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Genetic Markers at the Leptin (*OB*) Locus Are Not Significantly Linked to Hypertension in African Americans

Kelly L. Onions, Steven C. Hunt, Mark P. Rutkowski, Charles A. Klanke, Yan Ru Su, Max Reif, Anil G. Menon

Abstract—Increased body mass index (BMI) has been correlated with increased blood pressure in human populations. To examine the role of the leptin gene (*OB*) in essential hypertension in African Americans, we performed affected sib pair analysis on a set of 103 hypertensive African American sibships using four highly polymorphic markers at the human leptin locus. No evidence of linkage was detected between these markers and the phenotype of essential hypertension either in these sibships or in a severely obese subset of 46 sibships in which each sibling had a BMI \geq 85th percentile for the US population. Using BMI rather than hypertension as a quantitative trait, we found significant linkage for the marker D7S504 ($P=0.029$) but not for the other markers. Significance strengthened in the overweight subset of sibships for this marker ($P=0.001$), and there was a trend of lower P values for the other three markers. However, multipoint analysis with the use of all four markers simultaneously to estimate linkage between BMI and the leptin locus did not demonstrate a statistically significant relationship. Analysis of the coding region of the leptin gene (exons 2 and 3) by single-strand conformational polymorphism revealed a rare Ile-Val polymorphism at amino acid 45 but revealed no other alterations. These results suggest that the *OB* gene is not a major contributor to the phenotype of essential hypertension in African Americans, although a minor contribution to the phenotype of extreme obesity in this group cannot be ruled out. (*Hypertension*. 1998;31:1230-1234.)

Key Words: obesity ■ hypertension, essential ■ leptin ■ body mass index

Essential hypertension and obesity both result from multiple environmental and genetic determinants. These disorders are closely linked in epidemiological studies,¹ with high BMI strongly correlated with increased blood pressure^{2,3} and lean individuals with elevated blood pressure showing a predisposition to becoming obese.³ While environmental determinants certainly account for some of this epidemiological linkage, shared genetic determinants may also contribute to the association between hypertension and obesity. For example, monozygotic twins have a higher concordance rate for the joint occurrence of hypertension and obesity (31%) than dizygotic twins (15%), and multivariate genetic modeling using this twin data suggests a common latent factor that is, in part, likely to be genetically determined.⁴

The cloning of the *ob* gene in 1994 was an important advance in the study of obesity.⁵ Indeed, *ob/ob* mice are severely obese due to mutations in the *ob* gene that result in the lack of secretion of functional leptin protein, the product of the *ob* gene.⁵ In mice, this obese phenotype can be corrected by administration of recombinant leptin protein.^{6,7} Leptin is a 16-kD protein expressed predominantly in adipose tissue and is thought to act as a satiety signal in a feedback mechanism involving a target receptor in the hypothalamus.⁵

The end result of this feedback loop is the regulation of body fat stores. Unlike the *ob/ob* mice, a deficiency of circulating leptin has not been identified in obese humans and, in fact, *OB* mRNA levels from adipose tissue as well as serum leptin levels are elevated.⁸⁻¹¹ These findings suggested that it was possible for obesity in humans to be caused by mutations in the *OB* gene if altered expression or functionally defective leptin protein resulted from such mutations.¹²⁻¹⁴ Interest in identifying the loci that contribute significantly to human obesity and hypertension is based on the significant impact of such findings on the design of pharmacological intervention strategies for both hypertension and obesity.

Because there is a higher prevalence of both hypertension² and obesity¹⁵ in African Americans, they represent an excellent population in which to identify potential genetic factors that contribute to both traits. In this study we report the results of sib pair linkage analysis and mutational analysis of the coding portion of the leptin gene in a population of hypertensive African Americans. We report the extent of allele sharing at highly polymorphic loci within and around the *OB* gene in African American sib pairs, using either the phenotype of essential hypertension or the phenotype of obesity as measured by BMI and the absence of significant nucleotide

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Selected Abbreviations and Acronyms

BMI = body mass index
MIM = multipoint IBD (identity by descent) method
PCR = polymerase chain reaction
SSCP = single-strand conformational polymorphism

sequence variation in the coding region of the leptin gene in the DNA of these individuals.

