Brief Genetics Report

Common Polymorphisms in the Promoter of the Visfatin Gene (*PBEF1*) Influence Plasma Insulin Levels in a French-Canadian Population

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The adipokine visfatin (*PBEF1*) exhibits insulin-mimetic effects and correlates strongly with visceral adiposity. We sequenced visfatin gene exons and 1,480 bp of the promoter in 23 individuals, including 18 individuals from the Quebec Family Study (QFS) with varying degrees of abdominal visceral fat, assessed by computed tomography, and 5 individuals from the Saguenay-Lac-Saint-Jean region of Québec. We identified a synonymous polymorphism in exon 7 (SER301SER) but no nonsynonymous mutations. We observed an additional 10 polymorphisms, including 5 intronic, 4 within the promoter, and 1 within the 3′ untranslated region. Further promoter sequencing (816 bp) identified five additional single nucleotide polymorphisms (SNPs) in the QFS population. To investigate the role of visfatin gene variants in obesity-related phenotypes, we genotyped a total of 13 SNPs in the promoter region of the gene. From these, we analyzed the seven common SNPs in the QFS sample (918 participants from 208 families). A significant association was found between two SNPs (rs9770242 and rs1319501), in perfect linkage disequilibrium, and fasting insulin levels ($P = 0.002$). These SNPs were also associated with fasting glucose ($P \leq 0.02$). In addition, a more distal SNP (rs7789066) was significantly associated with the apolipoprotein B component of VLDL ($P = 0.012$). *Diabetes* 55:2896–2902, 2006

One of the biggest threats to global health is the rising epidemic of obesity. Observed first in the wealthier Western world (1), it has now been shown to accompany the Westernization of many rising economies (2). Obesity frequently occurs with a constellation of clinical parameters that are all poor prognostic indicators for diabetes and coronary heart disease (CHD). Obesity can be defined as an excessive amount of body fat. However, the distribution of fat may also be important for determining risk. The existence of an association between visceral adipose tissue accumulation and an increased risk of type 2 diabetes and CHD is well established (3). Visceral obesity is one of several features of the metabolic syndrome, a clustering of risk factors associated with CHD, which includes insulin resistance, dyslipidemia, and hypertension (4).

Insulin resistance plays a central role in the pathogenesis of the metabolic abnormalities associated with visceral adiposity. Chemical messengers secreted by adipocytes, termed adipokines, are thought to modulate insulin action (5). The newly identified adipokine visfatin, also known as pre–B-cell colony enhancing factor (*PBEF1*) (6), was discovered to be preferentially expressed in visceral as opposed to subcutaneous adipose tissue by use of a subtraction library (7). However, visfatin/*PBEF1* is expressed in a variety of other tissues and is especially high in the liver (6).

Intriguingly, not only were circulating levels of visfatin shown to correlate strongly with visceral adiposity, but visfatin also exhibited insulin-mimetic effects that were mediated by the insulin receptor and resulted in lower blood glucose levels in mice (7). In addition, chronic administration of visfatin, in mice, lowered both plasma glucose and insulin levels (7). Visfatin stimulated triglyceride synthesis and accumulation in preadipocytes (7) and appears to be regulated by other adipokines, namely interleukin-6 (8) and tumor necrosis factor-α (9).

These findings make visfatin a strong candidate for mediating the complex interplay between visceral obesity and associated metabolic complications. Thus, we hypothesized that genetic variants at the visfatin gene locus may

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apoB, apolipoprotein B; CHD, coronary heart disease; Coup-TF, chick ovalbumin upstream promoter–transcription factor; DR, direct repeat; HRE, hormone response element; LD, linkage disequilibrium; PPAR, peroxisome poliferator–activated receptor; SNP, single nucleotide polymorphism; QFS, Quebec Family Study.

