

Biological Pathway-Based Genome-Wide Association Analysis Identified the Vasoactive Intestinal Peptide (VIP) Pathway Important for Obesity

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Recent genome-wide association (GWA) studies have identified a number of novel genes/variants predisposing to obesity. However, most GWA studies have focused on individual single-nucleotide polymorphism (SNPs)/genes with a strong statistical association with a phenotypic trait without considering potential biological interplay of the tested genes. In this study, we performed biological pathway-based GWA analysis for BMI and body fat mass. We used individual level genotype data generated from 1,000 unrelated US whites that were genotyped for ~500,000 SNPs. Statistical analysis of pathways was performed using a modification of the Gene Set Enrichment Algorithm. A total of 963 pathways extracted from the BioCarta, Kyoto Encyclopedia of Genes and Genomes (KEGG), Ambion GeneAssist, and Gene Ontology (GO) databases were analyzed. Among all of the pathways analyzed, the vasoactive intestinal peptide (VIP) pathway was most strongly associated with fat mass (nominal $P = 0.0009$) and was the third most strongly associated pathway with BMI (nominal $P = 0.0006$). After multiple testing correction, the VIP pathway achieved false-discovery rate (FDR) q values of 0.042 and 0.120 for fat mass and BMI, respectively. Our study is the first to demonstrate that the VIP pathway may play an important role in development of obesity. The study also highlights the importance of pathway-based GWA analysis in identification of additional genes/variants for complex human diseases.

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INTRODUCTION

Obesity is a serious public health problem that has a strong genetic determination (1,2). Recent powerful genome-wide association (GWA) studies have identified >20 novel genes (e.g., *FTO*, *MC4R*, and *CTNBL1*) that were associated with obesity and related phenotypes (1). These findings have dramatically increased our knowledge of the genetic architecture of obesity.

Most GWA studies performed to date have used a stringent genome-wide level of statistical significance (e.g., P value of 10^{-7}) to identify individual variants (e.g., single-nucleotide polymorphisms (SNPs) and/or copy number variants that are most strongly associated with obesity. It is well known, however, that genes and their products often work together, interacting in functional physiological groups or pathways, and that these combined effects may contribute to susceptibility to a disease. Unfortunately, single-marker GWA analysis generally neglects the potential interplay of the tested genes. It is

therefore possible that genes/variants that exert sizeable effects on obesity through interactions with one another in a specific biological pathway(s) might be missed in single-marker analysis due to relatively modest association evidence when each of these genes are analyzed individually. Consequently, alternative approaches that complement single-marker GWA methodologies are necessary to identify additional genes/variants and biological pathways predisposing to obesity.

In this study, we performed pathway-based GWA analysis for BMI and body fat mass using the data generated from 1,000 US whites that were genotyped for ~500,000 SNPs. Statistical analysis was performed using a modification of the Gene Set Enrichment Algorithm (3). Our results showed that the vasoactive intestinal peptide (VIP) pathway was significantly associated with both BMI and fat mass, suggesting that the VIP pathway plays an important role in the development of obesity. Our study also highlights the importance of pathway-based

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GWA analysis in identifying genes/variants and biological pathways predisposing to complex human diseases.

METHODS AND PROCEDURES

Subjects

This sample contains 1,000 unrelated adults, which was randomly selected from our established and expanding genetic repertoire currently containing >10,000 subjects. All the study subjects were US whites of northern European origin living in Omaha, Nebraska and its surrounding areas. This US white population is relatively homogeneous due to a fairly stable population in which whites have predominated as the major ethnic group in this area for an extended period. Potential population stratification of this sample has been tested and ruled out previously (4). The study subjects were normal healthy subjects defined by a comprehensive suite of exclusion criteria (4). Briefly, subjects with chronic diseases and conditions involving vital organs (heart, lung, liver, kidney, and brain) and severe endocrine, metabolic, or nutritional diseases that might affect fat metabolism were excluded from this study. The age range of the subjects was 19.10–87.23 years, with a mean of 57.99 for males and 58.10 for females, respectively.

BMI was calculated as body weight (in kg) divided by the square of height (in meters). Weight was measured in light indoor clothing without shoes, using a calibrated balance beam scale, and height was measured using a calibrated stadiometer. Body fat mass was measured by a Hologic 4500 dual energy X-ray absorptiometry system (Hologic, Bedford, MA). The short-term reproducibility (coefficient of variation) of BMI and fat mass measurements is on average 0.2 and 1.1%, respectively. The general relevant characteristics of the study subjects are shown in **Table 1**. Measurement of body fat mass by dual energy X-ray absorptiometry is considered to be highly accurate (5). The correlation between BMI and fat mass was 0.85 ($P < 0.01$) in this sample.

