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Genome Wide Association Study and Follow-Up Analysis of Adiposity Traits in Hispanic-Americans: the IRAS Family Study

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Abstract

We investigated candidate genomic regions associated with computed tomography (CT)-derived measures of adiposity in Hispanic from the IRAS Family Study. In 1190 Hispanic individuals from 92 families from the San Luis Valley, CO and San Antonio, TX, we measured CT-derived visceral adipose tissue (VAT); subcutaneous adipose tissue (SAT); and visceral: subcutaneous ratio (VSR). A genome-wide association study (GWAS) was completed using the Illumina HumanHap 300 BeadChip (~317K single nucleotide polymorphisms (SNPs)) in 229 individuals from the San Antonio site (Stage 1). Two hundred ninety-seven SNPs with evidence for association with VAT, SAT, or VSR, adjusting for age and sex ($p<0.001$), were genotyped in the remaining 961 Hispanic samples. The entire Hispanic cohort (n = 1190) was then tested for association, adjusting for age, sex, site of recruitment and admixture estimates (Stage 2). In Stage 3, additional SNPs were genotyped in four genic regions showing evidence of association in Stage 2.

Several SNPs were associated in the GWAS ($p<1\times10^{-5}$) and were confirmed to be significantly associated in the entire Hispanic cohort ($p<0.01$), including: rs7543757 for VAT; rs4754373, and rs11212913 for SAT; and rs4541696, and rs4134351 for VSR. Numerous SNPs were associated with multiple adiposity phenotypes. Targeted analysis of four genes whose SNPs were significant in Stage 2 suggest candidate genes for influencing the distribution (*RGS6*) and amount of adiposity (*NGEF*).

Several candidate loci, including *RGS6* and *NGEF*, are associated with CT-derived adipose fat measures in Hispanic Americans in a three-stage genetic association study.

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Keywords

genetic association; visceral fat; subcutaneous fat; obesity; body mass index

Introduction

Although body mass index (BMI) and the amount of fat stored in specific depots are highly heritable phenotypes(1,2), investigations into the genetic etiology of adiposity have not yielded many replicated genes. In 2007, the *FTO* gene was identified as the first locus influencing fat mass and obesity on a population level have replicated this association(3), and large-scale GWAS and meta-analyses confirmed this association in several populations(4–6). These same studies revealed and confirmed that common variants near *MC4R* are also associated with fat mass, weight and risk of obesity (4–6). Several GWA studies have also found novel genes that may underlie obesity risk, such as *INSIG2*(7,8), *PFKP*(3), and TMEM18, KCTD15, GNPDA2, SH2B1, MTCH2, and NEGR1(5,6), although their functional importance needs to be established. Other GWAS have identified a small number of additional gene variants associated with adiposity-related phenotypes(9–11).

Many recent studies have noted that visceral adipose tissue may be more detrimental to one's health than simply an obese phenotype(12,13). To date, no GWAS have been conducted for adiposity phenotypes such as abdominal visceral and subcutaneous fat. The IRAS Family Study obtained abdominal computed tomography (CT)-derived measures of visceral and subcutaneous adipose tissue, in addition to anthropometric measures such as waist and hip circumference and BMI in Hispanic-American subjects. In this report, we describe a three-stage genetic association study for adiposity phenotypes in Hispanic-Americans.

METHODS AND PROCEDURES

IRAS Family Study (IRASFS)

Study design, recruitment and phenotyping for IRASFS have been described in detail(14). Briefly, the IRASFS is a multi-center study designed to identify the genetic determinants of quantitative measures of glucose homeostasis and adiposity. Members of large families of self-reported Hispanic ancestry (n=1190 individuals in 92 pedigrees; San Antonio, TX; San Luis Valley, CO) were recruited. African-American families were recruited in Los Angeles, CA, but are not the subject of this report. The institutional review boards at each participating analysis and clinical site approved the study protocol; and all participants provided their written informed consent. A clinical examination was performed that included an interview, a frequently sampled intravenous glucose tolerance test (FSIGT), anthropometric measurements, blood chemistry and biomarker analysis, and evaluation of fat distribution using computed tomography.

Measurement of obesity phenotypes—A CT scan was performed to estimate visceral and subcutaneous fat area (cm^2). The procedure consisted of a single scout of the abdomen followed by two 10mm thick axial images. Axial images were obtained at the L2–L3 and L4–L5 disc spaces, using a standard protocol. CT images were transferred to magnetic tape and sent to a centralized CT reading center at the University of Colorado Health Sciences Center for analysis. The phenotypes of visceral adipose tissue (VAT), subcutaneous adipose tissue (SAT), and the ratio between the two (visceral-to-subcutaneous ratio; VSR) were computed from these data as previously described(2). Several conventional measures of adiposity were also obtained at the IRAS Family Study clinic visit, including body mass index (BMI, kg/m^2), and waist circumference (minimum, between 10th rib and the iliac crest, in cm).

Genome-Wide Association Study (GWAS) in Subset (Stage 1)

GWAS Study Subjects—A subset of IRAS Family Study Hispanics (*n*=229 from 34 families in the San Antonio study sample) were chosen for an exploratory GWAS analysis. These individuals were chosen from non-diabetic participants with complete data for adiposity phenotypes, with age, BMI, and gender composition similar to that of the overall IRAS Family Study population. Individuals chosen represented a genetically homogenous population as assessed from Structure analysis(15) of the microsatellite markers from the Mammalian Genotyping Service (Marshfield, WI) linkage panel of markers genotyped on IRAS Family Study samples previously(2). Individuals were chosen that had Structure analysis scores greater than 0.90 (from a possible range of 0.0–1.0) on the Hispanic axis.