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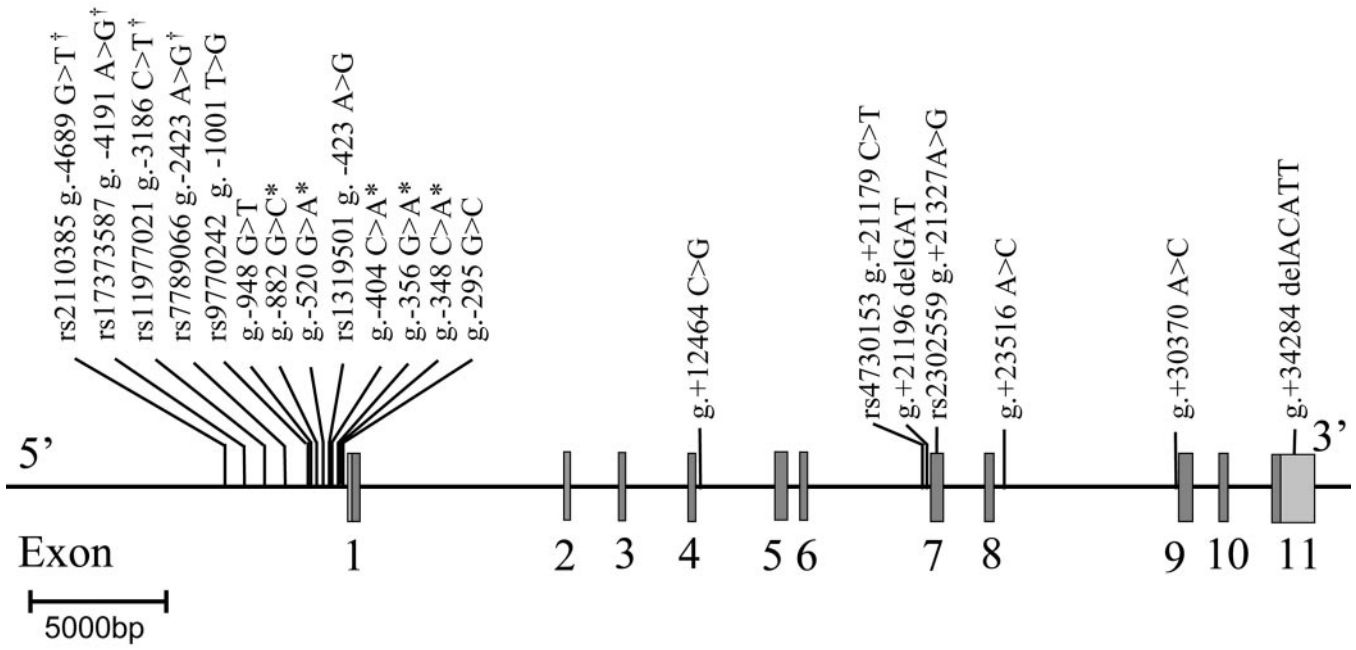

FIG. 1. SNPs in the visfatin gene. Schematic representation of the human visfatin (*PBEF1*) gene locus showing the exon structure and the location of 20 genetic variants. *Discovered during subsequent sequencing. †Chosen from a database search. Relative positions are based on contig map NT_00793.

contribute to interindividual variation in visceral obesity or insulin resistance.

RESULTS

We undertook a thorough examination of the visfatin gene locus for coding and noncoding variants. In the polymorphism discovery phase of this study, we sequenced all coding regions and 1,480 bp of the promoter of the visfatin gene in 18 individuals with varying degrees of visceral adipose tissue (assessed by computed tomography) from the Quebec Family Study (QFS) (10), as well as 5 individuals with various waist-to-hip ratios selected from ongoing CHD studies in the Saguenay-Lac-Saint-Jean region of Québec. Our initial sequencing identified a total of 11 polymorphisms (Fig. 1 and online appendix Table 1 [available at http://diabetes.diabetesjournals.org]). We observed one synonymous polymorphism in exon 7 (g.21327 A>C, SER301SER, rs2302559) but no nonsynonymous polymorphisms. This is in accordance with dbSNP, which lists the SER301SER variant but no other coding single nucleotide polymorphisms (SNPs). Two deletions were discovered: one in intron 6 (g.21196 delGAT) and the other in the 3′ untranslated region (g.34283 delACATT). We also found four SNPs within the proximal promoter (g.-1001 T>G [rs9770242], g.-948 G>T, g.-423 A>G [rs1319501], and g.-295 G>C), while the remaining polymorphisms were intronic: one in intron 4 (g.12464 C>G), one in intron 6 (g.21179 T>C), and two in intron 8 (g.23516 A>C and g.30370 A>C). Minor allele frequencies varied from 0.02 to 0.50 (online appendix Table 1).

