% (Jurkat), 30% (HEK293), and 38% (HepG2) increase of transcriptional activity compared with the T promoter (Fig. 2B1) in an orientation-dependent manner. Thus, the GATA-binding site of the C promoter acted as a promoter but not as an enhancer element. No difference was found in HeLa cells, which do not express GATA proteins. Cotransfected with a hGATA-3 expression vector, the C promoter was more than fivefold activated by

TABLE 4
Genotype counts (SNP rs361072 or -359 C/T SNP), Hardy-Weinberg tests, and failed genotype rate

Cohorts	TT	CT	CC	f(C)*	pHWE†	Missing‡
Obgen obese children	84	179	95	0.52	0.99	8 (2)
Saint Vincent obese children	105	187	105	0.50	0.26	10 (2.5)
Lille obese children	89	179	82	0.49	0.69	6 (1.7)
Napoli obese children	143	307	157	0.51	0.81	13 (2.1)
Ile-de-France obese children	47	78	39	0.48	0.6	3 (2.4)
Leangen non-obese children	157	291	158	0.50	0.32	9 (1.5)
Growthgen non-obese children	198	455	231	0.52	0.38	16 (2)

*Frequency of C allele. †P value for Hardy-Weinberg disequilibrium test. ‡n (%) of failed genotypes.