Methods

Subjects

The population base consisted of 103 hypertensive sibships (consisting of 24 half sibships and 79 full sibships) that came from 73 families and included 167 individuals. The number of sib pairs analyzed for each marker used in the analysis is shown in Table 2. Subjects were recruited from the Hypertension Clinics of the University of Cincinnati and the Veterans Administration Hospital, as well as from the community at large. The study was approved by the Institutional Review Board of the University of Cincinnati. All subjects underwent a brief clinical evaluation and provided a blood sample for plasma electrolytes and DNA isolation. Subjects were seated for 5 minutes, after which blood pressure readings were done in triplicate with the use of a mercury manometer with the appropriate cuff size based on the upper midarm circumference. Korotkoff phase V was used to define the diastolic pressure. Height was self-reported, but weight was measured with street clothing.

Treated hypertensive subjects were included if they reported the onset of hypertension before age 60 years and were undergoing continuous treatment with antihypertensive medication for the previous 6 months. In hypertensive subjects not on antihypertensive treatment, average diastolic blood pressure was confirmed to be >90 mm Hg on a second visit a week later. All index patients met the following criteria: no reported history of secondary hypertension, serum potassium level >3.5 mEq/L unless on thiazide or loop diuretics, serum creatinine <1.6 mg/dL for men and <1.4 mg/L for women, alcohol intake <1.5 oz of ethanol per day, and no use of steroids or estrogen before diagnosis of hypertension. In index subjects with non-insulin-dependent diabetes mellitus, the diagnosis of hypertension had to precede the diagnosis of diabetes mellitus by at least 5 years.

In this study two different traits were analyzed for excess allele sharing at the leptin locus. First, essential hypertension was analyzed as a qualitative trait with the use of the previously described inclusion criteria for hypertensive subjects because actual blood pressures could not be reliably used since many subjects were already on antihypertensive medications. Second, we analyzed BMI as a quantitative trait. A subset of sibships was analyzed separately that included only those sib pairs in which both sibs had a BMI \geq 85th percentile for the US population (27.8 kg/m² for men and 27.3 kg/m² for women).¹⁵ Although the terms obesity and overweight are often used interchangeably, obesity implies excess body fat, which is not always correlated with being overweight. It should be emphasized that in this study BMI rather than excess fat was measured.

Clinical characteristics of the study population are summarized in Table 1. The range of BMIs in the entire group was 18.1 to 56.8 kg/m², with the BMI <27 in 26 subjects, \geq 27 but <37 in 99 subjects, and \geq 37 in 42 subjects.

Analysis of Sib Pair Relationships

Establishment of full or half sibling relationships was based on questionnaires to the study subjects as well as PCR genotyping of DNA from these subjects with the use of 17 unlinked highly polymorphic markers. These markers were CD4, D16S408, D16S420, AGT, AE3, D7S504, D14S59, D6S282, D1S188, D14S81, D1S162, D10S179, LA4, SPN, RPN, NHE5, and D15S126. The identity-by-descent assumptions of full sibships sharing 50% of

TABLE 1. Clinical Characteristics of Hypertensive African American Siblings

	Men	Women
n	57	110
Age, y	50 \pm 11	50 \pm 11
Age Dx, y	39 \pm 12	36 \pm 11
BMI, kg/m ²	32 \pm 7	33 \pm 7
SBP, mm Hg	148 \pm 20	143 \pm 19
DBP, mm Hg	95 \pm 14	90 \pm 12
Rx, %	90	92
DM, %	12	25

Age Dx indicates age at diagnosis of hypertension; SBP, systolic blood pressure; DBP, diastolic blood pressure; Rx, subjects on antihypertensive medications; and DM, subjects with non-insulin-dependent diabetes mellitus. Values are mean \pm SD.

their genomes and half sibships sharing 25% of their genomes were transformed to identity-by-state expectations with the use of the Weeks and Lange equation.¹⁶ We determined 95% confidence intervals for each sibship using genotype information to calculate variance with (N-1) degrees of freedom, where N is the number of markers. The average observed number of alleles shared for these markers was calculated and compared with the 95% confidence intervals calculated for full and half sibships. Only sibships falling within the 95% confidence interval for a full sibling pair or a half sibling pair were included in the analysis.