Because no nonsynonymous polymorphisms were discovered, we initiated a study of common (minor allele frequency >0.05) promoter polymorphisms (g.-1001 T>G [rs9770242], g.-948 G>T, and g.-423 A>G [rs1319501]), which may influence expression of the visfatin gene. Since these SNPs are in close proximity to one another, we genotyped them by sequencing. In addition, a comprehensive search of publicly available databases (HapMap and Perlegen) was performed, and four additional common SNPs, from the more distal promoter, were chosen for genotyping (rs2110385, rs1737358, rs11977021, and rs7789066). During the follow-up sequence-based genotyping, we discovered five rare SNPs (g.-882 G>C, g.-520 G>A, g.-404 C>A, g.-356 G>A, and g.-348 C>A) with allele frequencies between 0.002 and 0.009 (Table 1, Fig. 1). None of the common visfatin SNPs deviated significantly from Hardy-Weinberg equilibrium (these calculations were not performed for rare SNPs) (Table 1).

To examine whether the common promoter SNPs of the visfatin gene explain some of the interindividual variation of clinical measures of obesity and related traits, we conducted a family-based association test on the QFS sample. Characteristics of the study subjects are shown in Table 2. Using a variance-components approach with a dominance parameter included in the model (adjusting for age and sex), a significant association was found between fasting plasma insulin levels and two SNPs, rs9770242 and rs1319501 ($P = 0.002$ for both SNPs) (Table 3). This association remained significant when corrected for the multiple testing of seven SNPs (empirical P value of 0.011 for both rs9770242 and rs1319501). In addition, an association was observed for both variants and fasting glucose levels ($P = 0.020$ and 0.017, respectively). Thus, individuals homozygous for the G-allele, of either the rs1319501 and rs9770242 variants, had lower fasting plasma insulin levels and lower fasting glucose levels. These two SNPs appear to be in perfect linkage disequilibrium (LD) with each genotype, predicting the other in all individuals for which genotype information was available. The discrepancy in the observed P values is a consequence of the distribution of missing genotypes.

We also observed a significant association between the rs7789066 variant and the apolipoprotein B (apoB) component of VLDL particles ($P = 0.012$) (Table 3). This result was comparable between the two models tested (Table 3). In addition, the rs11977021 variant was found to be

TABLE 1
Genotype distribution of SNPs in the QFS

SNP	MAF	Homozygous major allele (%)	Heterozygous (%)	Homozygous minor allele (%)	Hardy-Weinberg
rs2110385	0.41	33.6	50.2	16.2	NS
rs1737358	0.42	32.9	50.9	16.2	NS
rs11977021	0.17	68.0	29.3	2.7	NS
rs7789066	0.08	86.3	12.3	1.3	NS
rs9770242	0.26	54.7	37.9	7.3	NS
g.-948 G>T	0.14	74.1	23.8	2.1	NS
g.-882 G>C	0.002*	>99	<1	0	NA
g.-520 G>A	0.009	>99	<1	0	NA
rs1319501	0.26	55.8	37.1	7.1	NS
g.-404 C>A	0.002	>99	<1	0	NA
g.-356 G>A	0.004	>99	<1	0	NA
g.-348 C>A	0.002	>99	<1	0	NA
g.-295 G>C	0.002*	>99	<1	0	NA