Genotyping

Genomic DNA was extracted from whole human blood using a commercial isolation kit (Gentra Systems, Minneapolis, MN) following the protocols detailed in the kit. Genotyping with the Affymetrix Mapping 250k Nsp and Affymetrix Mapping 250k Sty arrays was performed using the standard protocol recommended by the manufacturer. Genotyping calls were determined from the fluorescent intensities using the dynamic model (DM) algorithm with a 0.33 P value setting (6) as well as the Bayesian-Robust linear model with mahalanobis distance classifier algorithm (7). DM calls were used for quality control whereas the B-RLMM calls were used for all subsequent data analysis. B-RLMM clustering was performed with 94 samples per cluster.

In GWA genotyping experiments, following an Affymetrix guideline, we set a standard for the minimum DM call rate at 93% for a sample, considering all the SNPs in the two arrays (i.e., the 250k Nsp and 250k Sty arrays). Of all of the 1,000 subjects, 990 met this call rate standard. The remaining 10 samples did not meet this standard, but they had one hybridized array reach this call rate standard (93%). Hence, the genotype data in the array (with the higher call rate) for these 10 samples were retained in the dataset for GWA analysis. For all the 1,000 subjects, the average DM call rate reached >95%.

The final average B-RLMM call rate across the entire sample reached the high level of 99.14%. However, out of the initial full-set of 500,568

SNPs, we discarded 32,961 SNPs with sample call rates <95%, another 36,965 SNPs with allele frequencies deviating from Hardy–Weinberg equilibrium ($P < 0.001$). We further discarded SNPs with minor allele frequency <5% and SNPs which are >500 kb away from any gene. The Affymetrix annotation file was used to assign SNPs to genes. A physical distance of 500 kb was used to link a SNP and a gene because most enhancers and repressors are <500 kb away from genes, and most linkage disequilibrium blocks are <500 kb (3). In total, 312,172 SNPs which covered 14,585 genes, were assayed in the pathway-based GWA analyses.

Pathway databases

Four public databases were explored to generate a collection of curated gene sets and pathways for testing in this study, which include BioCarta pathway database (<http://www.biocarta.com/genes>), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (<http://www.genome.ad.jp/kegg/pathway.html>), Ambion GeneAssist Pathway Atlas (<http://www.ambion.com/tools/pathway>), and Gene Ontology (GO) database (<http://www.geneontology.org>). There were 260, 190, and 380 annotated pathways retrieved from the BioCarta pathway database, KEGG pathway database, and Ambion GeneAssist Pathway Atlas, respectively. To integrate the GO information into our study, GO annotation files for human genes were downloaded. We processed the GO annotation files and generated gene sets on the basis of GO level 4 annotations in biological process and molecular function. Genes whose GO annotations are in level 5 or lower in the GO hierarchy were assigned to their ancestral GO annotations in level 4. In the analysis, we only tested pathways/gene sets in which at least 85% (and in number of 10–200) of the genes were included in our GWA data set. We also limited testing to those pathways that contained between 10 and 200 genes in our GWA data set. This was done in order to alleviate the multiple-testing problem by avoiding testing too narrowly or too broadly defined functional categories. In total, 963 pathways/gene sets were analyzed in this study.

Statistical analysis

Pathway-based GWA analysis was performed using the algorithm developed by Wang *et al.* (3). This method implements the existing Gene Set Enrichment Algorithm (8). The major analytical procedures adopted are as follows:

1. *Generation of statistic value of gene–phenotype association:* Suppose there are N genes each gene with x_i ($i \leq N$) typed SNPs. For a specific gene G_i , the highest SNP–phenotype association statistic value among all the x_i SNPs, denoted as r_i , was selected to represent the gene.
2. *Ranking statistic values of gene–phenotype association:* All the genes were ranked, denoted as $L(r_1, r_2, \dots, r_N)$, by sorting their statistic values from the largest to smallest.
3. *Enrichment score (ES) calculation:* For each given pathway/gene set S which is composed of N_S genes, a statistic ES was calculated. ES is a weighted Kolmogorov–Smirnov-like running-sum statistic that reflects the overrepresentation of genes within S at the top of the entire ranking list of genes in the genome. The score was calculated by walking down the gene list L , increasing a running-sum statistic when encountering a gene in S and decreasing it when encountering a gene not in S . The magnitude of the increment depends on the correlation of the gene with the phenotype. ES is the maximum deviation from zero encountered in the random walk.

$$ES^S = \max_{1 \leq i \leq N} \left\{ \sum_{G_i \in S, i \leq i} \frac{|r_{(i)}|^p}{N_R} - \sum_{G_i \notin S, i \leq i} \frac{1}{N - N_S} \right\},$$

where $N_R = \sum_{G_i \in S} |r_{(i)}|^p$ and p (designated as 1 here) is a parameter that gives higher weight to genes with extreme statistic values.