Genotyping

Subjects were genotyped at four closely linked short tandem repeat polymorphisms: one in the 3' UTR of the *OB* gene (*OB*-tet),¹⁷ one telomeric to the *OB* locus (D7S1875), and two centromeric to the *OB* locus (D7S635 and D7S504).¹⁸ These markers are known to be within a 1-cM segment of chromosome 7 based on the high-density genetic map of this chromosome available through the Whitehead/Massachusetts Institute of Technology Center for Genome Research. Primers for *OB*-tet were designed on the basis of the published genomic sequence.¹⁹ Primers for markers D7S1875, D7S635, and D7S504 were obtained from Research Genetics Inc and end-labeled with ³²P with the use of standard protocols. PCR was performed in accordance with the manufacturers' suggested conditions in a PTC 100 thermal cycler from MJ Research Inc. PCR products were resolved on 6% denaturing polyacrylamide gels and autoradiographed with the use of Kodak XAR film, and alleles were sized relative to markers of known size.

SSCP Analysis

SSCP analysis was performed on exons 2 and 3, which represent the entire protein-coding region of the *OB* gene. Exon 3 is a large exon and was therefore divided between two sets of primers. Each 15- μ L SSCP PCR reaction contained 100 ng of human genomic DNA template, a primer concentration of 0.25 pmol/ μ L, and 0.1 μ L [α -³²P]dATP. PCR products were denatured and then resolved on nondenaturing MDE gels that were subsequently vacuum dried and autoradiographed. Abnormal conformers were cut out from gels, eluted in double-deionized water, and reamplified. Both strands were sequenced without subcloning with the use of an automated ABI 377 sequencer.

Statistical Analysis

The most recent update of the sib pair linkage program SIBPAL version 2.7 (1996) of the program package Statistical Analysis for Genetic Epidemiology was used to calculate linkage. SIBPAL performs a linear regression between the estimated proportion of alleles a sib pair shares identical by descent at a locus and the square of the sib pair trait difference. (For a qualitative trait, the trait

TABLE 2. Affected Sibling Pair Linkage Analysis Between Markers Near the Leptin Locus and Hypertension or BMI in African Americans

Marker	No. Full Sib Pairs	Mean*	No. Half Sib Pairs	Mean*	Hypertension		BMI	
					<i>t</i>	<i>P</i>	<i>t</i>	<i>P</i>
D7S1875	70	0.496	24	0.243	-0.21	1.00	-1.45	0.08
<i>OB</i> -tet	60	0.501	19	0.291	0.64	0.26	-0.63	0.27
D7S504	72	0.525	21	0.197	-0.50	1.00	-1.93	0.03
D7S635	69	0.510	21	0.242	0.11	0.46	-0.88	0.19

*Mean of the estimated proportion of marker alleles shared identical by descent. Under the null hypothesis of no linkage, the expected mean is 0.50 for full sib pairs and 0.25 for half sib pairs.

difference is 0 for sibs who share the same trait and 1 for sibs who do not share the same trait.) When parental data are missing, as in our study, this program uses the phenoset for all possible genotypes. Allele frequencies were calculated with the use of the genotype of one member from each family. The logarithm of BMI was used because of the positive skew in the distribution of BMIs to facilitate data handling. The inclusion of the covariates age and gender in the BMI analysis did not significantly change results (data not shown).

MIM Program

The multipoint IBD (identity by descent) method (MIM)^{20,21} was also used to estimate linkage between BMI as a quantitative trait and the *OB* locus. Assumed distances of 0.3 cM between D7S1875 and *OB*-tet, 0.3 cM between *OB*-tet and D7S504, and 0.3 cM between D7S504 and D7S635 were used in the analysis since all four markers are known to be within a 1-cM segment of chromosome 7 based on the high-density genetic map of this chromosome available through the Whitehead/Massachusetts Institute of Technology Center for Genome Research. Since this program only handles full siblings, the half siblings were not included in this analysis. The log of BMI was standardized to a mean of 0 and SD of 1. This method estimates the proportion of genetic variance for BMI that is attributable to the *OB* gene region encompassed by these four markers. This method also requires either an estimate of the proportion of total variance in BMI that is due to additive genes or the use of multiple estimates spanning a reasonable range of proportions. Most studies have estimated that the additive genetic variance for BMI ranges from 20% to 40%. Therefore, we tested for linkage assuming 10%, 20%, 30%, and 50% total genetic variance. The null hypothesis is that the proportion of additive genetic variance due to the marker loci is 0. Deviation from this hypothesis was tested with the use of a χ^2 test with 1 *df*. *P* values from this test are shown in Table 4.

