

A Common Promoter Variant of the Leptin Gene Is Associated With Changes in the Relationship Between Serum Leptin and Fat Mass in Obese Girls

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Mutations in the leptin gene lead to rare obese syndromes of Mendelian inheritance in humans and rodents. However, no relevant mutations are found in the coding region of leptin gene DNA in patients with common multifactorial obesity. These obese patients tend to have an elevation of serum leptin proportional to their adiposity but with a rather wide dispersion of leptin levels for a given body fat content, which in part is attributable to sexual dimorphism. The current study, performed in two independent Caucasian cohorts of obese girls, shows that a frequent promoter variant of the leptin gene is associated with changes in the relationship between serum leptin and body fatness. Girls of comparable adiposity have different circulating leptin levels, depending on their genotype at this locus. Girls with the $-/-$ Lep $-2,549$ genotype have 25% lower mean leptin levels than the girls with other genotypes, as reflected by differences in the regression slopes of leptin-to-fat mass. Therefore, genetic factors related to the leptin gene may be important in defining the set point of obese individuals (i.e., the circulating leptin level for a given degree of body fatness). This definition may be of both physiological and therapeutic relevance, although a phenotypic association with an individual single-nucleotide polymorphism is not sufficient to assign function to this particular nucleotide site. *Diabetes* 49:2196–2200, 2000

Rare massive obesity syndromes are associated with mutations of the leptin gene in humans (1,10) and several animals models (2,3,11). No mutations are found, however, in the coding region of this gene in patients with more common forms of obesity (4–6,12–14). Obese patients have elevated levels of leptin in serum and of leptin gene mRNA in white adipocytes

(7–9,15), the major leptin-producing cells. Circulating leptin levels correlate with body fat according to a well-established linear relationship (7–9). This relationship is of physiological importance, because it allows leptin to act as a quantitative endocrine signal that ensures that target cells (mainly in hypothalamic areas) are informed of the amount of fat stored in adipose tissue.

Because of hyperleptinemia, most humans with multifactorial obesity, including our young patients (9), are considered resistant to leptin (16,17). Of obese humans, however, 5–10% have relatively low leptin levels (7–9). Based on results in a transgenic mouse model (18), it has been postulated that a decreased production of leptin by the adipose tissue can cause the obese state in these “low-leptin” individuals (18).

To address the question of underlying individual differences in the regulation of circulating of leptin levels in humans, we studied Caucasian girls in the dynamic phase of early-onset obesity, spanning a wide range of body fat content. These obese children underwent measurement of fasting serum leptin and were genotyped for a single-nucleotide polymorphism (SNP) within the 5' untranslated region (UTR) of the leptin gene, as described by Mammès et al. (19), which is immediately adjacent to sequences implicated in the regulation of leptin gene transcription (20) (Fig.1). Because of its location in the regulatory region of the leptin gene, we postulated that this SNP has the potential to influence leptin expression or serve as a surrogate for unrecognized neighboring functional SNPs. After obtaining evidence of an association between this variant and leptin levels in a first cohort, we safeguarded against the risk of false positivity by replicating the results in a second independent cohort of obese girls.

RESEARCH DESIGN AND METHODS

We recruited 140 Caucasian girls (cohort I), aged (mean \pm SD) 11.5 \pm 2.9 years (range 5.0–19.3), whose natural history of obesity was still progressing at the time of study. The following inclusion criteria were ascertained through study of their weight and height curves: having onset of obesity (>85th percentile of BMI) before 6 years of age, being in a dynamic phase of fat accumulation, having not yet attempted any weight reduction, showing a continuously accelerated gain of weight, having no diseases and taking no disease medication, and having parents without diabetes or endocrinopathy. Puberty was assessed according to the Tanner stages. The protocol was approved by the Cochin Ethics Committee (Paris), and informed consent was obtained from patients and parents.

To replicate the results obtained in cohort I, a group of 93 obese girls (cohort II) was recruited according to the same inclusion criteria.

We restricted our study to girls because our recruitment did not allow us to find and evaluate sufficient numbers of young obese boys.

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ANCOVA, analysis of covariance; ANOVA, analysis of variance; QTL, quantitative trait locus; SNP, single-nucleotide polymorphism; UTR, untranslated region.