4. *Permutation and nominal significance assessment:* To estimate the significance level of ES, permutation was performed to create the

Table 1 Basic characteristics of study subjects

Trait	Male (n = 500)	Female (n = 500)
Age (years)	59.77 (14.9)	58.10 (15.8)
Height (cm)	1.77 (0.07)	1.64 (0.06)
Weight (kg)	89.07 (15.44)	71.38 (15.887)
BMI (kg/m ²)	28.92 (4.30)	27.29 (5.98)
Fat mass (kg)	23.48 (8.88)	26.92 (10.33)

The presented data are mean (s.d.) of raw values.

null distribution of ES for each pathway/gene set. The phenotype data of sampled subjects were shuffled. Then, the previous three steps were repeated to calculate ES for the pathway/gene set in each permutation. A total of 1,000 permutations were performed. A distribution for ES (ES_{null}^S) was generated for each pathway/gene set S . The significance of an observed ES^S for a pathway/gene set (nominal P value) was estimated as the percentage of permutations whose ES_{null}^S were greater than the observed ES^S .

5. *Multiple testing adjustments*: To compare the significance among pathways with different numbers of genes, a normalized ES was first constructed based on the observed ES, mean and s.d. of ES_{null}^S .

$$NES^S = \frac{ES^S - \text{mean}(ES_{null}^S)}{SD(ES_{null}^S)}$$

False-discovery rate (FDR) was then calculated to adjust for multiple-hypothesis testing. For a pathway/gene set, $NES_{observe}^S$ denotes the normalized ES in the observed data. FDR q value (denoted as q_{fdr}^S) was calculated as the ratio of the fraction of all permutations with $NES \geq NES_{observe}^S$ to the fraction of observed pathways/gene sets with $NES \geq NES_{observe}^S$.

$$q_{fdr}^S = \frac{\% \text{ of permutations with } NES_{null}^S \geq NES_{observe}^S}{\% \text{ of observed } S \text{ with } NES_{observe}^S \geq NES_{observe}^S}$$

In SNP association analysis, age, age², sex, age-by-sex interaction, and age²-by-sex interaction were included to adjust the BMI and fat mass data. Association analysis for each SNP was performed using software package PLINK using “-assoc” command which conducts Wald test for an additive model.

RESULTS

Among all of the 963 pathways analyzed, the VIP pathway showed the most significant association with obesity phenotypes (Figure 1). It was the pathway that was most strongly associated with body fat mass (nominal $P = 0.0009$) and was the third most strongly associated pathway with BMI (nominal $P = 0.0006$). After multiple correction, the VIP pathway achieved FDR q values of 0.042 for fat mass and 0.120 for BMI. Among the top 10 ranking pathways (based on nominal P value) for BMI and fat mass, the VIP pathway is the only one overlaps between BMI and fat mass. After adjusting for multiple testing using FDR, only the VIP pathway turned out to be significant. In Table 2, we list the five top-ranking pathways based on nominal P value for BMI and fat mass, respectively.

The VIP pathway, which contains a total of 23 genes, is curated by the BioCarta pathway database (Figure 2). The individual genes in the VIP pathway and the associated P values for BMI and fat mass are summarized in Table 3. Of all the genes in the VIP pathway, the individual gene with the most significant P value was VIP ($P = 4.74 \times 10^{-4}$ for BMI and $P = 9.50 \times 10^{-4}$ for fat mass). A number of other genes in the VIP pathway also contributed positively to the ES (i.e., the genes that ranked before or at the point of ES, also denoted as leading-edge genes) for fat mass and/or BMI (Figure 3). These leading-edge genes along with their representative SNPs, their positions, and their minor allele frequency are presented in Table 4. It can be seen that the majority of the representative SNPs are common variants with minor allele frequency $\geq 5\%$ in our sample.