GWAS Genotyping—Samples chosen for genotyping were from DNA derived from transformed lymphoblastoid cell lines to facilitate high quality genotyping. GWAS genotyping was performed using 1.5 μg of genomic DNA (15 ul of 100 ng/ul stock) using Illumina Infinium II HumanHap 300 BeadChips at Cedars-Sinai Medical Center using the Illumina Infinium II assay protocol (16). Genotypes were called based on clustering of the raw intensity data for the two dyes using Illumina Bead Studio software. Consistency of genotyping was checked using 96 repeat samples. Repeat genotyping of DNA samples was performed once if the overall call rate was < 98%; and the sample was rejected if there was no improvement in call rate. Genotypes with GenCall scores < 0.15 were set to missing (0.25%). Across all individuals and GWAS SNPs, a total of 2258 Mendelian inconsistencies were identified using PedCheck(17). These inconsistencies spanned 1657 SNPs and these genotypes were converted to missing.

SNPs that were not in Hardy-Weinberg Equilibrium (HWE) or had a minor allele frequency (MAF) < 0.05 were excluded from analysis. A total of 309,200 SNPs met all quality control criteria and were evaluated for association with VAT, SAT, and VSR. The Quantile-Quantile (Q-Q) plot for the stage 1 GWAS, representing a function of the expectation under the null distribution versus the observed distribution after adjustment of age and sex, is shown in the Supplemental Online Figure 1. These plots compare the observed versus expected values of the *Z* test statistics under the null hypothesis of no association across the genome. As expected, the majority of SNPs exhibit a −log10(P-value) less than 2, and the observed distribution of P-values matches expectation for the majority of the observed data, but departs from the null distribution at $P < 10^{-3}$.

GWAS Analysis—To test for association between individual SNPs and the continuous adiposity phenotypes, while accounting for the correlations among family members in pedigrees of arbitrary size and complexity, variance component analysis was performed using SOLAR(18). To best approximate the test's distributional assumptions of conditional normality and homogeneity of variance, we square root transformed VAT and SAT and natural log transformed VSR. We tested for association, adjusting for age and sex. The additive genetic model is our primary inferential test, unless there were less than 10 individuals homozygous for the minor allele and then the dominant model was considered. These results were used to rank and prioritize results for selecting SNPs for testing in the entire Hispanic cohort (Stage 2).

Association Study in the entire IRAS Family Study Hispanic Cohort (Stage 2)

SNPs with evidence of association in the Stage 1 GWAS were genotyped in the entire IRASFS Hispanic-American cohort (n=1190) (Stage 2). SNP selection for Stage 2 was based upon the most strongly associated SNPs for VAT, SAT, and VSR ($p < 0.001$ in Stage 1). A total of 297 SNPs were included in a 1536 custom chip; 79 SNPs were selected for VAT, 89 for SAT, and 129 for VSR. Genotyping was performed at Cedars-Sinai Medical Center using the Illumina Golden Gate Assay. Samples with call rates below 95% were excluded and SNPs with call

frequency below 0.95 were reviewed and manually re-clustered where necessary (~15% of SNPs).

Maximum likelihood estimates of allele frequencies were computed using the largest set of unrelated Hispanic American individuals and then genotypes were tested for departure from Hardy-Weinberg Equilibrium (HWE) expectations. SNPs selected for analysis were those with less than 5% missing genotypes, no evidence of a difference in VAT, SAT, or VSR values between individuals with and without missing genotype data (p-value > 0.05), and no evidence of departure from HWE (p-value > 0.0001). The remaining SNPs were tested for association but their evidence of association was considered relative to neighboring SNPs that met the above criteria.

A collection of 80 ancestry-informative markers (AIMs) (14 on the × chromosome) were selected from the literature for Hispanic populations were also included in the 1536 custom chip. The CEPH and Yoruban HapMap data were merged with the study population and a principal component analysis was computed to estimate ancestry proportions. The first three principal components (PCs) from this analysis explained 10.3% (PC1), 4.8% (PC2), and 1.9% (PC3) of the variation across the 80 AIMs. PC2 was the best at distinguishing the parent populations of Hispanic-Americans in IRASFS and therefore was selected to adjust for admixture in these analyses.

As in Stage 1, SOLAR was used to test for association using the additive genetic model, adjusting for age, sex, recruitment site (San Antonio or San Luis Valley), admixture (using PC2), and where indicated, BMI. A minimum of 10 homozygotes of the minor allele was required for this test, otherwise the dominant genetic model was tested. We chose to combine the GWAS sample with the remaining Hispanic sample rather than analyzing the remaining Hispanic sample separately as a form of replication, because methodological studies by Skol *et al*(19) have suggested that an approach using joint analysis of data is more efficient than replication-based analysis for two-stage genome-wide association studies. Under an additive model, MAF=0.15, and α=0.0001 we have estimated power of 0.90 of detecting a 0.30 standard deviation change in the genotypic means in the 1190 Hispanic-Americans in IRASFS.