Results

Affected Sib Pair Analysis

In this study two different traits, one qualitative and one quantitative, were analyzed for genetic linkage determined by excess allele sharing at the leptin locus. The first was hypertension as a qualitative trait; actual blood pressure could

not be used because most subjects were already on antihypertensive treatment. The second was obesity, measured by BMI, as a quantitative trait. Subjects were genotyped at four closely linked short tandem repeat polymorphisms: D7S1875, D7S635, D7S504, and *OB*-tet, which is actually part of the *OB* gene. These markers were chosen because of their highly polymorphic nature, with heterozygosity scores of 0.894, 0.854, 0.797, and 0.853, respectively. Sib pair linkage was first used to calculate linkage with hypertension. Table 2 shows results of the analysis with four different polymorphic markers at the leptin locus. Linkage was not detected between the phenotype of essential hypertension (considered a qualitative trait) and the leptin locus in African Americans. Marker D7S504 showed the greatest excess sharing of alleles for full sibs (0.525) but also showed less than expected sharing for the half sibs (0.197). *OB*-tet had nonsignificant excess sharing for the half sibs but no excess for the full sibs. Therefore, there was no consistent evidence for any trend toward linkage. The results did not change when the sib pairs being analyzed were limited to those whose BMI was ≥ 85 th percentile (Table 3). Therefore, our analysis suggests that the human leptin locus does not play a major role in the etiology of essential hypertension in African Americans, although it does not formally exclude a minor role for this locus in hypertension.

Using sibling pair linkage analysis with these same markers, we detected weak evidence for genetic linkage between BMI (considered a quantitative trait) and the leptin locus in this same group of African Americans with hypertension ($P=0.08$ for D7S1875, $P=0.27$ for *OB*-tet, $P=0.03$ for D7S504, and $P=0.19$ for D7S635) (Table 2). The evidence for this linkage was slightly stronger when the analysis was restricted to a subset of 46 sibships in which each sibling had a BMI ≥ 85 th percentile for the US population ($P=0.08$ for

TABLE 3. Affected Sibling Pair Linkage Analysis Between Markers Near the Leptin Locus and Hypertension or BMI in African Americans With BMI ≥ 85 th Percentile for the US Population

Marker	No. Full Sib Pairs	Mean*	No. Half Sib Pairs	Mean*	Hypertension		BMI	
					<i>t</i>	<i>P</i>	<i>t</i>	<i>P</i>
D7S1875	44	0.511	12	0.264	0.37	0.36	-1.41	0.08
<i>OB</i> -tet	42	0.460	10	0.327	0.14	0.45	-1.41	0.08
D7S504	47	0.512	11	0.170	-0.74	1.00	-3.20	0.001
D7S635	46	0.532	12	0.236	0.43	0.33	-1.58	0.06

*Mean of the estimated proportion of marker alleles shared identical by descent. Under the null hypothesis of no linkage, the expected mean is 0.50 for full sib pairs and 0.25 for half sib pairs.

TABLE 4. Multipoint (MIM) Analysis

Marker	Percentage of Additive Variance of the Trait			
	10%	20%	30%	50%
D7S1875	0.52	0.35	0.24	0.11
D7S504	0.63	0.45	0.31	0.15
D7S635	0.46	0.30	0.22	0.17
<i>OB</i> -tet	0.91	0.89	0.87	0.84
Multipoint	0.51	0.34	0.22	0.10

Values are *P* values (χ^2 test). Distances between markers were assumed to be 0.3 cM between *OB*-tet and D7S1875, 0.3 cM between *OB*-tet and D7S504, and 0.3 cM between D7S504 and D7S635 since all markers are known to be within 1 cM of each other.

D7S1875, *P*=0.08 for *OB*-tet, *P*=0.001 for D7S504, and *P*=0.06 for D7S635) (Table 3).

Since the results became more consistent across markers when analysis included only those subjects with a BMI \geq 85th percentile, it was important to perform a multipoint analysis of the data. This method estimates the proportion of genetic variance for BMI that is attributed to the *OB* gene region encompassed by these markers. The program MIM (University of Utah, Salt Lake City) was used to perform this analysis on each marker individually and on all markers simultaneously. As can be seen in Table 4, the MIM results for each individual marker were not significant for any estimate of total additive variance. The multipoint analysis did not show significance and resulted in little increase of information above that contained by the marker D7S1875 alone.