Table 2 The five top-ranking pathways for BMI and fat mass

Phenotype	Pathway	P value (nominal)	FDR q value	Database
BMI	NADPH binding	0.0002	0.269	GO
	IL-12 pathway	0.0002	0.642	Biocarta
	VIP pathway	0.0006	0.120	Biocarta
	Hematopoietic cell lineage	0.0007	0.767	KEGG
	Interleukin binding	0.0013	0.504	GO
Fat mass	VIP pathway	0.0009	0.042	Biocarta
	Calcium ion transporter activity	0.0009	0.642	GO
	Cysteine-type peptidase activity	0.0049	0.234	GO
	Inhibition of matrix metalloproteinases	0.0049	0.491	Ambion
	Aromatic amino acid family metabolism	0.0059	0.536	GO

FDR, false-discovery rate; GO, Gene Ontology; IL-12, interleukin-12; KEGG, Kyoto Encyclopedia of Genes and Genomes; NADPH, nicotinamide adenine dinucleotide phosphate-oxidase; VIP, vasoactive intestinal peptide.

We used the FASTSNP program (<http://fastsnp.ibms.sinica.edu.tw>) to analyze potential functions for SNPs in VIP and other leading-edge genes. The SNPs for which a potential function could be surmised include rs555985 and rs10169164. SNP rs555985 is located at the 3' UTR of the *VIP* gene and an A to C change at this locus will delete a binding site for the transcription factor CdxA. The SNP rs10169164 is located in the promoter region of the *CALM2* gene and it is reasonable to speculate that this SNP may regulate gene transcription.

DISCUSSION

GWA has become a powerful tool for identifying genes associated with common human complex diseases such as obesity. However, GWA studies usually focus on the most significant individual variants without considering potential functional interactions of the genes/variants tested. Our study, via pathway-based analysis of GWA data, suggests for the first time the potential importance of genes in the VIP pathway genes to the development of obesity. Importantly, none of the individual genes in the VIP pathway reached the genome-wide significance level in single-marker GWA analysis, so the potential importance of the VIP pathway would not have been recognized without pathway-based analysis.

The pathway-based strategy is receiving increasing attention in genetic research of human diseases. For example, a genomic pathway approach was recently employed to mine a GWA dataset for SNPs that were within brain-expressed, axon-guidance pathway genes and found that the axon-guidance pathway might play an important role in Parkinson's disease (9); however, a follow-up study in two independent sample sets could not replicate the initial findings (10). Among many possible reasons for lack of replication, an important contributing factor is the different SNP selection methods in

