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ORIGINAL ARTICLE

A combined linkage and association strategy identifies a variant near the *GSTP1* gene associated with BMI in the Mexican population

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Obesity is a major public health concern in Mexico and worldwide. Although the estimated heritability is high, common variants identified by genome-wide association studies explain only a small proportion of this heritability. A combination of linkage and association strategies could be a more robust and powerful approach to identify other obesity-susceptibility variants. We thus sought to identify novel genetic variants associated with obesity-related traits in the Mexican population by combining these methods. We performed a genome-wide linkage scan for body mass index (BMI) and other obesity-related phenotypes in 16 Mexican families using the Sequential Oligogenic Linkage Analysis Routines Program. Associated single-nucleotide polymorphisms (SNPs) were tested for associations in an independent cohort. Two suggestive BMI-linkage peaks (logarithm of odds ⩾ 1.5) were observed at chromosomal regions 11q13 and 13q22. Only rs614080 in the 11q13 region was significantly associated with BMI and related traits in these families. This association was also significant in an independent cohort of Mexican adults. Moreover, this variant was significantly associated with *GSTP1* gene expression levels in adipose tissue. In conclusion, the rs614080 SNP near the *GSTP1* gene was significantly associated with BMI and *GSTP1* expression levels in the Mexican population.
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INTRODUCTION

Obesity is a major risk factor for a number of chronic diseases, such as type 2 diabetes, dyslipidemias, cardiovascular disease, non-alcoholic fat liver disease and several cancer types.[1] Obesity has become an important public health problem in both developing and developed countries.[2] Specifically, Mexico has experienced a rapid increase in obesity prevalence in recent decades. The last National Health and Nutrition Survey indicates that approximately one-third of Mexican adults aged above 20 years were obese in 2012,[3] and projections made for 2050 indicate that if recent trends continue, by 2050 up to half (54%) of the Mexican adult population could be obese.[4]

Family and twin studies have shown that obesity is a highly heritable trait; heritability (H2r) is estimated between 40 and 70%.[5,6] Family studies have reported several loci linked to common forms of obesity, although in most cases it has not been possible to identify the causal variant or gene. In addition, genome-wide association studies have identified a great number of single-nucleotide polymorphisms (SNPs) contributing to variance of body mass index (BMI) and increased risk of obesity mainly in European populations.[7] The association of several of these genetic variants with obesity has been replicated in the Mexican population.[8–10] However, common genetic variation identified to date explains only a small fraction (5–10%) of BMI variance,[7,10] and other yet unidentified DNA variants could contribute substantially to modulate BMI and related phenotypes. Thus, a combination of linkage and association methodologies could be a more robust and powerful approach to identify and characterize other obesity-

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susceptibility variants.[11] The purpose of this study was to find genetic variants linked and associated with BMI and related traits in Mexican families using this combined approach.

MATERIALS AND METHODS

Mexican obesity families

The study included 16 families recruited from a population-based adult cohort (described below), who met the following criteria: at least 10 individuals aged over 18 years distributed in three or more generations; an obese proband with at least one first-degree relative with obesity (BMI $\geqslant 30\,kg\,m^{-2}$); and all participants identified themselves as Mexican Mestizos. A total of 172 individuals were included in the study. Ethics Committees of participating Institutions approved this project and all subjects provided written informed consent.

Population-based study

A total of 1499 unrelated Mexican Mestizo subjects aged 18–68 years were recruited from several governmental Institutions in Mexico City. A nested case–control analysis was performed including 577 of these subjects (301 non-diabetic normal weight and 276 obese individuals). Because only 68 of these individuals had class II/III obesity, we extended this group recruiting 85 additional class II/III obese patients from the obesity clinic of the Hospital Rubén Leñero in Mexico City.