Minor allele frequencies (MAFs) were calculated in founders. Hardy-Weinberg calculations were performed on common variants only. *Estimated. NA, not applicable; NS, not significant ($P > 0.05$).

significantly associated, under the additive model of inheritance, with total plasma cholesterol levels and LDL cholesterol ($P = 0.03$ and $P = 0.049$, respectively) (Table 3). Finally, the g.-948 G>T variant was found to be significantly associated, when a dominance parameter was included in the model, with fasting insulin levels ($P = 0.011$) and total apoB and LDL apoB levels ($P = 0.031$ and $P = 0.033$, respectively) (Table 3). (This variant is located between the rs9770242 and rs1319501 variants and is in significant LD with them [data not shown].)

DISCUSSION

Our sequencing of the coding regions of the visfatin gene in 23 unrelated individuals from Québec failed to identify any coding nonsynonymous variants. Human visfatin shares 95% sequence identity with both the rat and mouse protein sequences (data not shown). The absence of common missense mutations and the conservation of the visfatin protein suggests an important biological function, which is supported by the observation that homozygous knockout mice were embryonic lethals (7). The finding that certain 5′ flanking variants of the visfatin gene (rs1319501 and rs9770242) are associated with plasma insulin levels ($P = 0.002$) and with plasma glucose levels ($P < 0.02$) is consistent with its demonstrated insulin-mimetic properties (7). Interestingly, Berndt et al. (11) found a significant correlation between visfatin gene expression in visceral fat tissue and plasma insulin levels, although these same authors observed no correlation between plasma visfatin and plasma insulin levels. In a recent study (12), plasma visfatin levels were found to be increased in individuals with type 2 diabetes and there was a significant association between plasma visfatin levels and plasma insulin levels. In our study, we did not observe a significant association between any of the visfatin promoter SNPs and type 2 diabetes (data not shown). However, the number of individuals with type 2 diabetes in the QFS sample is low (51 individuals).

The change in plasma insulin levels associated with the rs1319501 and rs9770242 variants are consistent with the alteration of a transcription factor binding site at one of these SNPs that effects visfatin gene expression. Because of the perfect LD, it is not possible to say which is the functional variant. Of note, the G-allele of the rs9770242 (−1001 T>G) variant was recently associated with acute lung injury and sepsis, though a transfection assay did not reveal a significant change in visfatin/*PBEF1* gene expression in human microvascular endothelial cells from the lung cells (13). However, these authors did not investigate the rs1319501 variant, for which perhaps a much stronger argument can be made. This SNP occurs within an imperfect nuclear hormone response element (HRE), which contains a direct repeat (DR) of the concensus hexamer (A/G)GGTCA separated by a range of nucleotide spacings (Pu-GGTCAN$_i$Pu-GGTCA); in this case, a spacing of six nucleotides (DR6) (14,15). The receptor family that recognizes these HREs include the peroxisome poliferator–activated receptors (PPARs), the chick ovalbumin upstream promoter–transcription factors (Coup-TFs), and the vitamin D receptor (14,15).

In a recent study, PPAR-α and -γ agonists were shown to decrease plasma insulin levels and increase visfatin mRNA expression in OLETF rats, a model of type 2 diabetes (16). Interestingly, the Coup-TFs have been shown to repress the activity of other nuclear hormone receptors (15). They bind to HREs with variable-sized linkers with different binding affinities and have been shown to bind to the DR6 (15). A Coup-TF response element was found in the rat insulin II promoter (17), and when Coup-TFII, also known as apolipoprotein A1 regulatory protein-1, was conditionally knocked out in the pancreas, the mice had an altered insulin response to glucose (18). The vitamin D receptor has also been shown to activate DR6 (14).