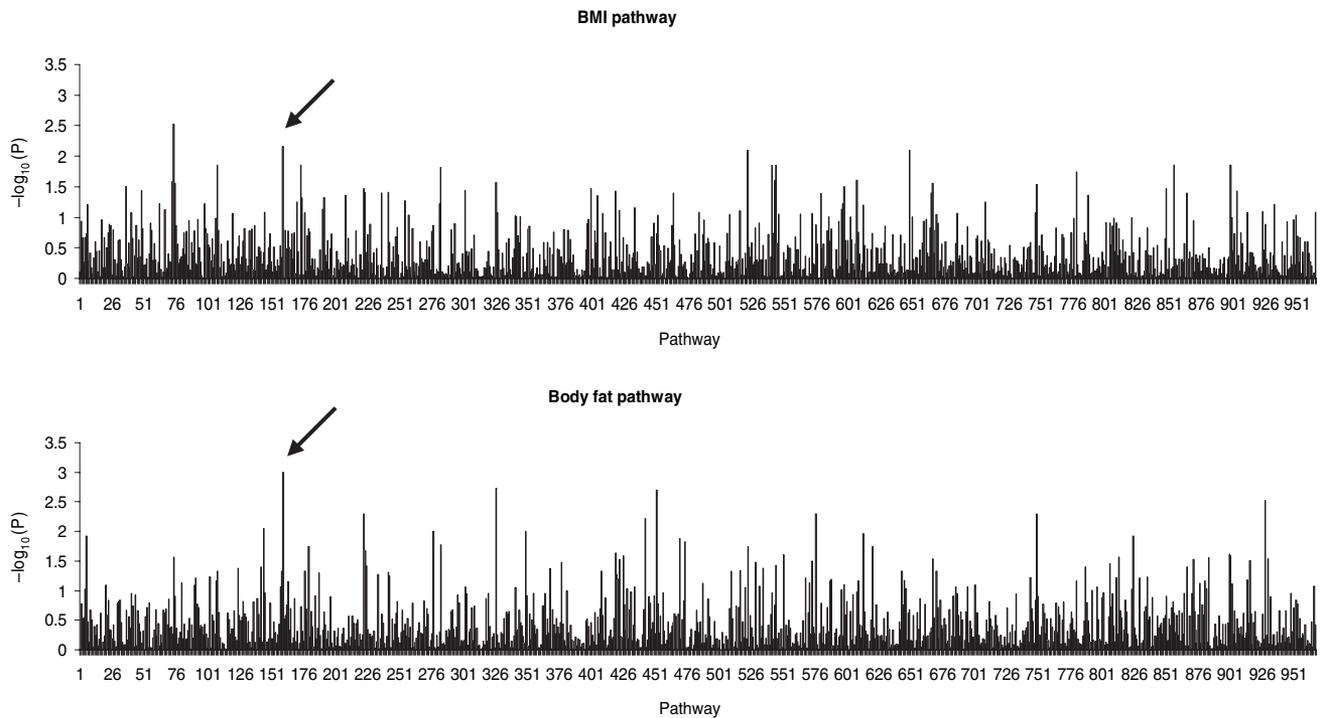


Figure 1 Pathway-based genome-wide association results for BMI and fat mass. The y axis is the $-\log_{10} P$ value of all the 963 pathways with BMI and fat mass. The arrow points to the VIP pathway. VIP, vasoactive intestinal peptide.

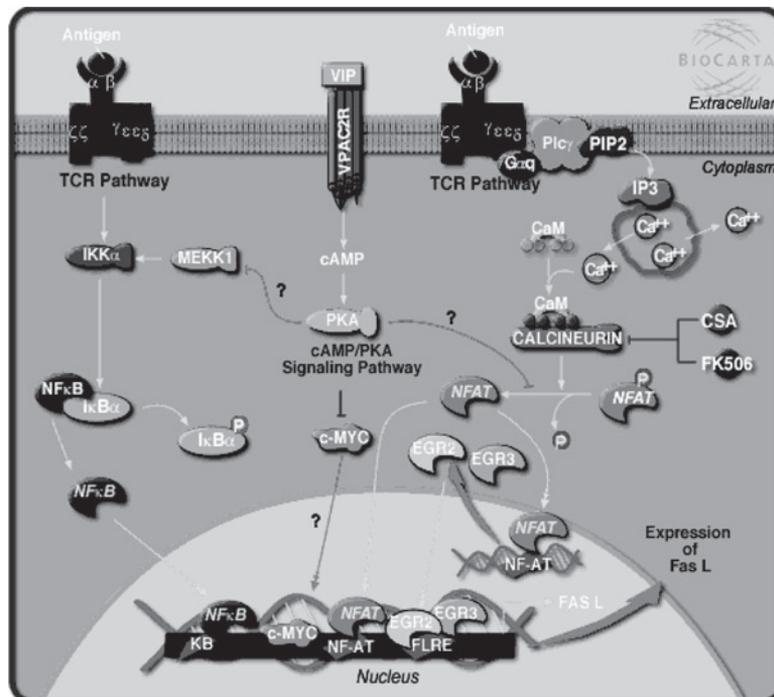


Figure 2 Functional interactions among the genes in the VIP pathway. VIP, vasoactive intestinal peptide.

two studies for the axon-guidance pathway genes (10). In our study reported here, we did not focus on any specific pathway but systematically analyzed a large number of biological pathways which do not necessarily have known functional importance to obesity. Notably, the VIP pathway that we identified as significantly associated with body fat mass and BMI has not

been previously recognized as a pathway that has a sizeable genetic effect on body fat. Thus, pathway-based strategies, which examine groups of genes (i.e., genes sharing a biochemical or cellular function, and chromosomal location or regulation) that make modest contributions to disease risk, may use GWA data in a more efficient way and have the potential to

