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Normal Variation in Leptin Levels Is Associated with Polymorphisms in the Proopiomelanocortin Gene, *POMC**

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ABSTRACT

We previously reported that our genome-scanning initiative had detected a highly significant linkage (log odds ratio = 4.95; $P = 9 \times 10^{-7}$) between a quantitative trait locus (QTL) on chromosome 2 and leptin levels in Mexican American families. We now have typed additional microsatellite markers in this region, increasing this log odds ratio score to 7.46 ($P = 2 \times 10^{-9}$). This region of chromosome 2 contains a strong positional candidate gene, *POMC*. The *POMC* gene codes for POMC, the prohormone from which αMSH, ACTH, and β-endorphin are derived. Studies by others have shown that *POMC*-derived products are involved in the regulation of appetite and obesity. We have used polymorphisms in *POMC* to map its location within the 95% confidence interval of the peak for the linkage signal for the QTL. We also constructed *POMC* haplotypes using these polymorphisms and have found a significant association with normal variation in leptin levels ($P = 0.001$). We conclude that variation in *POMC* is associated with normal variation in serum leptin levels, providing further evidence that *POMC* may be the leptin QTL previously identified in Mexican American families. (*J Clin Endocrinol Metab* **84:** 3187–3191, 1999)

THE CIRCULATING protein leptin is produced by adipocytes and appears to satisfy the requirements of an afferent signal in a negative feedback loop regulating fat mass (1). Previous work by ourselves and others has shown that variation in leptin levels has a strong genetic component, with estimates of genetic heritability ranging from 0.40–0.60 (2, 3). Further evidence for the influence of genes on the expression of leptin is provided by our identification of a quantitative trait locus (QTL) on chromosome 2 [log odds ratio (LOD) = 4.95; $P = 9 \times 10^{-7}$] in the San Antonio Family Heart Study (SAFHS) (2). This QTL has the highest LOD score yet published from a genome scan for quantitative trait in humans and has been replicated in two other studies, one in African Americans (4) and one in a French population (5).

This region of chromosome 2 containing the major leptin QTL also contains a strong positional candidate gene for human obesity, *POMC* [previously mapped to 2p23 by *in situ* hybridization (6, 7)], which codes for the prohormone POMC. POMC is the precursor for several peptide hormones that are produced by posttranslational processing, some of which are involved in energy homeostasis, including αMSH, ACTH, and β-endorphin (8). POMC is highly expressed in neuronal cells of the arcuate nucleus, a region of the hypothalamus that is involved in the regulation of energy homeostasis (8).

In this report, we present further studies of the major QTL for leptin levels in SAFHS families, including placement of additional microsatellite markers in this region of chromosome 2. We also examined genetic variation in *POMC* to determine the relationship of this strong positional candidate gene to the major leptin QTL. We identified and typed polymorphisms to more precisely map *POMC* relative to microsatellite markers and the major leptin QTL. In addition, we used the *POMC* polymorphisms for statistical analysis to directly test for association with leptin levels in the SAFHS families.

Materials and Methods

The population

Subjects in this study included 337 Mexican Americans (153 males and 184 females) distributed in 10 families ranging in size from 35–71 individuals. These families are all participants in the San Antonio Family Heart Study, a broader project designed to investigate the genetics of risk factors for atherosclerosis, noninsulin-dependent diabetes mellitus, and obesity (9, 10). Probands and family members for this analysis were ascertained without regard to obesity or any other preexisting medical conditions. However, on the basis of the most recent criteria (11), 72% of these SAFHS participants would be classified as overweight (body mass index, >25), and 38% would be classified as obese (body mass index, ≥30). Age in this sample ranges from 18–92 yr, with an average age of 39 yr. Although Mexican Americans constitute an admixed population, the degree of admixture and interindividual variation in admixture are predominantly functions of socio-economic status, with low income individuals exhibiting higher levels of Amerind admixture (12). We limited the study population to low income families to minimize genetic heterogeneity due to differential admixture. All protocols have