The finding that the rs7899066 variant was suggestively associated with the apoB component of VLDL ($P = 0.012$) is intriguing because diabetic patients and insulin-resistant individuals have increased VLDL apoB and triglyceride levels (19). The rs7899066 SNP is located within a nuclear factor of activated T-cells (NFAT) binding sequence ([T/A]GGAAAA) (20). The NFAT transcription factors are involved in adipocyte differentiation (21,22) and can regulate the expression of fatty acid–binding protein aP2 (21), PPAR-γ2 (22), and insulin (23).

Finally, the rs11977021 variant, which was suggestively associated with total cholesterol and LDL cholesterol levels ($P < 0.049$), is also located within an HRE sequence; in this case, a perfect DR separated by four nucleotides (DR4), which is a binding site for the thyroid hormone receptor, and is another member of the nuclear receptor family mentioned above (14). In addition, the g.-948 G>T

TABLE 2
QFS clinical characteristics from 208 families

	Founders			Nonfounders		
	Males (n = 136)	Females (n = 173)	Total (n = 309)	Males (n = 261)	Females (n = 348)	Total (n = 609)
Age (years)	57.5 ± 9.9 (136)	57.7 ± 13.1 (173)	57.6 ± 11.8 (309)	30.5 ± 12.8 (261)	32.5 ± 13.4 (348)	31.7 ± 13.2 (609)
Weight (kg)	82.2 ± 18.3 (127)	69.4 ± 20.2 (155)	75.2 ± 20.3 (282)	80.8 ± 22.4 (256)	70.8 ± 22.5 (341)	75.1 ± 23 (597)
Height (cm)	170.3 ± 5.8 (127)	157.2 ± 7 (155)	163.1 ± 9.2 (282)	173.7 ± 7.9 (256)	161.4 ± 6.4 (341)	166.7 ± 9.3 (597)
BMI (kg/m^2)	28.3 ± 6.1 (127)	28.1 ± 7.9 (155)	28.2 ± 7.1 (282)	26.7 ± 6.8 (256)	27.2 ± 8.6 (341)	27 ± 7.9 (597)
Body fat (%)	26.8 ± 7.3 (96)	36.1 ± 8.9 (100)	31.6 ± 9.4 (196)	21.1 ± 9.7 (221)	30.4 ± 10.7 (297)	26.5 ± 11.2 (518)
Fat mass (kg)	23.2 ± 11.5 (96)	26.9 ± 13.2 (100)	25.1 ± 12.5 (196)	18.7 ± 14.5 (221)	23.1 ± 15.6 (297)	21.3 ± 15.3 (518)
Waist girth (cm)	99.1 ± 14.4 (122)	87.5 ± 18.1 (137)	93 ± 17.4 (259)	90 ± 17.5 (256)	82.1 ± 18.2 (339)	85.5 ± 18.3 (595)
Hip girth (cm)	102.1 ± 10.9 (121)	106.3 ± 16.1 (136)	104.3 ± 14 (257)	99.8 ± 12.6 (254)	104 ± 17.1 (339)	102.2 ± 15.4 (593)
Waist-to-hip ratio	1 ± 0.1 (121)	0.8 ± 0.1 (136)	0.9 ± 0.1 (257)	0.9 ± 0.1 (254)	0.8 ± 0.1 (339)	0.8 ± 0.1 (593)
Fasting glucose (mmol/l)	5.7 ± 1.3 (99)	5.2 ± 1.3 (127)	5.4 ± 1.4 (226)	5.2 ± 0.7 (221)	5.1 ± 0.8 (298)	5.1 ± 0.7 (519)