Follow-up Association Study of SNPs in Selected Genes (Stage 3)

Four genic regions (e.g. *ASB18, NGEF, RGS6* and *VAV2*) with evidence of association in both Stages 1 and 2, association with multiple adiposity phenotypes and potential biological rationale, were targeted for additional genotyping using tag SNPs. This genotyping was performed using iPLEX Gold SBE assays on the Sequenom MassArray Genotyping System. Locus-specific primers were designed using the MassARRAY Assay Design 3.0 software (Sequenom, Cambridge, MA). The resulting mass spectrograms were analyzed using MassARRAY TYPER software (Sequenom). Fifty-one blind duplicate samples were included to evaluate genotyping accuracy. SNPs were selected to capture common variation within LD haplotype blocks as defined by the CEPH (CEU) population of the International HapMap project(20), which was selected as the best ancestral model for the IRASFS Hispanic population. Specifically, genotype data from the genomic interval containing the candidate gene +/−5 kb were exported from the HapMap database and imported into Haploview(21). SNP selection within larger candidate genes (e.g., *RGS6, NGEF* and *ASB18*) attempted to focus on the LD block that the associated SNP or SNPs were within as per HapMap. For the candidate gene with lower LD (e.g. *VAV2*), a small genomic region 20kb around the associated SNP was used for SNP selection. HapMap YRI tagging SNPs were selected using the aggressive tagging algorithm, a minimum minor allele frequency (MAF) of 10%, and a minimum r^2 of 0.8. Selected YRI tag SNPs were force included, to the exclusion of all other tag SNPs, in the HapMap genotype dataset for CEUs for the same genomic region. The percentage of variation in the genomic region captured was considered adequate if it was estimated as >75% in both

the YRI and CEU HapMap cohorts. Only those SNPs that were in HWE and had a MAF > 0.10 were included in the analysis.

As in Stages 2 and 3, SOLAR was used to test for association using the additive genetic model, adjusting for age, sex, site (San Antonio or San Luis Valley), admixture (using PC2), and where indicated, BMI. Further adjustments for multiple comparisons were not performed due to selection of SNPs based on *a priori* hypotheses regarding the selected candidate genes.

RESULTS

As described above, we carried out a 3-stage genetic association study of adiposity phenotypes, with Stage 1 being a GWAS in a subset of 229 Hispanic IRASFS participants, Stage 2 being analysis of significant SNPs from the GWAS in all 1190 Hispanics in the IRASFS, and Stage 3 being analysis of additional SNPs in significant gene regions identified in Stage 2. Comparisons between the GWAS sample (used in Stage 1) and the entire IRASFS Hispanic cohort (used in Stages 2 and 3) with respect to demographic and adiposity measures show that the GWAS sample was representative of the entire Hispanic cohort (Table 1).

Stage 1 and 2: Pilot GWAS and Association Study on Entire IRASFS Hispanic Cohort

Those SNPs that were selected from the pilot GWAS (Stage 1) because they were significantly associated (at $p < 0.001$) with either VAT, SAT, or VSR, and then confirmed to be statistically significant in the entire Hispanic cohort of the IRASFS (Stage 2) (at $p < 0.01$ level) for that same adiposity phenotype are displayed in Table 2–Table 4. P-values from association analysis of these SNPs in the Hispanics that were not included in the GWAS are reported in Online Supplemental Table 1.

Visceral Adipose Tissue Area (VAT)—Of the 79 SNPs that were significant ($p<0.001$) in the pilot GWAS for VAT, 7 are significant ($p<0.01$) in the entire Hispanic cohort. (Table 2). The most significantly associated SNP, rs4785644, (Stage 1 $p=7.9\times10^{-5}$, Stage 2 $p=6.9\times10^{-4}$) is in a non-genic region, 25.8 kb from *ZNF778* and 12.7 kb from *ANKRD11* on chromosome 16. SNP rs870583 in the Na+/K+ transporting ATPase interacting 2 (*TCBA1*) gene is significant in Stages 1 ($p= 7.3\times10^{-4}$) and 2 ($p= 4.5\times10^{-3}$).

Subcutaneous Adipose Tissue Area (SAT)—Of the 89 SNPs that were significant in the pilot GWAS for SAT, 7 are significant in the entire Hispanic cohort (Table 2). The most significant findings are three SNPs on chromosome 11 in a non-genic region between *DDX10* and *LOC399947*. The LD between these SNPs was moderate: rs4754373-rs1509729 $r^2=0.44$ and rs1509729-rs11212913 $r^2=0.51$. SNP rs12193017, which is associated with SAT (Stage 1 $p=2.8\times10^{-5}$, Stage 2 $p=6.5\times10^{-3}$), was also associated with VAT (see Table 2). A SNP (rs4973062) in the neuronal guanine nucleotide exchange factor (NGEF) was associated with SAT in Stage 1 ($p=1.3\times10^{-4}$) and Stage 2 ($p=2.0\times10^{-4}$).

Visceral to Subcutaneous Adipose Tissue Ratio (VSR)—Of the 129 SNPs that were significant in the pilot GWAS for VSR, 12 are significant in the entire Hispanic cohort (Table 2). The most significant association in the entire cohort is rs7086207 in a non-genic region 165.8 kb from AK123440 and 35.5 kb from MYO3A (Stage 1 $p=1.2\times10^{-4}$, Stage 2 $p=3.9\times10^{-5}$). SNPs in several genes are associated with VSR in both Stages 1 and 2. Rs12707628 in the sema domain, immunoglobulin domain, short basic domain, secreted (semaphorin) 3A (*SEMA3A*) was associated with VSR in Stages 1 ($p=8.3\times10^{-4}$) and 2 ($p=6.7\times10^{-4}$). Rs666432, in the tripartite motif-containing 29 gene (*TRIM29*), was also associated with VSR in both Stage 1 ($p=2.5\times10^{-5}$) and Stage 2 ($p=8.8\times10^{-4}$). Two SNPs in the Regulator of G-protein signaling 6 (RGS6) gene (rs2239227 and rs2239247, $r^2=0.28$) are

associated with VSR. Rs2015983 in the ASB18 gene and rs2240003 in the *ASB4* gene were associated with VSR in Stages 1 and 2. *ASB18* and *ASB4* are members of the ankyrin repeat and SOCS box-containing (ASB) family of proteins, which contain ankyrin repeat sequence and SOCS box domains. Rs669443 of *VAV2*, the second member of the VAV guanine nucleotide exchange factor family of oncogenes, is associated with VSR (Stage 1 $p=2.5\times10^{-5}$, Stage 2 $p=3.1\times10^{-3}$).