Anthropometric and biochemical parameters

Anthropometric measurements, including height, weight, waist and hip circumference, were collected following the procedures recommended by Lohman *et al.*[12] Body fat percentage was measured by electrical bioimpedance (BIA 101 RJL Systems, Clinton Township, MI, USA). BMI was calculated as weight in kilograms divided by the square of height in meters. Obesity status was defined according to the World Health Organization criteria:[13] class I obesity as BMI $\geqslant 30$ and $<35\,kg\,m^{-2}$; class II as BMI $\geqslant 35$ and $<40\,kg\,m^{-2}$; and class III as BMI $\geqslant 40\,kg\,m^{-2}$. Normal weight was defined as BMI >18.5 and $<25\,kg\,m^{-2}$. Blood samples were collected from participants after an overnight fast, and lipoproteins (total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, glucose and insulin) were measured as previously described.[14]

Genotyping

DNA was extracted from blood using a commercial kit based on the salt fractionation method (QIAmp 96 DNA Blood Kit, Quiagen, Hilden, Germany) according to the manufacturer's instructions. Genome-wide genotyping was performed in 172 Mexican family members using the Infinium Human Linkage-12 Genotyping Bead-Chip (Illumina, San Diego, CA, USA), which includes 6090 SNPs. Two duplicate samples were included for quality control, showing 99% concordance of genotypes.

Rs614080 and rs1695 (Ile105Val) SNPs were genotyped using TaqMan probes (Applied Biosystems, Foster City, CA, USA) both in a case–control study and a population-based cohort utilizing a LightCycler 480 instrument II (Roche, Rotkreuz, Switzerland). Call rate exceeded 99%, with no discordant genotypes in 10% of duplicate samples. Finally, a set of 95 ancestry informative markers distributed across the genome was genotyped using a GoldenGate BeadArray (Illumina). The ancestry informative markers discriminated ancestry mainly between three continental populations (American, European and African).[15]

Quantitative trait loci expression

Thirty-two human subcutaneous adipose tissue biopsies from obesity class II/III patients were obtained during bariatric surgery. Gene expression was analyzed using Affymetrix Human Gene 2.0 ST array (Affymetrix, Santa Clara, CA, USA). Briefly, total RNA was purified in a fraction of each adipose tissue biopsy using RNeasy Lipid Tissue Mini Kit (Qiagen GmbH, Hilden, Germany). Procedures for hybridization to the microarrays were performed according to the Affymetrix protocol.

mRNA expression levels of *GSTP1* gene were validated in 83 additional subcutaneous adipose tissue biopsies by quantitative PCR using the LNA TaqMan hydrolysis probe #56 from the Universal Probe Library (Roche), in combination with the following primers (forward 5′-TCTCCCTC ATCTACACCAACTATG-3′ and reverse 3′-AGGTCTTGCCTCCCTGGT-5′), in a LightCycler 480 II instrument (Roche). Gene expression values were normalized to the value of the housekeeping gene β-actin (*ACTB*) using the following primers (forward 5′-CCAACCGCGAGAAGATGA-3′ and reverse 3′-CCAGAGGCGTACAGGGATAG-5′), probe #64. The relative expression level was determined on the basis of the comparative $2^{-\Delta\Delta CT}$ method and presented in arbitrary units (a.u.) as mean mRNA levels.

Statistic analyses

Heritability. The heritability of BMI and obesity-related traits, including height, weight, waist circumference, hip circumference and body fat percentage, were estimated using the maximum-likelihood variance decomposition method assuming a polygenic model implemented in Sequential Oligogenic Linkage Analysis Routines (SOLAR).[16] Age, sex and BMI were included as covariates when was adequate. Heritability was estimated as the proportion of the total phenotypic variability attributable to additive genetic effects.

Linkage and association analyses. A total of 6009 (98.7%) SNPs met quality control criteria, which included minor allele frequency >5%, call rate >99% and no deviation from Hardy–Weinberg equilibrium ($P>1.0\times10^{-6}$). Mendelian and gender inconsistencies were evaluated using PLINK; no discordant data were observed. Relatedness of individuals was verified based on Identity by descent estimation. Two-point and multipoint genome-wide linkage analyses were performed for BMI, weight, body fat percentage, waist and hip circumference. Body fat percentage, waist and hip circumference were log-transformed for the analysis. A total of 10 000 permutation simulations were used to calculate the empirical *P*-value of logarithm of odds (LOD) scores for all traits using the SOLAR program. Linkage was considered as 'suggestive' when the LOD score was $\geqslant 1.5$.