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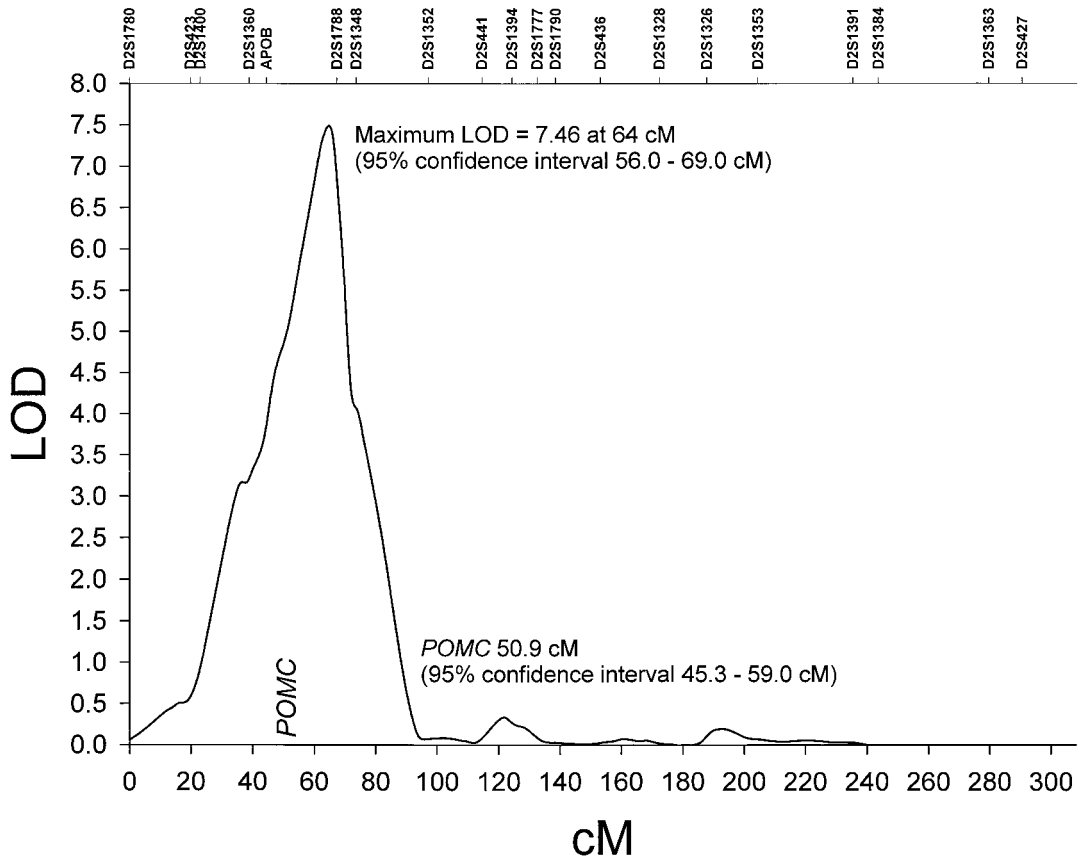

FIG. 1. Estimated LOD function obtained from multipoint QTL analysis of serum leptin levels and showing the map placement of *POMC* to 50.9 cM (95% confidence interval, 45.3–59.0 cM), within the 95% confidence interval for the maximum LOD score of 7.46 for the QTL (56.0–69.0 cM).

been approved by the institutional review board at the University of Texas Health Science Center (San Antonio, TX).

The phenotype

We measured leptin levels by RIA in serum samples collected after an overnight (~12-h) fast. The assay was conducted using a commercially available RIA kit (Linco Research, Inc., St. Louis, MO) (13). The interassay coefficient of variation for this assay was 7.4%, a value within the range reported by the manufacturer (13).

Genotyping

SAFHS family members were typed for 6 additional microsatellite markers (*D2S423*, *D2S1400*, *D2S1352*, *D2S1394*, *D2S1777*, and *D2S1790*) to the 15 previously typed as part of our 20-centrimorgan (cM) genome scan set located in the region of chromosome 2 that contains our peak linkage signal for leptin levels (2). We used lymphocyte DNA samples for PCR with fluorescently labeled primers from the MapPairs Linkage Screening Sets 6 and 8 (Research Genetics, Inc.).