Analysis of Multiple Adiposity Phenotypes—IRAS Family Study also measured BMI and waist, in addition to the phenotypes of VAT, SAT, and VSR, that were used to perform Stages 1 and 2. While all of these adiposity phenotypes are correlated (the spearman correlation was 0.64–0.69 between VAT and BMI, 0.76–0.77 for VAT and Waist, 0.89 for SAT and BMI, and 0.87-0.85 for SAT and Waist in Hispanic IRASFS men and women, respectively(22), the different measures represent different types of fat and patterns of fat distribution. Therefore, we took the SNPs found to be associated with VAT, SAT, and VSR in Stages 1 and 2, and tested these in the entire IRASFS Hispanic cohort for associations with every adiposity phenotype, including BMI, Waist, and BMI-adjusted VAT, the latter being a derived phenotype to examine genetic influences on depot of fat rather than overall adiposity (Table 3). Statistical significance, which was based on the p-value from the admixture-adjusted additive model, is depicted in Table 3 by intensities of shading, to allow investigation of patterns of association that might provide clues as to whether the SNP was associated with amount or distribution of adiposity. Five SNPs are associated with both VAT and Waist. There are 4 SNPs that are associated with Waist, SAT, and BMI. Similarly, VSR and BMI-adjusted VAT, are most often associated with the same SNP in 6 instances. Four SNPs, rs1509729, rs7258003, rs6745724 (*NGEF*), and rs2400963 (*RGS6*) are associated with 4, 4, 4, and 5 adiposity phenotypes, respectively, suggesting that the genes that these SNPs mark may have either very basic or pleiotropic effects on adiposity.

Stage 3. Follow-up Association Study of SNPs in Selected Genes

Based on strength of association, association with multiple adiposity phenotypes, and potential biological rationale, the following four genes were selected for additional focused genotyping and analysis: *NGEF, RGS6, VAV2*, and *ASB18*. Sixteen additional SNPs within a ~46 Kb region were typed in *NGEF* (Table 4), and 6 of these SNPs were associated with VAT (p-values ranging from 0.005 to 0.037), 5 were associated with Waist (p-values ranging from 0.0007 to 0.039), 7 were associated with SAT (p-values ranging from 0.0002 to 0.01), and 4 were associated with BMI (p-values ranging from 0.002 to 0.04). The SNP associations with VAT became non-significant after adjusting VAT for BMI, suggesting that *NGEF* may be related to overall adiposity or amount of adiposity rather than adiposity depot. The genotypic means for associated *NGEF* SNPs with BMI, VAT and SAT are presented in Table 5.

Of the 16 additional SNPs typed within an ~100kb region of *RGS6*, which incorporated rs2239227 and rs2239247, 4 of these SNPs were associated with VAT, 3 were associated with Waist, 2 were associated with SAT, 5 were associated with BMI, and 8 of these SNPs were associated with VSR (Table 4). Eleven of these 16 SNPs were associated with BMI-adjusted VAT (P-values ranged from 0.000048 to 0.039). Several of the SNPs in *RGS6* became more significant when VAT was adjusted for BMI (compared with unadjusted VAT), and several of these same SNPs were significantly associated with VSR rather than SAT and VAT individually, suggesting that *RGS6* may be related to distribution of fat rather than amount of fat. The genotypic means for associated *RGS6* SNPs with BMI-adjusted VAT and VSR are presented in Table 5.

The Stage 3 analysis of the two remaining genes, *ABS18 and VAV2*, which showed less remarkable results (data not shown).

DISCUSSION

The measurement of adiposity phenotypes derived from CT scans provides information regarding amount, depot and relative distribution of fat, which may have different genetic etiologies as well as different effects on overall health(12,13). These adiposity phenotypes have varying heritability estimates, from h^2 of 0.72 and 0.63 for BMI and SAT, respectively, to h^2 of 0.38 for VAT, to h^2 of 0.29 for BMI-adjusted VAT in IRASFS Hispanics(2). Here, we report the first genome-wide association study of these CT-derived measures of adiposity in a Hispanic sample. This exploratory study has identified multiple candidate regions for further study and is an initial step towards teasing out the potentially different genetic influences on amount and distribution of fat.