The association analyses of regions with suggestive linkage were performed using the family-based association test (FBAT) v2.0.3 software package.[17,18] The FBAT Min-p correction for multiple testing was performed on the 25 SNPs spanning the 11q13 region.[19]

Replication in population-based study. Association analyses of rs614080 with BMI and other obesity-related traits were performed by linear regression under an additive model adjusting by age, sex and ancestry. Differences among continuous variables between groups were tested using the *U* Mann–Whitney test. Association with obesity was tested with a logistic regression analysis under an additive model adjusted for the same covariates using SPSS statistical software v20.0 (SPSS Inc., Chicago, IL, USA). Population admixture proportions were determined with a Bayesian clustering algorithm implemented in the STRUCTURE program, considering genotypes from three parental populations (Native American, European and African).[20,21]

Transcript units were logarithmically transformed for the statistical analysis. Multiple linear regressions were used to test the associations between mRNA expression and SNP rs614080 under an additive model. These associations were adjusted for multiple testing using Bonferroni correction.

RESULTS

Linkage and association analysis

The clinical characteristics of all members of the 16 families are detailed in Supplementary Table 1. The overall prevalence of obesity in these families was 54.6%. Supplementary Table 2 shows that 5/6 quantitative obesity-related traits exhibited statistically significant heritability ($P\leqslant0.05$). The heritability estimate for BMI was 40% ($P=0.001$).

Multipoint linkage analysis for BMI identified only two chromosomal regions with a LOD score >1.5; one at 11q13 (maximum LOD score = 1.7) and the other at 13q22 (maximum LOD score 1.9; Figure 1). Of note, we observed that the linkage peak for BMI in the 11q13 region overlapped with the only suggestive linkage peak observed for weight (LOD score = 1.50; Supplementary Figures 1 and 2).

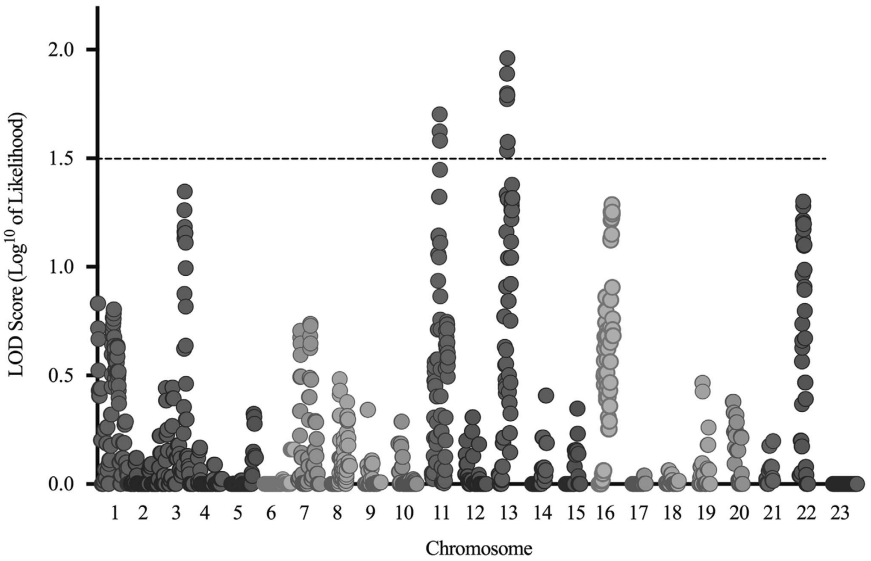

Figure 1 Multipoint logarithm of odds (LOD) scores from the genome-wide linkage analyses for body mass index in 16 multigenerational families. Two linkage regions on 11q13.3 and 13q22 reached the maximum estimated LOD score $\geqslant 1.5$ indicated by the dotted line. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