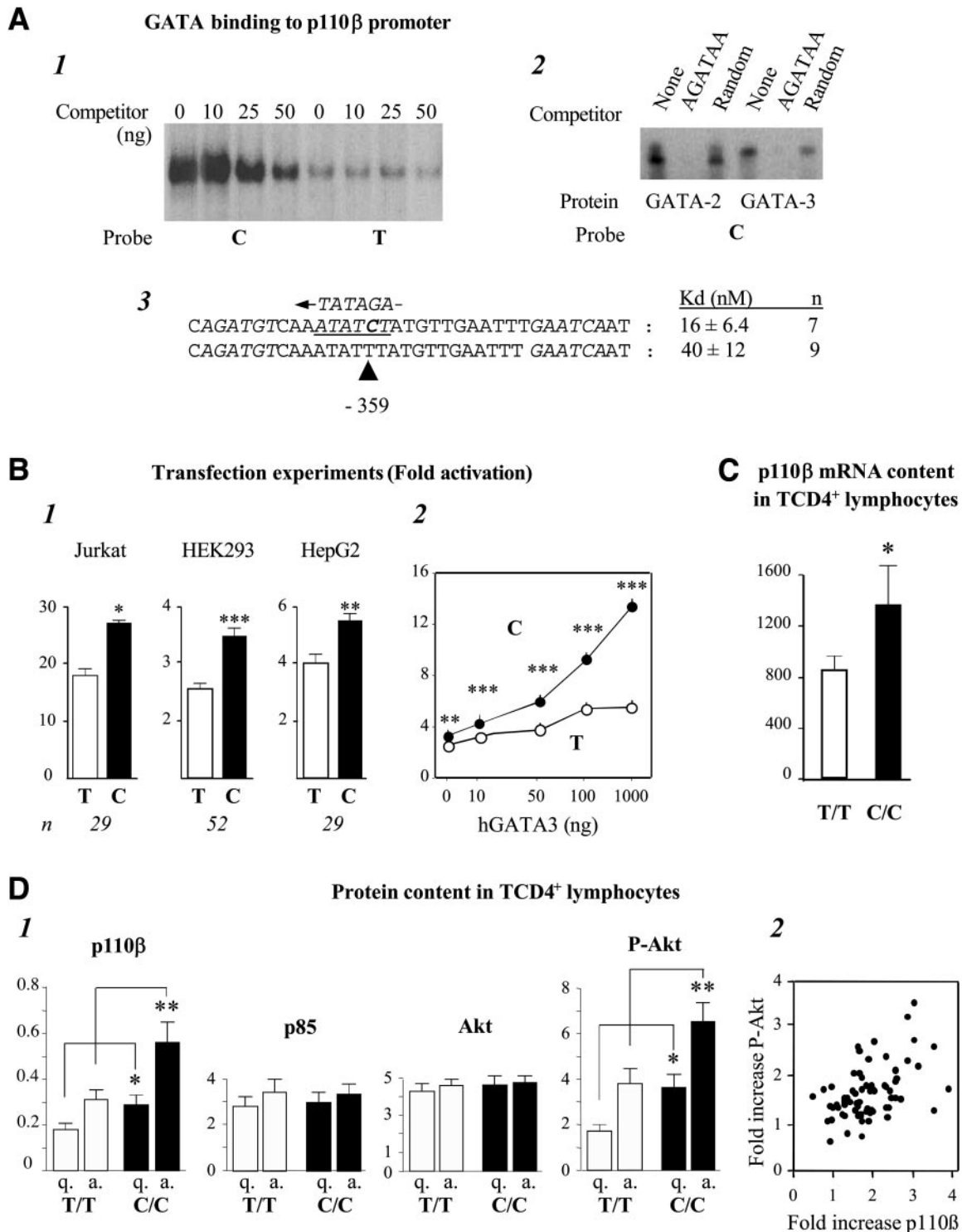


FIG. 2. The C allele of the $-359C/T$ SNP creates a GATA-binding site associated with increased transcriptional activity of the p110 β promoter and increased Akt phosphorylation. **A:** GATA proteins bind the GATA-binding site created by the C variant. **1)** Specific binding of GATA to the C probe. EMSAs were performed using the C or T probe and Jurkat T cell nuclear extracts. Competition using increasing amounts of a consensus GATA-binding site TGATAG competitor showed the specificity of the binding. **2)** hGATA-3 and cGATA-2 bind specifically to the C probe. The DNA binding domains of chicken GATA-2 and human GATA-3 were tested for binding to a probe containing only the C site, with a canonical GATA site or a random oligonucleotide as competitors. **3)** Sequences of the C and T alleles flanking the $-359C/T$ polymorphism on the p110 β promoter, with their dissociation constants. **B:** Increased transcriptional activity of p110 β promoter sequence carrying the $-359C$ allele. **1)** Results (mean \pm SE) of transfection experiments in Jurkat cells, HEK293, and HepG2 cells. $*P < 0.02$, $***P < 0.0002$. **2)** Effect of cotransfected hGATA-3 protein on the transcriptional activity of the C or T promoters, expressed relative to empty vector. Each value (mean \pm SE) is the average of 58 independent experiments ($***P < 0.0001$). **C:** C/C subjects have increased levels (mean \pm SE) of p110 β mRNA in their TCD4⁺ lymphocytes. $*P < 0.05$. **D:** p110 β and effectors of the PI 3-kinase-Akt pathway in TCD4⁺ lymphocytes from obese children. **1)** Comparison of p110 β , p85 α , Akt, and P-Akt protein content (mean \pm SE) normalized to actin in quiescent (q) and activated (a) C/C and T/T lymphocytes. $*P < 0.03$, $**P < 0.01$. **2)** Relative increases in p110 β and P-Akt protein levels are correlated in activated lymphocytes ($r = 0.50$, $P < 0.001$).

hGATA-3 and never reached a plateau, whereas the T promoter showed only a modest increase (Fig. 2B2).

We next studied the *in vivo* effects of the C allele on the PI 3-kinase-Akt pathway in lymphocytes from obese children. Although not metabolic targets of insulin, circulating TCD4⁺ lymphocytes, known to contain high levels of GATA-3, were used because of practicability and reliability to reflect insulin effects (40). Basal and activated C/C lymphocytes showed higher p110 β mRNA (Fig. 2C) and protein content (Fig. 2D1), whereas p85 α protein levels were similar (Fig. 2D1). Although having comparable Akt content (Fig. 2D1), C/C lymphocytes displayed greater levels of P-Akt protein than T/T lymphocytes (Fig. 2D1). The relative increase in p110 β and P-Akt were correlated (Fig. 2D1). p110 β content in TCD4⁺ lymphocytes and HOMA-IR of OGEN patients were inversely related following the equation $y = -1.7x + 3.5$ ($r = 0.37$, $P < 0.01$).

DISCUSSION

To limit the risk of reporting a false association, the current study attempted to match strict guidelines for genotype-phenotype associations (41), including the demonstration of consistent genetic and functional effects of rs361072. Although positive associations need confirmation by multiple replications and other investigators, we believe that our observation is safeguarded from false positivity by the statistical strength of the rs361072 association with HOMA-IR, by the replication in independent cohorts, by the lack of demographic stratification, and by the demonstration of a cis-regulatory function for the variant in transfected cell lines and in lymphocytes from the patients.