To carry out this study we chose a research design in which a 318K SNP GWAS analysis was performed on 229 Hispanic subjects from one clinical center (San Antonio). From the analysis results, a set of 1536 high scoring SNPs were chosen for genotyping and analysis in the IRASFS Hispanic cohort in which we have high quality adiposity measures. While we ideally would have carried out the GWAS on the entire cohort, this was not financially possible. Our purpose was to identify some potential polymorphisms, given this is the first such study, and was motivated by the success of a number of small GWAS studies. For example, the complement factor H gene association with macular degeneration, with 224 cases and 134 controls(23), NOS1AP gene association with cardiac repolarization with 200 subjects(24), TNFSF15 conferring susceptibility to Crohn's disease with 94 subjects(25), and the recent report of association to coronary heart disease near the CDKN2A and CDKN2B genes on chromosome 9 based initially on results from a GWAS analysis of 322 cases and 312 controls, with only 75,000 SNPs(26). We acknowledge that the associations reported herein do not meet genome-wide significance, and we consider the loci reported here as candidates for future detailed evaluation rather than confirmed adiposity genes.

It is a difficult process to identify a targeted set of genomic regions for subsequent study in a GWAS. One way to limit the SNPs to follow up is to choose only those that meet some genome-wide threshold of significance. An alternative way is to examine SNPs that do not quite reach the genome-wide significance threshold but have independent supporting evidence of importance, such as association with multiple related phenotypes, biological plausibility based on review of the literature, and lying within a region of linkage in that population. We have compared associated SNPs in Stage 2 to the results of our whole genome linkage scan analysis of these same adiposity phenotypes in Hispanics in the IRAS Family Study. As shown in Online Supplemental Table 2, several SNPs that were significant in Stages 1 and 2 also lie under a linkage peak (LOD > 1.0) for an adiposity trait, lending additional support to these SNPs and providing an argument for further follow-up.

No SNPs in either *FTO* or *MC4R* were found to be significantly associated with SAT, VAT, or VSR in the IRAS Family Study GWAS (Stage 1). *FTO* has been found to be associated with obesity in another Hispanic-American population(27), and *MC4R* has only been analyzed in populations of European descent(4). One possible explanation for this lack of association is that if *FTO* and *MC4R* were primarily associated with BMI and fat mass, and not with SAT, VAT, or VSR , SNPs in these genes would not have been selected for confirmation, as we did not use BMI as a phenotype in the GWAS to choose SNPs for Stage 2 of our study. We have independently assessed the influence of FTO polymorphisms in this sample (Wing et al, submitted) in which we have observed nominal evidence for association with BMI and SAT, but with levels of significance less than reported here. Moreover, none of the novel SNPs recently reported for BMI (5) or BMI and weight (6) in GWAS in primarily European ancestry populations were significantly associated with SAT, VAT or VSR in the Hispanics in IRAS Family Study GWAS (Stage 1).

Four loci in genic regions that were significant in both Stages 1 and 2,had evidence of association across multiple adiposity phenotypes, and potential biological rationale received additional follow-analysis in Stage 3. This follow-analysis revealed two possible candidate genes for adiposity, *NGEF* and *RGS6*. *NGEF* was selected for follow-up genotyping due to the association of rs4973062 with multiple adiposity phenotypes (VAT, SAT, Waist, and BMI) in Stage 2. *NGEF* is a downstream signaling component of the ephrin A (EphA4) tyrosine kinase receptor, and is important for the appropriate formation of neural networks. *NGEF* serves to either promote neural outgrowth or neural growth cone collapse(28,29). Genetic polymorphisms within or near *NGEF* may affect the well-documented feedback mechanisms between the central nervous system and adipose tissue or impede normal neural network formation. This is intriguing given the recent identification of several loci associated with BMI suggesting a possible neuronal influence on body weight regulation(5,6). As shown in Table 4, evidence of association with NGEF is largely observed with whole body adiposity measures, which greatly diminishes when adjusting for BMI. These results suggest that *NGEF* may be a total fat-contributing gene. The strongly BMI- and SAT-associated SNP rs6745724 was highly correlated with the previously associated (in Stage 1) SNP rs4973062, with an inter-SNP r^2 of 1. However, on average, inter-SNP linkage disequilibrium was low (mean D' was .264) and inter-SNP correlation was likewise low (mean r^2 was .025). This would imply that additional genotyping of *NGEF* variants may be informative.

The *RGS6* gene is one of the R7 subfamily of regulators of G-protein signaling, which has slow-acting GTPase activity(30,31). *RGS6* (and other R7 proteins) shows an ability to regulate opioid receptor agonist tachyphylaxis and acute tolerance at μ-opioid receptors, and may thus contribute to the modulation of opioid and opioid receptor function(31). The connection between stress and diet-induced obesity may be mediated in large part by the potent hormone, cortisol, which can promote changes in energy resource management by modulating lipid metabolism, insulin sensitivity, insulin secretion, leptin sensitivity, and leptin secretion that favor obesity(32–38). In addition, humans display comparable eating behaviors in response to chronic stress(38–40), such as the choice of foods with a high sugar or fat content, which likewise cause opioid release(38,41,42). Therefore, it is possible that a polymorphism near or within *RGS6* alters the expression or function of the gene, which could favor increased adiposity by altering the opioid response, and thus cortisol secretion and feeding behavior. An increase in visceral adiposity would specifically be favored by abnormal cortisol secretion. The profile of association, and the potential functional relevance of *RGS6* as described above, are highly suggestive of a gene that contributes to obesity and obesity-related pathogenesis by increasing visceral adiposity. Inter-SNP correlation between the genotyped BMI-adjusted VAT and VSR associated tagging SNPs and rs2239227 and rs2239247 was inconsistent. On average, inter-SNP linkage disequilibrium was high (Mean D'= .787) and inter-SNP correlation was low (Mean r^2 = .178). This would imply that most SNPs in the tagged region are inherited together more often than was seen with *NGEF*, but no definitive conclusion can be drawn. However, it seems that we have found a region of interest that contributes to increased visceral adiposity within the second intron of *RGS6*.