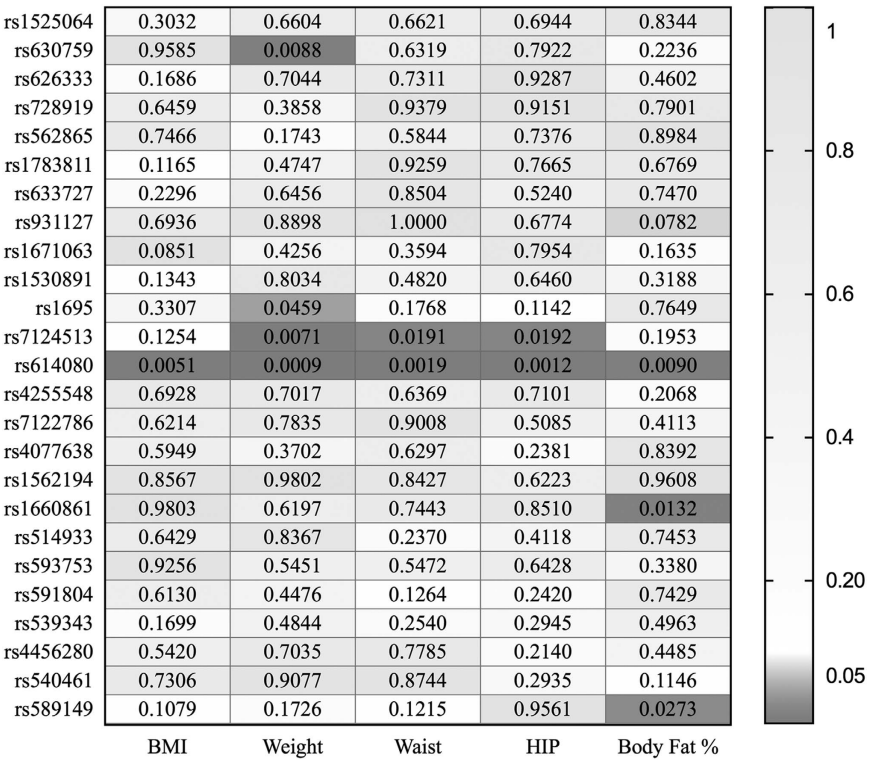

Figure 2 Heat map of *P*-values for associations of 11q13 SNPs with obesity-related traits in Mexican families. All traits were adjusted for sex and age. Significant uncorrected *P*-values are shaded. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

SNPs contained in both linked regions (25 SNPs for11q13 and 20 SNPs for 13q22) were tested for association analyses in families with obesity-related traits. Only one SNP in the 11q13 region (rs614080) was significantly associated with BMI, weight, waist and hip circumference, and body fat percentage (uncorrected $P \leqslant 0.01$; Figure 2). Permutation testing using FBAT Min-p showed that all associations, except body fat percentage, remained significant after correction for multiple testing ($P < 0.05$). We subsequently reanalyzed our data by performing linkage analysis conditional on BMI association, and determined that the rs614080 SNP explained about 29% of BMI linkage evidence in the 11q13 region. Therefore, additional variants in this region also could influence BMI.

Replication of the association of rs614080 with BMI in an independent cohort

Consistent with the family-based associations, SNP rs614080 was significantly associated with increased BMI and weight in an independent cohort of 1499 Mexican adults ($P<0.05$ adjusted for age, sex, and Native American ancestry; Table 1). Moreover, rs614080 was significantly associated with a higher risk of class II/III obesity in Mexican adults ($P=0.023$; Table 2). Because the number of individuals with class II/III obesity was small ($n=68$), 85 more class II/III obesity individuals were included in the analysis, confirming the association with class II/III obesity (odds ratio = 1.723; 95% confidence interval: 1.262–2.351; $P=0.001$). This SNP was not significantly associated with biochemical parameters (Supplementary Table 3).

Although rs1695 SNP has been previously associated with obesity,[22] this SNP was not significantly associated with obesity or related traits in the family analyses (Figure 2) or in the independent cohort (Supplementary Tables 4 and 5).

Expression quantitative trait loci analyses

Rs614080 is located in an intergenic region. In order to identify the gene or genes responsible for its association with BMI, we compared the quantitative expression of all 19 genes located within the 11q13 linkage region in 32 subcutaneous adipose tissue samples of morbidly obese individuals with different rs614080 genotypes. Multiple lineal regression analyses demonstrated a significant association of the rs614080 'A' allele with *RPS6KB* and *GSTP1* mRNA levels ($P\leqslant0.05$). However, after correction for multiple testing, only the association with *GSTP1* mRNA levels remained significant ($P=1.9\times10^{-05}$; Figure 3). To confirm the expression quantitative trait loci analyses results, we compared *GSTP1* mRNA according to genotype in 83 subcutaneous adipose tissue samples obtained from obesity class III individuals. *GSTP1* mRNA levels were significantly higher in individuals with AA genotypes as compared with those with GA and GG genotypes ($P=0.013$ and 0.001, respectively; Figure 4).