Table 3 Genes in the VIP pathway and the associated *P* values

Gene symbol	Gene ID	Genome location	Full name	<i>P</i> value for BMI	<i>P</i> value for fat mass
<i>VIP</i>	7,432	6q25	Vasoactive intestinal peptide	4.742×10^{-4}	9.499×10^{-4}
<i>CALM2</i>	805	2p21	Calmodulin 2 (phosphorylase kinase, delta)	4.795×10^{-4}	1.767×10^{-3}
<i>NFATC2</i>	4,773	20q13.2-q13.3	Nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 2	1.930×10^{-3}	1.150×10^{-2}
<i>CHUK</i>	1,147	10q24-q25	Conserved helix-loop-helix ubiquitous kinase	2.448×10^{-3}	9.670×10^{-3}
<i>PPP3CC</i>	5,533	8p21.3	Protein phosphatase 3 (formerly 2B), catalytic subunit, γ isoform	2.773×10^{-3}	3.363×10^{-2}
<i>EGR3</i>	1,960	8p23-p21	Early growth response 3	7.943×10^{-3}	6.642×10^{-3}
<i>PPP3CA</i>	5,530	4q21-q24	Protein phosphatase 3 (formerly 2B), catalytic subunit, α isoform	9.806×10^{-3}	3.413×10^{-2}
<i>PRKACB</i>	5,567	1p36.1	Protein kinase, cAMP-dependent, catalytic, β	9.911×10^{-3}	4.493×10^{-2}
<i>EGR2</i>	1,959	10q21.1	Early growth response 2 (Krox-20 homolog, <i>Drosophila</i>)	1.202×10^{-2}	9.049×10^{-3}
<i>PRKAR2B</i>	5,577	7q22	Protein kinase, cAMP-dependent, regulatory, type II, β	1.379×10^{-2}	2.081×10^{-2}
<i>NFKBIA</i>	4,792	14q13	Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α	1.719×10^{-2}	1.966×10^{-2}
<i>NFKB1</i>	4,790	4q24	Nuclear factor of κ light polypeptide gene enhancer in B-cells 1	2.531×10^{-2}	5.929×10^{-2}
<i>MYC</i>	4,609	8q24.21	v-myc myelocytomatosis viral oncogene homolog (avian)	2.887×10^{-2}	1.969×10^{-2}
<i>NFATC1</i>	4,772	18q23	Nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1	3.309×10^{-2}	2.537×10^{-2}
<i>VIPR2</i>	7,434	7q36.3	Vasoactive intestinal peptide receptor 2	3.614×10^{-2}	1.291×10^{-1}
<i>GNAQ</i>	2,776	9q21	Guanine nucleotide binding protein (G protein), q polypeptide	9.709×10^{-2}	9.682×10^{-2}
<i>CALM3</i>	808	19q13.2-q13.3	Calmodulin 3 (phosphorylase kinase, delta)	1.369×10^{-1}	2.944×10^{-1}
<i>PRKAR1B</i>	5,575	7p22	Protein kinase, cAMP-dependent, regulatory, type I, β	1.449×10^{-1}	5.223×10^{-1}
<i>PRKAR1A</i>	5,573	17q23-q24	Protein kinase, cAMP-dependent, regulatory, type I, α (tissue-specific extinguisher 1)	1.503×10^{-1}	8.732×10^{-2}
<i>RELA</i>	5,970	11q13	v-rel reticuloendotheliosis viral oncogene homolog A (avian)	4.500×10^{-1}	4.121×10^{-1}
<i>PPP3CB</i>	5,532	10q21-q22	Protein phosphatase 3 (formerly 2B), catalytic subunit, β isoform	5.809×10^{-1}	7.905×10^{-1}
<i>PRKAR2A</i>	5,576	3p21.3-p21.2	Protein kinase, cAMP-dependent, regulatory, type II, α	6.310×10^{-1}	6.664×10^{-1}
<i>PLCG1</i>	5,335	20q12-q13.1	Phospholipase C, γ 1	6.741×10^{-1}	5.636×10^{-1}

cAMP, cyclic adenosine monophosphate; VIP, vasoactive intestinal peptide.

help reveal interconnected pathophysiological networks for a spectrum of common human diseases of major public health importance.

VIP is a neuropeptide hormone acting as a neurotransmitter and neuromodulator in practically all tissues, resulting in a wide variety of biologic effects (11,12). VIP was originally isolated as a vasodilator peptide (13). Subsequently, its signature features as a neuropeptide became consolidated (14). More recent research demonstrates that VIP may serve as a cytokine-like molecule that modifies immune function (14). Studies of the VIP pathway with relevance to human obesity have been scarce. In animal models, it has been reported

that VIP receptor 2 deficient mice exhibited growth retardation, increased basal metabolic rates, decreased fat mass, and increased lean mass (12). These findings suggest that VIP plays an important role in growth and energy homeostasis (15). Although little is known about the direct biological/physiological importance of the VIP pathway to obesity risk, based on our knowledge, we can envision four potential mechanisms by which the VIP pathway may exert its effects on body fat.

First, the VIP pathway plays an important role in regulation of pancreatic endocrine function by stimulating insulin and glucagon secretion (16,17), which may ultimately affect energy metabolism and body fat storage. Studies found that