We identified and typed *POMC* polymorphisms in lymphocyte DNA samples from SAFHS family members. A known polymorphic *Rsa*I site in the 5′-flanking region was typed using Southern blots with a *POMC* complementary DNA probe (American Type Culture Collection, Manassas, VA; clone plP2) according to the method of Feder and colleagues (14). We used a PCR-based method to type a cryptic trinucleotide repeat polymorphism in exon 3 as previously described (15). We found two new polymorphisms by direct sequencing of exon 3 sequences from 20 SAFHS participants [C→T at position 7284, C→T at position 7566; numbered according to Takahashi and colleagues (16)]. For association studies, we simultaneously typed both polymorphisms in the SAFHS samples using direct nucleotide sequencing. Exon 3 sequences were amplified from lymphocyte DNA samples using PCR with a forward primer (5′-AGCCCCGCAGCGATGGT-3′) and a reverse primer (5′-TCCATGCTGCTGTTATTTGACGGC-TACG-3′). PCR conditions included denaturation at 97 C for 5 min, followed by 30 cycles of annealing at 63 C for 40 s, elongation at 74 C for 40 s, denaturation at 97 C for 40 s, and a final elongation step at 74 C for 5 min. The PCR products were directly sequenced on an automated DNA Sequencer (ABI model 377) using a reverse primer (5′-CTGCTGTTATTTGACGGCTACG-3′) with the ABI Prism Cycle Sequencing kit (Perkin Elmer Corp., Foster City, CA). The C→T polymorphism at position 7566 can also be typed by treatment with the restriction enzyme *Ear*I.

Statistical analysis

We performed variance component linkage analysis as described by Almasy and Blangero (17) and implemented in the program package SOLAR. Map distances between markers were estimated using CRI-MAP (18). We used a measured genotype analysis (19, 20) that has been implemented in the program package SOLAR (17) to look for evidence of association between serum leptin levels and polymorphisms in *POMC*. Specifically, we compared a model in which the genotype-specific means were forced to be equal across the *POMC* genotypes (*e.g.* $\mu_{AA} = \mu_{Aa} = \mu_{aa}$) to a model in which each of these parameters was estimated separately. We also adjusted for the effects of age and sex on leptin levels by simultaneously estimating their effects in each of the models evaluated in the association analysis. The comparison of the competing models was performed by means of the likelihood ratio test that yields a test statistic asymptotically distributed as a χ^2 with degrees of freedom equal to the difference in the number of independent parameters being estimated in the two models.

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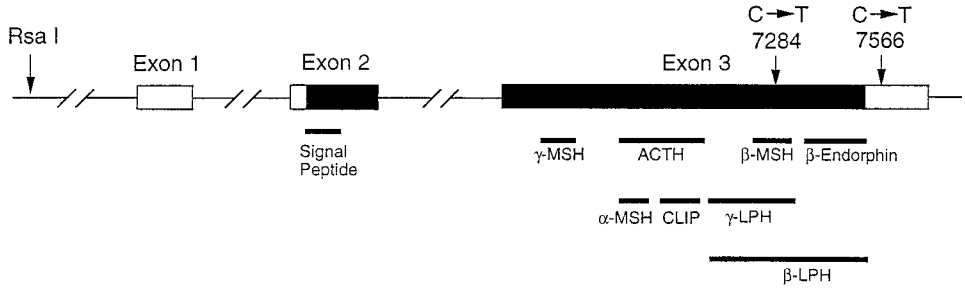

FIG. 2. Location of polymorphisms in *POMC*. This schematic representation of *POMC* shows the exons by *boxes* (*open boxes* are untranslated regions, *closed boxes* are coding sequences). The polypeptide products of *POMC* are shown *below the map* according to Takahashi *et al.* [γMSH, αMSH, ACTH, corticotropin-like intermediate lobe peptide (CLIP), γ-lipotropin (γ-LPH), and α-lipotropin (α-LPH)]. *Vertical arrows above the map* show the positions of POMC polymorphisms.

Results

Further mapping of the major QTL for leptin levels on chromosome 2

To further refine the localization of the leptin QTL (2), we typed family members for six additional microsatellite markers located in this region of chromosome 2 and performed multipoint QTL analysis of leptin levels in the extended families. Figure 1 shows the multipoint linkage curve for serum leptin levels using the additional microsatellite markers. The peak LOD score increased to 7.46 ($P = 2 \times 10^{-9}$) at 64 cM (95% confidence interval, 56.0–69.0 cM), which is substantially higher than our previously reported peak LOD score of 4.95 (2).