Table 1 Association of SNP rs614080 with obesity-related traits in Mexican adults ($n=1499$)

	Effect (s.e.)	P-*value*[a]
Anthropometric traits		
BMI (kg m^{-2})	0.409 (0.183)	**0.026**
Weight (kg)	1.112 (0.529)	**0.036**
Height (cm)	0.000 (0.003)	0.916
Waist circumference (cm)	0.814 (0.465)	0.080
Hip circumference (cm)	0.612 (0.439)	0.163

Abbreviations: BMI, body mass index; SNP, single-nucleotide polymorphism.
Effect values are presented as effect size per risk allele copy (Padd). *P*-values were calculated by linear regression analysis.
[a]*P*-values were adjusted for age, sex and admixture.
Bold text indicates statistical significance with a *P*-value $\leqslant0.05$.

DISCUSSION

Although obesity heritability has been estimated as high as 70%,[5,6] common variants explain only a very small proportion of the heritability of this trait.[23] In this study, the heritability estimate for BMI in our Mexican families was 40%, comparable with the 36–70% range reported in families from different ethnic groups.[24]

Importantly, we identified suggestive linkage on chromosomes 11q13 and 13q22 for BMI. These loci overlap with linkage regions previously identified in a genome-wide linkage meta-analysis for BMI and obesity.[25] We thus sought genetic associations for SNPs within both these loci. Interestingly, only rs614080 within the 11q13 locus showed significant association with BMI. This SNP also showed suggestive linkage and a significant association with weight in the Mexican families. Furthermore, the association of rs614080 with BMI and class II/III obesity was replicated in an independent cohort of Mexican Mestizo adults.

There are no evident functional candidate genes for obesity or related phenotypes in the 11q13 chromosomal region. However, using expression quantitative trait loci analyses of human subcutaneous adipose samples, we identified *GSTP1* as a putative functional gene in this locus. It is noteworthy that *GSTP1* has not been previously associated with obesity in genome-wide association studies.[23,26] Because the vast majority of genome-wide association studies have been performed in populations of European origin, this finding may be related with ethnicity. In this regard, there is no experimental evidence supporting a functional effect for rs614080. However, in the Mexican population, this SNP was found to be in linkage disequilibrium ($D'\geqslant0.99$) with two other *GSTP1* promoter and 5′-untranslated region SNPs predicted to be functional (rs8191438 and rs8191439). Interestingly, the latter SNPs have much higher minor allele frequencies in Mexicans than in other ethnic groups (0.34 in Mexicans vs 0.017 in Europeans), and explained ~41% of variation *GSTP1* mRNA expression in Mexican-Americans.[27] Therefore, these SNPs in linkage disequilibrium with rs614080 could be responsible for the association with *GSTP1* expression levels and obesity found in the Mexican population. Further studies analyzing rs8191438 and rs8191439 in populations with Native American ancestry are necessary to confirm this finding.

GSTP1 belongs to the glutathione *S*-transferase superfamily, a major group of enzymes responsible for the detoxification of a wide range of xenobiotics, specifically catalyzing the nucleophilic attack of reduced glutathione (GSH) on electrophilic compounds, protecting the cells to free radical-mediated damage.[28] The glutathione *S*-transferase pathway is known to regulate GSH homeostasis. GSH levels and glutathione

Table 2 Association of rs614080 with obesity

	Genotype, n *(%)*				*Additive model*	
	n	*GG*	*GA*	*AA*	*A-allele frequency (%)*	*OR (95% CI)*	P-*value*
Lean	301	142 (47.2)	130 (43.2)	29 (9.6)	31.22		
Obese	276	127 (46.0)	121 (43.8)	28 (10.1)	32.06	1.129 (0.865–1.474)	0.371
Class II/III obese	68	27 (39.7)	29 (42.6)	12 (17.6)	38.97	1.613 (1.068–2.436)	**0.023**

Abbreviations: CI, confidence interval; OR, odds ratio.
P and OR values were calculated by logistic regression analysis using additive models. *P*-values were adjusted for age, sex and admixture.
Bold text indicates statistical significance with a *P*-value $\leqslant0.05$.