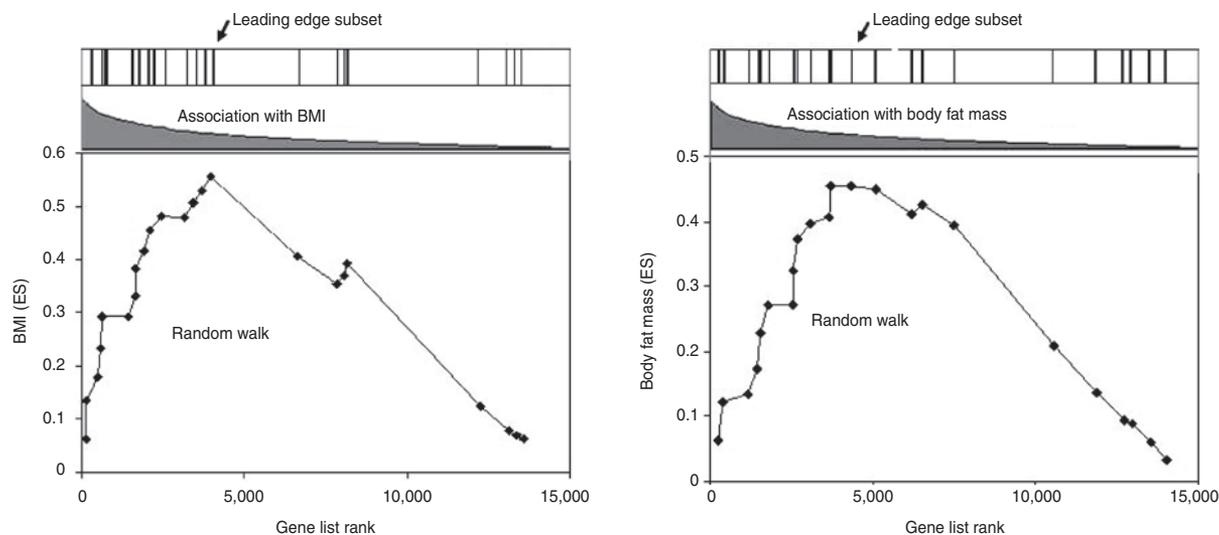


Figure 3 Running-sum plot for the VIP pathway. The figure includes the location of the maximum enrichment score (ES) and the leading-edge subset. The x axis is the rank of the 23 genes in the VIP pathway in the whole gene list generated by ranking all the genes by their association significance with BMI and fat mass from the largest to smallest. The y axis represents the running enrichment score. ES is the maximum deviation from zero achieved in the running-sum walk. The bar plot at the top of the figure also shows the distribution of the 23 genes from the VIP pathway in the whole gene list with the leading-edge genes indicated. VIP, vasoactive intestinal peptide.

Table 4 The most significant SNPs mapped to the leading-edge genes of the VIP pathway

dbSNP	Genes	Position (bp)	Allele	MAF	Role
rs555985	<i>VIP</i> ^{a,b}	153260415	A/C	0.484	3' UTR
rs10169164	<i>CALM2</i> ^{a,b}	47332295	A/T	0.457	Promoter
rs16995730	<i>NFATC2</i> ^{a,b}	49148516	C/T	0.116	3' UTR
rs17112751	<i>CHUK</i> ^{a,b}	101969243	C/T	0.027	Intron 5
rs896379	<i>PPP3CC</i> ^{a,b}	22430145	C/T	0.441	Intron 6
rs10104039	<i>EGR3</i> ^{a,b}	22594329	A/G	0.379	3' UTR
rs2850973	<i>PPP3CA</i> ^{a,b}	102410610	C/G	0.104	Intron 1
rs12031680	<i>PRKACB</i> ^{a,b}	84307885	A/C	0.297	Promoter
rs2842275	<i>EGR2</i> ^{a,b}	64493977	C/G	0.050	Promoter
rs1981696	<i>PRKAR2B</i> ^{a,b}	106581336	C/T	0.198	Intron 8
rs1951273	<i>NFKBIA</i> ^{a,b}	34876720	C/T	0.287	3' UTR
rs13115343	<i>NFKB1</i> ^a	103584555	A/C	0.306	Promoter
rs4130120	<i>MYC</i> ^{a,b}	128714137	C/T	0.092	Promoter
rs12959273	<i>NFATC</i> ^{a,b}	75378707	C/T	0.374	Intron 9
rs3850493	<i>VIPR2</i> ^a	158447009	C/T	0.029	3' UTR

bp, base pair; dbSNP, single-nucleotide polymorphism database; MAF, minor allele frequency in our sample; UTR, untranslated region.

^aLeading-edge genes for BMI. ^bLeading-edge genes for body fat mass.

VIP and a homologous neuropeptide, pituitary adenylate cyclase-activating polypeptide (PACAP), are expressed in islets and in islet parasympathetic nerves (18). Both peptides bind to their common G-protein-coupled receptors (VPAC1, VPAC2, and PAC1) which are expressed in islets. VIP and PACAP stimulate insulin secretion in a glucose-dependent manner and they both also stimulate glucagon secretion (18). This action is achieved through increased formation of cyclic adenosine monophosphate after activation of adenylate cyclase and stimulation of extracellular calcium uptake (18).