Though these results are encouraging, and we appear to have localized genomic regions of interest within *RGS6* and *NGEF*, additional follow-up genotyping is necessary to more finely resolve the linkage disequilibrium structure and narrow the region of interest. The common SNPs that we chose for fine mapping were chosen using a SNP-centric approach without regard to linkage disequilibrium in the region; therefore more SNPs should be genotyped to completely cover common variation across the region. Further resolution of the haplotype block structure in these regions will enable a more informative haplotype analysis. Successful replication of these *NGEF* and *RGS6* associations in additional and ethnically distinct cohorts is important to determine the extent to which these genes may contribute to human obesity.

In summary, using a three stage GWAS, validation and follow-up approach, we have identified several genic and non-genic loci that are candidates for association with multiple adiposity phenotypes in Hispanic Americans. Two candidate genes, *NGEF* and *RGS6*, have been identified for additional follow-up and replication.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Demographic and Adiposity Characteristics of the Hispanic Subjects in the IRAS Family Study. Mean ± Std (Median) is reported unless otherwise noted

	GWAS Hispanic Sample	Entire Hispanic Cohort
	N=229	N=1190*
Sex (n, % Male)	83 (36.2%)	493 (41.4%)
Age (years)	41.3 ± 13.9 (40.3)	42.8 ± 14.5 (41.3)
Type 2 Diabetes (n, % DM)	0%†	147 (12.4%)
BMI (kg/m^2)	29.4 ± 5.9 (28.5)	29.0 ± 6.2 (28.1)
Waist (cm)	90.9 ± 13.6 (91.0)	90.0 ± 14.4 (89.4)
WHR	0.85 ± 0.08 (0.85)	0.86 ± 0.09 (0.85)
VAT (cm^2)	115.0 ± 61.9 (102.7)	114.7 ± 62.3 (105.7)
SAT (cm^2)	341.8 ± 151.6 (311.6)	339.7 ± 155.7 (313.7)
VSR	0.37 ± 0.21 (0.32)	0.37 ± 0.22 (0.32)

*includes the GWAS sample

†Diabetic subjects were excluded from GWAS sample.

Table 2

SNPs Significantly Associated with Visceral Adipose Tissue Area (VAT), Subcutaneous Adipose Tissue Area (SAT), and Visceral to Subcutaneous Fat Ratio (VSR) in Hispanics in Both the GWAS Sample (Stage 1) ($p < 1.0\times10^{-3}$) and the Entire Cohort (Stage 2) ($p< 1.0\times10^{-2}$) of the IRAS Family Study. SNPs are ordered in decreasing level of significance in the Stage 2.

Marker	Chr	Position	MAF *	Gene Name	Stage 1 GWAS Sample p-value† (n=229)	Stage 2 Entire Cohort p-value‡ (n=1190)	Genotypic Means in Entire Cohort Mean ± SD 1/1	1/2	2/2
Phenotype: Visceral Adipose Tissue Area (VAT)									
rs4785644	16	87848739	0.45		7.9×10^{-5}	6.9×10^{-4}	119.1± 60.7	119.9± 63.2	98.2± 54.8
rs10063941	5	91257403	0.24		6.0×10^{-4}	3.0×10^{-3}	109.2± 58.1	121.5± 62.3	127.6± 75.7
rs1026228	8	102076877	0.34		3.2×10^{-4}	3.7×10^{-3}	111.9± 59.2	117.5± 62.5	116.1± 64.1
rs6436346	2	223382566	0.41		2.9×10^{-5}	3.9×10^{-3}	110.0± 57.7	116.5± 62.9	121.1± 63.9
rs870583	6	125097228	0.21	TCBA1	7.3×10^{-4}	4.5×10^{-3}	117.6±62.1	110.9±61.2	105.4± 47.7
rs12193017	6	79198412	0.22		1.4×10^{-4}	5.2×10^{-3}	118.5± 62.5	111.8± 59.9	99.9± 57.4
rs7543757	1	105029437	0.34		4.6×10^{-6}	9.1×10^{-3}	110.4± 59.9	116.8± 62.5	127.1± 61.9
Phenotype: Subcutaneous Adipose Tissue Area (SAT)									
rs1509729	11	108432693	0.36		1.5×10^{-4}	1.5×10^{-5}	359.2± 162.0	335.6± 150.9	289.8± 141.9
rs4754373	11	108398056	0.27		9.5×10^{-7}	2.7×10^{-5}	358.4± 162.9	321.3± 143.4	301.3± 145.8
rs4973062#/ rs6745724	2	233566880/ 233562485	0.34	NGEF	1.3×10^{-4}	2.0×10^{-4}	356.9± 160.9	328.0± 146.8	297.2± 150.7
rs11212913	11	108452854	0.36		6.5×10^{-6}	3.5×10^{-4}	354.2± 163.1	340.5± 151.1	286.4± 135.2
rs399162	22	18426687	0.28	C22orf25	3.6×10^{-4}	7.6×10^{-4}	326.1± 146.8	353.7± 164.6	377.6± 159.5
rs11022428	11	12572786	0.19		8.2×10^{-4}	3.4×10^{-3}	333.0± 154.9	349.5± 154.6	394.0± 167.8
rs12193017	6	79198412	0.22		2.8×10^{-5}	6.5×10^{-3}	355.1± 162.5	323.0± 144.9	313.5± 142.4
Phenotype: Visceral to Subcutaneous Fat Ratio (VSR)									
rs7086207	10	26227683	0.25		1.2×10^{-4}	3.9×10^{-5}	0.39± 0.22	0.36± 0.20	0.32± 0.13