Fasting insulin (pmol/l)	81.1 ± 54.4 (97)	72.6 ± 48 (125)	76.3 ± 50.9 (222)	77.8 ± 61.6 (220)	76.6 ± 58.4 (294)	77.1 ± 59.8 (514)
L4L5: total adipose tissue surface area (cm^2)	407.9 ± 179.3 (80)	507.7 ± 239.6 (93)	461.5 ± 219 (173)	316.2 ± 210.1 (172)	416.3 ± 232.7 (248)	375.3 ± 228.9 (420)
L4L5: visceral adipose tissue area (cm^2)	172.4 ± 82.7 (80)	136.7 ± 72.8 (93)	153.2 ± 79.3 (173)	105.6 ± 81.9 (172)	86.9 ± 63.3 (248)	94.6 ± 72 (420)
L4L5: subcutaneous adipose tissue surface area (cm^2)	235.5 ± 116.7 (80)	371 ± 180.5 (93)	308.4 ± 168.1 (173)	210.6 ± 147.1 (172)	329.4 ± 185.8 (248)	280.7 ± 180.6 (420)
Total cholesterol (mmol/l)	5.4 ± 0.9 (82)	5.3 ± 1.5 (95)	5.3 ± 1.2 (177)	4.6 ± 0.9 (190)	4.6 ± 1 (258)	4.6 ± 1 (448)
VLDL cholesterol (mmol/l)	0.6 ± 0.4 (82)	0.6 ± 0.4 (95)	0.6 ± 0.4 (177)	0.5 ± 0.4 (190)	0.4 ± 0.4 (258)	0.5 ± 0.4 (448)
LDL cholesterol (mmol/l)	3.7 ± 0.8 (82)	3.4 ± 1 (95)	3.6 ± 0.9 (177)	3 ± 0.8 (190)	3 ± 0.8 (258)	3 ± 0.8 (448)
HDL cholesterol (mmol/l)	1 ± 0.2 (82)	1.2 ± 0.3 (95)	1.1 ± 0.3 (177)	1 ± 0.2 (190)	1.2 ± 0.3 (258)	1.1 ± 0.3 (448)
HDL2 cholesterol (mmol/l)	0.3 ± 0.1 (82)	0.5 ± 0.2 (95)	0.4 ± 0.2 (177)	0.3 ± 0.2 (190)	0.5 ± 0.2 (257)	0.4 ± 0.2 (447)
HDL3 cholesterol (mmol/l)	0.6 ± 0.1 (82)	0.7 ± 0.2 (95)	0.7 ± 0.1 (177)	0.7 ± 0.1 (190)	0.7 ± 0.1 (257)	0.7 ± 0.1 (447)
Plasma triglycerides (mmol/l)	1.8 ± 1 (82)	2 ± 3.3 (95)	1.9 ± 2.5 (177)	1.5 ± 0.8 (190)	1.3 ± 0.6 (258)	1.4 ± 0.7 (448)
VLDL triglycerides (mmol/l)	1.2 ± 0.9 (82)	1 ± 0.7 (95)	1.1 ± 0.8 (177)	1 ± 0.7 (190)	0.8 ± 0.5 (258)	0.8 ± 0.6 (448)
LDL triglycerides (mmol/l)	0.3 ± 0.1 (82)	0.3 ± 0.2 (95)	0.3 ± 0.1 (177)	0.2 ± 0.1 (190)	0.3 ± 0.1 (258)	0.3 ± 0.1 (448)
HDL triglyceride (mmol/l)	0.2 ± 0.1 (82)	0.3 ± 0.1 (95)	0.3 ± 0.1 (177)	0.2 ± 0.1 (190)	0.3 ± 0.1 (258)	0.2 ± 0.1 (448)
Total apoB (g/l)	1.1 ± 0.2 (82)	1 ± 0.3 (95)	1.1 ± 0.2 (177)	0.9 ± 0.2 (190)	0.9 ± 0.2 (259)	0.9 ± 0.2 (449)
VLDL apoB (g/l)	0.1 ± 0.1 (82)	0.1 ± 0.1 (95)	0.1 ± 0.1 (177)	0.1 ± 0.1 (189)	0.1 ± 0.1 (258)	0.1 ± 0.1 (447)
LDL apoB (g/l)	1 ± 0.2 (82)	0.9 ± 0.2 (95)	1 ± 0.2 (177)	0.8 ± 0.2 (190)	0.8 ± 0.2 (258)	0.8 ± 0.2 (448)

Data are means ± SD (n). Total number of individuals included was 918. The average family size was 5.25 people. L4L5, intervertebral space between the fourth and fifth lumbar vertebrae.