Genetic mapping of POMC to the region of chromosome 2 containing the maximum LOD for the leptin QTL

We also investigated *POMC* as a positional candidate gene that may be responsible for the major QTL for leptin levels that was originally identified using random markers. We identified and typed polymorphisms in several regions of *POMC* in the SAFHS families. Figure 2 shows a schematic of *POMC* and the location of the polymorphisms. We typed a known polymorphic *Rsa*I site in the 5′-flanking region (14). The frequency of the R1 allele (absence of the site) was 0.16, and the frequency of the R2 allele (presence of the site) was 0.84. We also examined a known cryptic trinucleotide repeat polymorphism in exon 3, but a preliminary survey showed only limited variability in the SAFHS population (15). In addition to these known polymorphisms, we searched for new polymorphisms by direct sequencing of exon 3 in 20 SAFHS participants. Exon 3 contains the coding sequences for the various polypeptide products of *POMC*. We found two nucleotide substitutions, but only a C→T polymorphism at position 7566 (7566C/T) in the 3′-untranslated region was sufficiently common for further typing in the SAFHS population. The frequency of the C allele was 0.89, and the frequency of the T allele was 0.11.

Although previous cytogenetic studies had placed *POMC* in this region of chromosome 2, the location of *POMC* relative to other genetic markers was not known. Therefore, we used the *POMC* polymorphisms for marker to marker linkage analysis to more precisely map *POMC* relative to the microsatellite markers on chromosome 2. Figure 1 shows the location of *POMC* relative to the markers and the multipoint linkage curve for leptin levels. *POMC* mapped to 50.9 cM from the p-ter of chromosome 2, with a 95% confidence interval from 45.3–59.0 cM. The 95% confidence interval for the map placement of *POMC* overlaps with the 95% confidence interval for the leptin QTL, indicating that *POMC* is located within the region of chromosome 2 that shows the peak linkage signal for serum leptin levels (Fig. 1).

TABLE 1. Frequency of *POMC* haplotypes and their mean serum levels of leptin (nanograms per mL)

POMC haplotype[a]	Frequency	Mean leptin level (ng/mL)
R1-C/R1-C	0.02	25.6 ± 2.1
R1-C/R2-C	0.23	29.3 ± 2.1
R1-C/R2-T	0.05	30.2 ± 1.8
R2-C/R2-C	0.54	29.9 ± 4.8
R2-C/R2-T	0.15	34.1 ± 2.5
R2-T/R2-T	0.01	45.0 ± 5.0

[a] R1 and R2 refer to the absence (R1) or the presence (R2) of the polymorphic *Rsa*1 site in the 5′-flanking region. C and T refer to the C→T polymorphism at position 7566 in exon 3.

POMC haplotypes are significantly associated with serum leptin levels

We constructed *POMC* haplotypes using the *Rsa*I and 7566C/T polymorphisms and tested for association with leptin levels in the SAFHS participants. The haplotypes and their relative frequencies in the study group are given in Table 1. The *POMC* haplotypes were used to test for association with serum leptin levels using maximum likelihood-based statistical analysis. This analysis also included adjustment for significant covariates that affect leptin levels, including sex and age. The results showed that *POMC* haplotypes were associated with significantly different serum levels of leptin ($P = 0.001$; Table 1). The homozygotes for the R1-C haplotype had the lowest leptin level (25.6 ± 2.1 ng/mL). The homozygotes for the R2-T haplotype had the highest leptin level (45.0 ± 5.0 ng/mL), almost 2-fold higher than that in the R1-C homozygotes. In general, the intermediate haplotypes followed the same pattern, so that the R1 and C polymorphisms were associated with lower leptin levels, and the R2 and T polymorphisms were associated with higher leptin levels.

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Discussion

In this report, we have extended our studies of a major QTL for leptin levels on chromosome 2 that was previously identified in a genome scan of Mexican American families from SAFHS (2). Confidence in a putative QTL is increased by the consistency of results when new microsatellite markers are added to the region. Our results show that addition of six new microsatellite markers did not weaken support for the leptin QTL, but actually increased the LOD score (4.95 to 7.46) and further refined the position of this QTL. This QTL probably represents a common variant affecting serum leptin levels rather than a private polymorphism limited to only a few families, as this QTL has been replicated in two other large family studies (4, 5).