The C allele of rs361072 initially suspected to create a GATA-binding site in the promoter of *PIK3CB* was found to bind GATA peptides with high affinity and to activate p110 β transcription through a promoter effect, resulting in increased p110 β expression at the RNA and protein level. The *PIK3CB* region, which harbors three SNPs associated with *PIK3CB* expression, one of which is rs361072, can thus be considered as a eQTL. More specifically, the C allele of rs361072 can be considered both an expression quantitative trait nucleotide (eQTN) acting in cis on *PIK3CB* transcription and a QTN for insulin resistance. This is one of the few examples of a SNP cis-acting as a eQTL (or eQTN) on a complex trait in humans (42). We postulate that the effect of rs361072 on insulin resistance is directly related to the variation of p110 β abundance in insulin target cells and a resulting increase in PI 3-kinase heterodimers and increased Akt phosphorylation in lymphocytes from C/C obese children. This could explain why homozygous C/C have a trend for milder insulin resistance than T/T obese patients.

Our work certainly has a major weakness: the lack of direct studies in insulin target tissues. Because HOMA-IR reflects mostly the effect of fasting insulin on hepatic glucose production (43), it would have been important to document the functional effects of the variant in the liver, which could not be done for obvious reasons. For ethical and methodological reasons, it was not realistic either to consider sampling of adipose tissue or muscle biopsies in large numbers of children. Given the effect size of our rs361072 on *PIK3CB* transcription, it is unlikely that sampling a small number of obese children would yield a statistical power sufficient to test such a genotypic effect. Thus we can only postulate that p110 β expression and the

activity of the PI 3-kinase-Akt pathway studied in lymphocytes might reflect that of liver, muscle, or adipose tissue (40). Several observations support that rs361072 could affect the regulation by insulin of adipose tissue deposition and metabolism. Intra-myocellular and intra-abdominal lipid accumulation are linked to insulin resistance development in obese children (44), and p110 β is a major effector of insulin action on adipose tissue metabolism and adipogenesis (45) under GATA2 or GATA3 control (46). The GATA-regulated p110 β promoter variant could thus affect adipose tissue development and partition between visceral and subcutaneous depots (44), and the resulting changes in insulin resistance in liver and muscle (47,48). We favor this interpretation because it would help understand the association of rs361072 with BMI with respect to insulin resistance. The effects of the C allele of rs361072 can also occur primarily in the liver or muscle or in the hypothalamus (49) through interaction with other members of the GATA family, such as GATA 6 (50). In muscle biopsies from obese adults, Bandyopadhyay et al. (21) documented a large decrease in p110 subunit, a decrease in p85 subunit, and a decrease in insulin-stimulated PI3-kinase activity in IRS-1 immunoprecipitates, fully consistent with a decrease in formation of IRS-1/p85/p110 trimeric complexes. Decreased Akt phosphorylation was also reported in muscle biopsies of insulin-resistant obese subjects (21,51).

The association of rs361072 with HOMA-IR indicates that genetic markers could help identify obese children who are at greatest risk of developing insulin resistance. The different prevalence of rs361072 in Africans, Asians, and Europeans may influence the relationships between obesity and associated insulin resistance in obese children of these populations. Because only a marginal effect was detected in nonobese adolescents, we do not expect rs361072 to play a significant role in the insulin resistance of patients with type 2 diabetes who do not have marked obesity, nor a fortiori in the predisposition of nonobese patients to type 2 diabetes itself (52). Further studies are needed to delineate the role of rs361072 in the genetics of the relationships between obesity, insulin resistance, and obesity-related type 2 diabetes.

ACKNOWLEDGMENTS

This study has been supported by the Agence Nationale des Refusés 2005. C.T. has received support from the Intramural Research Program of the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health.

A. Millet did the first experiments long ago as a summer student. We thank B. Payrastré for the enzymatic assays of PI 3-kinase; F. Amalric for many discussions; A. Dermane for in-hospital patient investigation; C. Dos Santos, I. Vigon, and I. Max-Audit for technical help; and A. Rothenbuhler for editing support. We acknowledge the clinicians who provided the samples and phenotypes of their patients to D. Meyre and P. Froguel. We thank Valigen Biotech for preliminary work.

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