TABLE 3
QTDT software results

	rs2110385	rs1737358	rs11977021	rs7789066	rs9770242	g.-948 G>T	rs1319501
Additive model							
Weight							
BMI							
Body fat							
Fat mass	0.062						
Waist girth							
Hip girth							
Waist-to-hip ratio							
Fasting glucose							
Fasting insulin							
L4L5: total adipose tissue surface area							
L4L5: visceral adipose tissue area							
L4L5: subcutaneous adipose tissue surface area							
Total cholesterol			0.03				
VLDL cholesterol							
LDL cholesterol	0.092		0.049				
HDL cholesterol							
HDL2 cholesterol							
HDL3 cholesterol							
Plasma triglycerides							
VLDL triglycerides				0.067		0.079	
LDL triglycerides							
HDL triglyceride				0.061			
Total apoB	0.067						
VLDL apoB				0.012			
LDL apoB	0.074						
Dominance parameter							
Weight							
BMI							
Body fat							
Fat mass							
Waist girth							
Hip girth							
Waist-to-hip ratio							
Fasting glucose					0.02		0.017
Fasting insulin					0.002	0.011	0.002
L4L5: total adipose tissue surface area							
L4L5: visceral adipose tissue area							
L4L5: subcutaneous adipose tissue surface area					0.091		
Total cholesterol							
VLDL cholesterol							
LDL cholesterol							
HDL Cholesterol							
HDL2 cholesterol							
HDL3 cholesterol							
Plasma triglycerides							
VLDL triglycerides				0.069			
LDL triglycerides							
HDL triglyceride				0.035			
Total apoB						0.031	
VLDL apoB				0.032			
LDL apoB						0.033	

An orthogonal model was used to test for association. Age and sex were included in the model. For each test, 1,000 permutions were performed. Only $P < 0.1$ are shown. L4L5, intervertebral space between the fourth and fifth lumbar vertebrae.

variant was found to be associated with fasting plasma insulin levels, total serum apoB, and LDL apoB levels. These findings, while only suggestive, may indicate that visfatin is involved in other aspects of the dyslipidemia associated with insulin resistance, such as the presence of small dense LDL particles.

It is worth noting that we obtained a greater number of significant results when our model included a dominance parameter (Table 3). This included our best result, being the two SNPs that were strongly associated with fasting insulin. Because we have used a family-based design with families from Québec (the site of a well-documented founder effect), this study may have increased power to detect genetic effects that exhibit a dominance compo-

nent. Further, this could be due to the increased probability of sharing two alleles identical by descent.

Our results demonstrate a significant association between common promoter polymorphisms in the visfatin gene and quantitative measures of insulin resistance among French Canadians in Québec. While we did not see a direct association with type 2 diabetes; our power to detect an association is low in the QFS population. The association between fasting insulin and glucose levels and the rs9770242 and rs1319501 variants, as well as the association between the rs7899066 and rs11977021 variants and components of diabetic dyslipidemia, warrant additional genetic studies in larger obesity and type 2 diabetes population samples, as well as functional characterization of these promoter variants.

RESEARCH DESIGN AND METHODS

The QFS is a long-term study of French-Canadian families from Québec City and its surrounding area and has been described in detail (10). Briefly, the current study involved 208 families and a total of 918 individuals for which phenotypic information and visfatin genotypes were available from QFS. Though QFS contains some extended families, the majority (165 families) are nuclear families. The five unrelated individuals from the Saguenay-Lac-Saint-Jean region of Québec were selected from ongoing CHD studies.

Phenotypes. The biochemical phenotypes that were analyzed were obtained before the initiation of any pharmaceutical intervention, except in the case of 31 diabetic individuals. These individuals were genotyped, but their trait values were not included in the analysis. All clinical/biochemical measurements were taken after a fast of at least 12 h.