Deletion of PAC1 receptors or VPAC2 receptors results in glucose intolerance (19). These peptides may also be important in mediating prandial insulin secretion and the glucagon response to hypoglycemia (18).

Second, the VIP pathway may influence body fatness through mediating feeding behavior. The hypothalamic region of the brain in vertebrates is a center that plays an important role in feeding regulation. The effect of VIP and PACAP on food intake has been demonstrated in animal models. For example, intracerebroventricular injection of VIP or PACAP inhibits

feeding in mouse (20), chick (21), and goldfish (22). Further studies have suggested that VIP/PACAP are involved in the inhibitory regulation of appetite and food intake via the anorexigenic action by the α -melanocyte-stimulating-hormone or corticotrophin-releasing hormone neuronal pathways in vertebrates (23).

Third, the VIP pathway may influence energy metabolism and obesity risk through regulation of psychomotor behaviors in mammals. In a study using the goldfish, *Carassius auratus*, the investigators found that intracerebroventricular and intraperitoneal administration of VIP induced a significant decrease in locomotor activity (24), which in turn may result in increased fat deposition.

Fourth, VIP regulates circadian rhythmicity and synchrony in mammalian clock neurons, which may influence feeding behavior and potentially, fat deposition. The mammalian suprachiasmatic nucleus is a master circadian pacemaker. VIP and the VIP receptor VPAC2 (encoded by the gene *Vipr2*) may mediate rhythms in individual suprachiasmatic nucleus neurons, synchrony between neurons, or both. In a study, *Vip(-/-)* and *Vipr2(-/-)* mice showed two daily bouts of activity in a skeleton photoperiod and multiple circadian periods in constant darkness (25). Loss of VIP or VPAC2 also abolished circadian firing rhythms in approximately half of all suprachiasmatic nucleus neurons and disrupted synchrony between rhythmic neurons (25). The relationship between the circadian and metabolic systems and the implications for human diseases (such as obesity, cardiovascular disease, and diabetes) has been recognized. Emerging evidence shows that circadian regulation is intimately linked to metabolic homeostasis and that dysregulation of circadian rhythms can contribute to disease such as obesity (26). Conversely, metabolic signals also feed back into the circadian system, modulating circadian gene expression and behavior.

In our analysis of 963 different pathways, the VIP pathway was the pathway that was associated most strongly with fat mass, and was the third most strongly associated pathway with BMI. Compared to fat mass, BMI (weight/height²) is a relatively less homogeneous trait that is influenced by both body fat mass and body lean mass. The fact that the VIP pathway was strongly associated with both fat mass and BMI make it convincing that this pathway could play a role in obesity etiology.

There are several limitations to our study. First, the Gene Set Enrichment Algorithm used in this study only chose a single SNP as the best association statistic to represent a gene. This method might be limiting, as multiple functional variants may exist in a real disease causing gene. To address this limitation, it is necessary to develop an improved gene statistic which incorporates information for more than one variant or, ideally, all of the variants for each gene to be tested. In addition, the Affymetrix GeneChip 500K Array used in this study covers the whole genome, but not all genomic regions are equally well covered. We therefore cannot exclude other pathways for their significance for obesity even though they did not achieve statistical significance in our analyses. Second, although several

publicly available pathway databases were analyzed, we are aware that knowledge regarding these biological and disease pathways is limited, placing significant limitations on our analyses. Third, due to the current limitations of pathway-classification tools, some genes are inevitably shared by different pathways from the same or different databases (27). Although the overlapped genes among different pathways will not affect the pathways' relative ranking in terms of normalized ES values, interdependence between pathways will lead to a decreased power by affecting *FDR* when the causal genes are shared by multiple pathways (due to permutation procedure to adjust for multiple testing) (27). Finally, it should be noted that current pathway-based methods have some other limitations. For example, there are differences in outcome between different pathway methods even on the same dataset; analyzing randomly selected SNPs may result in significantly overrepresented pathways; and most genes in the human genome are not sorted out in any biological pathway (27,28).

In summary, using our available GWA data and a pathway-based analytical approach, we found that the VIP pathway was significantly associated with BMI and fat mass. Despite some potential limitations, the fact that the VIP pathway is a top-ranking pathway in this study, together with functional evidence from previous studies, support an important role for the VIP pathway in the development of obesity. Further functional studies of this pathway are required to discern the mechanism by which it contributes to the pathogenesis of obesity.

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DISCLOSURE

The authors declared no conflict of interest.

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