Marker	Chr	Position	MAF *	Gene Name	Stage 1 GWAS Sample	Stage 2 Entire Cohort	Genotypic Means in Entire Cohort Mean ± SD		
					p-value† (n=229)	p-value‡ (n=1190)	1/1	1/2	2/2
rs4134351	13	82851694	0.11		1.6×10^{-6}	1.9×10^{-4}	0.38± 0.21	0.37± 0.20	0.28± 0.17
rs12707628	7	83655831	0.27	SEMA3A	8.3×10^{-4}	6.7×10^{-4}	0.40± 0.22	0.36± 0.20	0.33± 0.18
rs666432	11	119508743	0.12	TRIM29	2.5×10^{-5}	8.8×10^{-4}	0.39± 0.21	0.34± 0.20	0.29± 0.10
rs7258003	19	20691124	0.49		7.9×10^{-5}	1.1×10^{-3}	0.39± 0.21	0.38± 0.21	0.36± 0.21
rs2239227	14	71899732	0.11	RGS6	2.7×10^{-4}	1.4×10^{-3}	0.39± 0.21	0.35± 0.19	0.33± 0.27
rs2015983	2	236814905	0.20	ASB18	1.0×10^{-3}	2.6×10^{-3}	0.39± 0.23	0.35± 0.18	0.38± 0.21
rs669443	9	135631534	0.25	VAV2	4.0×10^{-5}	3.1×10^{-3}	0.40± 0.22	0.36± 0.20	0.36± 0.19
rs4541696	5	634053	0.37		6.1×10^{-6}	3.6×10^{-3}	0.38± 0.21	0.38± 0.22	0.36± 0.19
rs2239247	14	71871989	0.23	RGS6	1.2×10^{-4}	4.0×10^{-3}	0.39± 0.21	0.36± 0.21	0.35± 0.19
rs885883	14	106345097	0.50		1.5×10^{-5}	5.6×10^{-3}	0.34± 0.19	0.39± 0.22	0.38± 0.21
rs2240003	7	95003210	0.25	ASB4	4.9×10^{-5}	6.2×10^{-3}	0.36± 0.20	0.39± 0.21	0.43± 0.24

*MAF are calculated from unrelateds in the entire Hispanic Cohort

†P-values from additive model, adjusting for age and sex. SNPs

‡P-values from additive model, adjusting for age, sex, site of recruitment and admixture.

#The typing of rs4973062 failed on the 1536 platform for a number of individuals. For Stage 2, we report the genotyping results of rs6745724, which has a D' of 1.0, and is perfectly correlated (r^2), with rs4973062.

TCBA1, Na+/K+ transporting ATPase interacting 2; ***NGEF***, neuronal guanine nucleotide exchange factor; ***C22orf25***, chromosome 22 open reading frame 25; ***ASB18***, ankyrin repeat and SOCS box-containing 18; ***ASB4***, ankyrin repeat and SOCS box-containing 4; ***SEMA3A***, sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A; ***VAV2***, vav 2 guanine nucleotide exchange factor; ***TRIM29***, tripartite motif-containing 29; ***RGS6***, regulator of G-protein signaling 6

Table 3

Loci Associated with Multiple Adiposity Phenotypes in 1190 Hispanic-Americans in the IRAS Family Study (Stage 2).

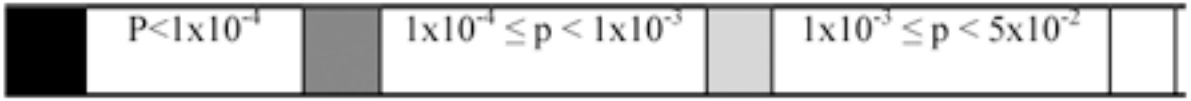
			Key					
			$p<1x10^{-4}$	$1x10^{-4} \leq p < 1x10^{-3}$	$1x10^{-3} \leq p < 5x10^{-2}$	ns		
			P-Value from Additive Model Adjusting for Age, Sex, Site and Admixture					
Chr	**SNP**	**Gene**	**VAT**	**Waist**	**SAT**	**BMI**	**VSR**	**BMI- adj VAT**
5	Rs10063941		*					
6	rs12193017		*					
6	rs870583	TCBA1	*					
8	rs1026228		*					
16	rs4785644		*					
2	rs6745724	NGEF			*			
11	rs1509729				*			
	rs4754373				*			
	rs11212913				*			
22	rs399162	C22orf25			*			
13	rs4134351						*	
14	rs2239227	RGS6					*	
	rs2239247						*	
2	rs2015983	ASB18					*	
9	rs669443	VAV2					*	
10	rs7086207						*	
19	rs7258003						*	

*Phenotype with which the SNP was originally associated in Stages 1 and 2.

Table 4

Follow-up SNP Genotyping in *NGEF* and *RGS6*. Stage 3 of IRAS Family Study Genetic Association Study of Adiposity.