Sequencing. PCR conditions were 10 ng genomic DNA, 0.5 units Qiagen HotStarTaq (Qiagen, Mississauga, ON, Canada) (1.5 mmol/l $MgCl_2$,), 0.5 μl of 10 mmol/l dNTPs, and 1 μl of 20 μmol/l primers in a 25-μl reaction. Primer annealing temperatures were 56–60°C. PCR products were purified (Multiscreen; Millipore, Bedford, MA), and sequencing was performed using BigDye Terminator (version 3.1) and analyzed on ABI 3730XL sequencers (Applied BioSystems, Foster City, CA). Data were processed using Sequencing Analysis software (version 5.1) and then aligned with PhredPhrap-Consed (24). Primers and specific annealing temperatures are in online appendix Table 2.

Genotyping. Genotyping was performed using either sequencing, restriction fragment–length polymorphism, or TaqMan assays. The following primers were used for the sequencing reaction to genotype nine of the promoter variants: left primer, 5′CACTTCTTTATTTTGGGGTTGC 3′; right primer, 5′GCAGTCTGGGAGCTCTGG 3′.

Restriction fragment–length polymorphisms. PCR conditions were 30–50 ng genomic DNA, 0.5 units of Qiagen HotStarTaq (Qiagen), 0.5 μl of 100 μmol/l dNTPs, and 0.5 μl of 25 μmol/l primers in a 25-μl reaction. Primer annealing temperatures were 58–60°C. PCR products were restriction digested overnight at 37°C. The rs2110385 primer sequences were as follows: left primer, 5′ TGCTAGCCCATATCAATGACTG 3′; right primer, 5′ AATGGGAGAAGAGGG GAAAA 3′. Digestions were overnight with 5 units of *Alu*I (Invitrogen, Carisbad, CA). The rs1737358 primer sequences were as follows: left primer, 5′ AATTTTGCTAATGGC 3′; right primer, 5′ AATAATACCCCTCCC 3′. Digestions were overnight with 0.5 units of *Af*lII (Invitrogen).

TaqMan assays. TaqMan (Applied BioSystems) assays were Assays-on-Demand C_11405260_10 (rs11977021) and C_29286200_10 (rs7789066) and were performed according to the manufacturer's instructions.

Statistical analysis. The genotype distribution of each common SNP was tested in the founders for its adherence to Hardy-Weinberg equilibrium by a χ^2 test with 1 degree of freedom. Mendelian inheritance of all alleles was confirmed using the Pedstats program (available at http://www.sph.umich.edu/csg/abecasis/pedstats/). Only one inconsistency was observed in the QFS sample, and all genotype information for the individual's family at that locus was removed. Association tests were performed using the QTDT software package (available at http://www.sph.umich.edu/csg/abecasis/QTDT/) (25). In this study, we used the orthogonal model of association within a variance component framework (age and sex were included in the model). To control for the possible nonnormal distribution of the traits, 1,000 permutations were performed for each test to assess significance. Identity by descent probability estimations were generated using the Simwalk2 software package (available at http://www.genetics.ucla.edu/software/).

SNP selection, transcription factor binding site identification, and protein sequence alignments. Distal promoter SNPs were chosen from a database search using both the HapMap (available at http://www.hapmap.org) and Perlegen Sciences (available at http://genome.perlegen.com/) datasets.

Potential transcription factor binding sites were identified on forward and backward strands of genomic DNA using the transcription element search system TESS program (available at http://cbil.upenn.edu/tess). Between-species protein sequence alignments were performed using the BLAST algorithm (available at http://www.ncbi.nlm.nih.gov/BLAST/).

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NOTE ADDED IN PROOF

After the current article was submitted, we became aware of similar work that also demonstrates a significant association between the visfatin promoter polymorphism (g.-948 G>T) and fasting plasma insulin levels (26).

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