We investigated the role of a positional candidate, *POMC*, which had previously been mapped to this region of chromosome 2 by *in situ* hybridization (7). By discovering new variants in *POMC* and typing known polymorphisms, we were able to apply marker to marker linkage analysis to genetically map *POMC* to the same region of chromosome 2 containing the maximum LOD of the leptin QTL. In general, genetic mapping is an important step in positional candidate gene analysis, because, like *POMC*, most such candidate genes have been mapped only by *in situ* hybridization, and because genetic map position may show natural variation due to different proportions of men and women among study populations. These mapping results provide further support for *POMC* as a positional candidate gene for the major leptin QTL on chromosome 2.

We also used the *POMC* polymorphisms to construct haplotypes for statistical analysis to test for direct associations with serum leptin levels in Mexican Americans. We found a significant association of *POMC* haplotypes with leptin levels ($P = 0.001$), with almost 2-fold differences between the most disparate homozygotes (R1-C/R1-C *vs.* R2-T/R2-T). It should be noted that this association study differs from those reported in previous candidate gene studies, because our first criterion for selection of *POMC* was its proximity to the leptin QTL on chromosome 2 (*i.e. POMC* is a positional candidate gene). Previous association studies have typically examined candidate genes based largely on *a priori* assumptions concerning their involvement in relevant physiological processes, and the results were often not replicated in different study populations. One reason for this inconsistency may be the reliance of association studies on linkage disequilibrium between candidate gene markers and a causative mutation that differs according to population histories (a genetic effect). Spurious associations may also arise as a statistical artifact caused by hidden stratification within study populations (a nongenetic effect). Our initial identification of the leptin QTL using linkage analysis that relies only on genetic effects indicates that this association of *POMC* with leptin levels is due to linkage disequilibrium rather than simply hidden stratification within the population.

In addition to its map position, *POMC* is a strong candidate gene for the leptin QTL because of the central role its multiple hormone products play in energy homeostasis. Recent studies by Krude and colleagues (21) have shown that rare mutations in *POMC* that abolish normal translation and processing of its hormone products cause a distinct form of early-onset human obesity. We do not yet know how normal genetic variation in *POMC* influences leptin levels, but numerous physiological studies may provide some clues. For example, ACTH, a product of *POMC*, acts on the adrenal cortex to stimulate the production of glucocorticoids. Previous studies have suggested a relationship of glucocorticoid levels with obesity (22, 23), and molecular studies have shown that glucocorticoids may directly regulate transcription of the *OB* gene that encodes leptin (24, 25). An alternative hypothesis comes from recent studies of the catabolic role of the hypothalamic melanocortin system, with αMSH strongly implicated in the control of food intake (26–32). αMSH is a high affinity ligand for both the melanocortin 3 and melanocortin 4 receptors, and deficiency of the melanocortin 4 receptor in mice leads to hyperphagia and obesity (27, 28, 33). Thus, this association of *POMC* haplotype with leptin levels might derive from variation in the control of food intake and body fat deposition via variations in melanocortin expression and activity (27, 32, 34–36).

As far as we know, neither of the polymorphisms used to construct the *POMC* haplotypes for the association analysis has any direct impact on the expression of *POMC* or the function its hormone products. The *Rsa*I substitution is located in the 5′-flanking region, but has not been localized with respect to known sequence elements that regulate *POMC* transcription. The 7566C/T substitution is located in the 3′-untranslated region and does not alter the amino acid sequence of *POMC*. It is more likely that these polymorphisms are not themselves responsible for the observed association with leptin levels, but are in linkage disequilibrium with another polymorphism that does affect *POMC* expression or function. Given that the chromosome 2 QTL and *POMC* haplotypes affect variation in levels of leptin, and changes in the *POMC*-coding sequences might affect multiple overlapping hormone products involved in other important physiological processes, the causative mutation seems more likely to occur in a regulatory region rather than in the coding sequence. We are now searching for such variants in the *POMC* regulatory elements in SAFHS subjects that could be responsible for this association with normal differences in serum leptin levels.

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