Gene Name	Marker	MAF‡	Key					
			$P<1x10^{-4}$	$1x10^{-4} \leq p < 1x10^{-3}$	$1x10^{-3} \leq p < 5x10^{-2}$			
			P-value from Additive Model, Adjusted for Age, Gender, Site and Admixture					
			VAT	WAIST	SAT	BMI	VSR	BMI-adj VAT
NGEF	**Rs11678490**							
	Rs3817321	0.39						
	Rs954526	0.47						
	Rs938572	0.46						
	Rs6745724	0.34						
	Rs13404304	0.23						
	Rs127471240	0.14						
	Rs884089	0.37						
	Rs6720354	0.48						
	Rs1104953	0.42						
	Rs10933423	0.18						
	Rs10169752	0.42						
	rs10933424	0.11						
	Rs13383577	0.44						
	Rs3811589	0.15						
	Rs3811588	0.12						
	Rs6725244	0.38						
RGS6	Rs860195	0.39						
	Rs2283393	**0.08**						
	Rs847354	**0.42**						

Gene Name	Marker	MAF‡	Key					
								
			P-value from Additive Model, Adjusted for Age, Gender, Site and Admixture					
			VAT	WAIST	SAT	BMI	VSR	BMI-adj VAT
	Rs847352	0.42						
	Rs2239250	**0.14**						
	Rs8018927	0.12						
	Rs7147236	0.11						
	Rs2238199	0.47						
	Rs2239247	**0.23**						
	Rs1402064	0.37						
	Rs2681749	0.42						
	Rs12892244	0.23						
	Rs847334	0.28						
	Rs847330	**0.29**						
	Rs2239227	**0.11**						
	Rs847228	0.23						
	Rs769148	**0.30**						
	Rs4903013	0.49						
	Rs10149207	0.27						
	Rs6574069	0.26						
	Rs2239223	0.48						
	Rs2239219	0.49						
	Rs10149848	0.12						

‡MAF was calculated from unrelateds in the entire Hispanic cohort

ns; non-significant.

SNPs in **bold** are those in the gene that were already typed in Stages 1 and 2.

Table 5

Genotypic Means of Selected Adiposity Phenotypes with *NGEF* and *RGS6* (Stage 3)

Phenotype	SNP	Position	Genotypic Means ± SD (n) 1/1	1/2	2/2	P-value †
			NGEF			
BMI						
	rs6745724	233562485	29.8 ± 6.5 (557)	28.3 ± 5.6 (540)	27 ± 5.7 (124)	0.002
	rs13404304	233572741	29.5 ± 6.4 (749)	28 ± 5.6 (422)	26.9 ± 5.3 (68)	0.008
	rs6720354	233575910	29.5 ± 6.53 (359)	28.9 ± 6.0 (617)	27.8 ± 5.8 (251)	0.04
	rs13383577	233582643	29.9 ± 6.4 (403)	28.5 ± 6.0 (620)	27.7 ± 5.7 (205)	0.008
SAT						
	rs6745724	233562485	357 ± 161 (535)	328 ± 147 (526)	297 ± 151 (115)	0.0002
	rs13404304	233572741	350 ± 158 (726)	322 ± 146 (403)	297 ± 144 (62)	0.003
	rs6720354	233575910	357 ± 166 (345)	340 ± 151 (594)	311 ± 144 (242)	0.003
	rs1104953	233578610	312 ± 147 (383)	353 ± 154 (584)	348 ± 168 (213)	0.007
	rs13383577	233582643	364 ± 159 (390)	331 ± 151 (596)	311 ± 152 (197)	0.0005
VAT						
	rs6745724	233562485	119 ± 60 (535)	111 ± 62 (526)	103 ± 58 (115)	0.019
	rs884089	233574273	109 ± 58 (476)	116 ± 63 (547)	121 ± 62 (162)	0.008
	rs6720354	233575910	119 ± 62 (345)	114 ± 60 (594)	104 ± 57 (242)	0.005
	rs10169752	233580293	111 ± 63 (394)	113 ± 59 (587)	120 ± 61 (206)	0.015
	rs13383577	233582643	119 ± 61 (390)	112 ± 61 (596)	109 ± 63 (197)	0.006
			RGS6			
BMI-adjusted VAT						
	rs8018927	71856196	116 ± 62 (881)*	109 ± 57 (278)*	96 ± 46 (28)*	0.001
	rs7147236	71857999	116 ± 63 (883)*	109 ± 57 (279)*	97 ± 46 (29)*	0.0005
	rs12892244	71891466	117 ± 63 (683)*	110 ± 60 (429)*	104 ± 47 (78)*	0.005
	rs4903013	71906670	108 ± 57 (371)*	116 ± 62 (555)*	119 ± 65 (272)*	0.003

Phenotype	SNP	Position	Genotypic Means ± SD (n) 1/1	1/2	2/2	P-value †
	rs6574069	71908853	116 ± 63 (630)*	111 ± 59 (466)*	110 ± 59 (103)*	0.0006
VSR						
	rs8018927	71856196	0.388 ± .214 (856)	0.342 ± .192 (264)	0.328 ± .263 (26)	0.002
	rs7147236	71857999	0.388 ± .216 (857)	0.345 ± .192 (265)	0.332 ± .259 (27)	0.002
	R s12892244	71891466	0.391 ± .213 (660)	0.359 ± .209 (413)	0.328 ± .187 (75)	0.007
	rs4903013	71906670	0.355 ± .208 (358)	0.379 ± .201 (533)	0.399 ± .237 (265)	0.008
	rs6574069	71908853	0.388 ± .215 (608)	0.369 ± .213 (450)	0.337 ± .187 (99)	0.005

† p-values from the additive model, adjusting for age, gender, site of recruitment and admixture (and BMI for the BMI-adjusted VAT phenotype).

* Mean VAT, unadjusted for BMI.