Detection of Variants

SSCP analysis was used to detect mutations in the human *OB* gene in the population. The *OB* gene is encoded by three exons. Exon 1, only 29 bp in size, is not part of the protein-coding region and was therefore excluded from the analysis. The entire coding sequence of the *OB* gene, represented by exons 2 and 3, was amplified through PCR with the use of three sets of primers. One conformational variant was found in exon 2, and sequencing revealed an ATT(Ile) to GTT(Val) transition. This polymorphism was not present in the corresponding sibling or in any other individual analyzed. No other variants were detected in the coding sequence of the *OB* gene.

Discussion

Essential hypertension and obesity are both thought to be caused by multiple environmental and genetic determinants. These two traits are closely linked in epidemiological studies, and essential hypertension is estimated to be up to three times more prevalent among the obese.¹⁵ This correlation suggests that essential hypertension and obesity may share genetic determinants in some individuals.

Experimental data in rodents have suggested that leptin, the serum protein encoded by the *ob* gene, plays a central role in regulating food intake and energy expenditure,⁶⁻⁸ and inactivation of this gene is observed to cause severe obesity in mice. To determine whether polymorphic markers at the

human *OB* gene were linked either to essential hypertension or to obesity, we performed linkage analysis in a hypertensive African American population. Affected sib pair analysis did not show any evidence of linkage to the trait of essential hypertension and showed only suggestive linkage to the phenotype of obesity (defined as BMI >27.8 kg/m² for men and BMI >27.3 kg/m² for women).¹⁵ Although analysis was performed with four different markers, including *OB*-tet, a marker within the 3' untranslated region of the *OB* gene, linkage was detected with only one marker, D7S504, which lies outside *OB*.

Part of the reason why the MIM results were not as significant as the SIBPAL results is that only full siblings could be used in MIM. This resulted in a loss of approximately one quarter of the total sib pairs and a corresponding loss of power. However, the fact that the multipoint analysis did not improve the overall significance level and that the *OB*-tet marker, which is located within the leptin gene, was not significant in this analysis diminishes the likelihood that the suggestive linkage by individual markers is real. Analysis of a larger population of sibships will therefore be required to increase the power of this study. Further patient recruitment is under way. Other groups have detected stronger genetic linkage between severe obesity and the human *OB* gene^{12,13} by using a higher BMI threshold (35 kg/m²) in the collection of sib pairs. Our study was directed at essential hypertension and the more commonly seen form of obesity (average BMI >27.3 kg/m² for women and >27.8 kg/m² for men),¹⁵ and our data suggest that *OB* is not a major contributor to either trait in this population of African Americans. Other studies suggest that genes other than *OB* may play the major role in human obesity. Comuzzie et al²² report the identification of a distinct locus on human chromosome 2 that confers susceptibility to obesity in white populations.²² As in our study of African Americans, Comuzzie et al found no evidence of linkage to obesity with markers on chromosome 7 in the region containing the leptin (*OB*) gene in Mexican Americans.

Although the sib pair method of analysis did not detect linkage, our study does not exclude the possibility that the *OB* gene is linked to BMI in the African American population. Complex disorders such as obesity may require very large numbers of sib pairs (reviewed by Schork²³) to provide adequate power to detect linkage and power calculations on our population well below the 80% threshold generally used in such studies. Further complications could include the problem of "admixture" in the African American population. Therefore, our findings can only be considered preliminary at this point.

To address the possibility that our sib pair analyses may have lacked the power to detect linkage between *OB* and hypertension and the inherent difficulties that are posed by genetic admixture in the study population, we performed mutational analysis of the human *OB* gene using DNA from individuals in this population. SSCP analysis of the 167 African American subjects in our study did not reveal any significant alterations in the coding region of the human *OB* gene. A single polymorphism was detected in exon 2, ATT(Ile) to GTT(Val), a conservative alteration in amino

acids not expected to significantly alter the function of the protein. This variant was not present in the corresponding sibling or in any other individual analyzed and is therefore more likely to be a neutral sequence variation than a mutant that alters leptin function. This absence of mutations is consistent with recent reports in other human populations²²⁻²⁶ and strengthens the absence of linkage in this study. Although we cannot exclude the possibility that alterations in the promoter or regulatory regions of this gene are affected in these individuals, our data and the data from other reports indicate that the leptin locus is not significantly linked to BMI and that polymorphic variants in the coding region of the leptin gene are not seen in individuals with high BMI.

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