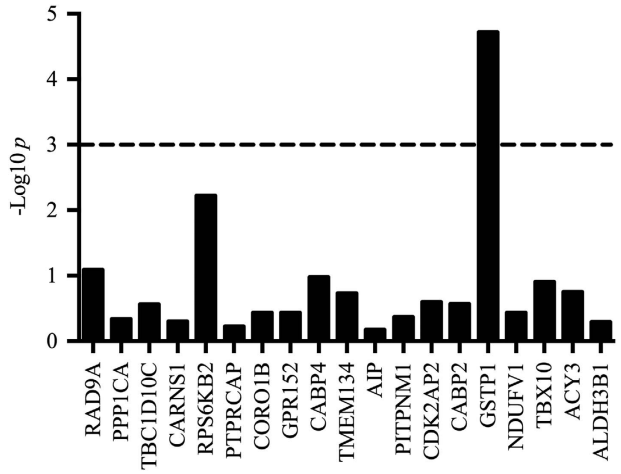

Figure 3 Expression quantitative trait loci analysis of 19 genes within the 11q13 locus according to rs614080 genotypes in 32 subcutaneous adipose tissue biopsies. Bars indicate the *P*-values and the dotted line indicates the threshold for statistical significance after correction for multiple testing ($P=0.0026$).

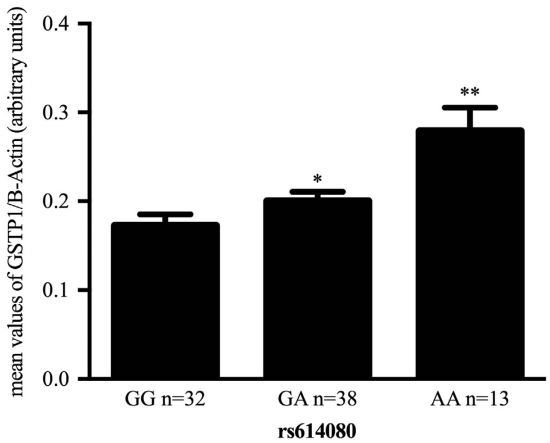

Figure 4 Expression quantitative trait loci analysis of the *GSTP1* gene stratified by rs614080 genotype in 83 subcutaneous adipose tissue biopsies using real-time PCR. **P*-value $\leqslant 0.05$ compared GA vs AA genotype. ***P*-value $\leqslant 0.005$ compared GG vs AA genotype.

systems are involved in the regulation of energy metabolism, inflammation, insulin resistance and obesity; and pharmacologic depletion of GSH prevents diet-induced obesity, increases energy expenditure and locomotor activity, and enhances insulin sensitivity in animal models.[29,30] Moreover, the *GSTP1* gene has been associated with diabetes and cardiovascular risk factors in humans,[31,32] although there is only one previous study reporting an association of *GSTP1* (Ile105Val) with obesity in the Egyptian population.[22] We provide further evidence that *GSTP1* genetic variation is associated with obesity, although rs1695 was not significantly associated with obesity or related traits in this study. Undoubtedly, the potential role of *GSTP1* gene in the pathophysiology of human obesity and related traits requires further research.

Certain limitations of the study should be addressed. First, the number of families was small, and scores were only suggestive of linkage. The linkage signal on chromosome 11q13 was not completely explained by rs614080 (*GSTP1* gene), suggesting that other genetic variants in this region may also influence BMI. Moreover, because GSH concentrations were not measured, we can only hypothesize that the rs614089 'A' allele and its association with higher *GSTP1* gene expression leads to higher GSH concentrations in obese subjects. However, it is important to point out the strengths of the study: in contrast with previous studies reporting loci linked or associated with BMI and obesity, the Mexican families included in this study were selected specifically for the obesity trait, and not for obesity-related metabolic traits.[24,33] In addition, the findings were replicated in an independent cohort, suggesting that rs614080 is associated with obesity in the Mexican population.

In conclusion, the approach of combining linkage and association strategies allowed us to identify rs614080 SNP in 11q13 region near *GSTP1* gene was associated with higher BMI in the Mexican population. This SNP was also associated with *GSTP1* expression levels in subcutaneous adipose tissue. Additional studies are needed to clarify the role of *GSTP1* in human obesity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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