TABLE 1
Characteristics of the studied genotype subgroups

Genotype	Cohort I			Cohort II		
	+/+	+/-	-/-	+/+	+/-	-/-
<i>n</i>	32	78	30	39	36	18
Age (years)	11 ± 0.4	11.7 ± 0.3	11.8 ± 0.6	11.7 ± 0.4	12 ± 0.4	10.9 ± 0.9
BMI (kg/m ²)	29 ± 1	30 ± 1	29 ± 1	30 ± 1	30 ± 1	29 ± 1.5
Fat mass (kg)	25.7 ± 1.6	28.1 ± 1.4	27.4 ± 2.3	27.1 ± 1.2	28.1 ± 1.5	26.5 ± 2.5
Leptin (ng/ml)	35 ± 3	35 ± 2	27 ± 2*	38 ± 3	37 ± 3	28 ± 3*
Leptin† (ng/ml per kg fat mass)	1.3 ± 0.1	1.2 ± 0.1	1.0 ± 0.1‡	1.4 ± 0.1	1.3 ± 0.1	1.1 ± 0.1*

Data are means ± SD. The mean leptin levels were 25% lower in the obese girls with -/- Lep-2,549 genotypes. This discrepancy was observed in both cohorts ($P < 0.05$) and in the pooled population of obese girls ($P = 0.016$). When normalized to fat mass, leptin levels showed comparable differences ($P = 0.017$ in the pooled cohorts). * $P < 0.05$; †normalized to body fat content; ‡ $P < 0.02$.

Procedural methods. For 3 days, subjects received meals consisting of a controlled in-hospital high-caloric standard diet (9). Obese children were then studied at 8.00 A.M., 12 h after the last meal. Their serum leptin levels were measured by radioimmunoassay (Linco Research, St. Charles, MO) (9). The mean intraindividual reproducibility of leptin measurements, ascertained through repeated sampling during the in-hospital study period, was 11 ± 1% (range 1–14.3%). Body composition was determined by bioelectric impedance (Eugenia, France) (9).

Genotyping of the promoter variant. Obese children were genotyped at the restriction site located at position -2,549 bp from the transcription initiation site of the leptin gene. Allele positivity (presence of a C at position -2,549 bp) and allele negativity (an A in the same position) were identified through the GCGC sequence recognized by *CfoI* (allele positive). Amplification was carried out in a volume of 50 µl containing 200 ng genomic DNA, 1 µmol/l of each primer (forward 5'-TTTCCTGTAATTTCCCGTGAG-3', and reverse 5'-AAAGCAAAGACAGGCATAAAAA-3'), 0.2 mmol/l of each dNTP, 1.5 mmol/l magnesium chloride, 10 mmol/l Tris-hydrochloric acid (pH 8.32), 50 mmol/l potassium chloride, 0.001% gelatin, and 1.25 U Taq polymerase (PerkinElmer, France). Thirty cycles were performed in a 9700 PerkinElmer thermocycler under the following conditions: initial denaturation 94°C for 5 min followed by 94°C for 45 s, annealing at 50°C for 45 s, extension at 72°C for 45 s, with a final extension at 72°C for 7 min. The amplified polymerase chain reaction products (242 bp) were digested with the addition of 1 U *CfoI* (Boehringer, France) for 1 h at 37°C. The digested samples were separated by electrophoresis through a 2% agarose gel. Digestion of the 242-bp product with *CfoI* produced fragments of the following sizes: 181 and 61 bp in homozygotes +/+; 242, 181, and 61 bp in heterozygotes; and 242 bp in homozygotes -/-. The -2,549 polymorphism allele and genotype frequencies met Hardy Weinberg equilibrium in the two cohorts.

Statistical analysis. Analysis of variance (ANOVA) was used to compare means of continuous variables in the three genotype subgroups. The relationship between serum leptin and fat mass (or BMI) was studied by Pearson's product-moment correlation. We calculated the slopes of regression of serum leptin (L) on fat mass as $L = a \times \text{fat mass} + b$, separately for each genotype group (a and b indicate regression constants). Analysis of covariance (ANCOVA) was performed to test the genotypic effect on serum leptin adjusted to fat mass. ANCOVA allowed us to test the homogeneity of these slopes for the genotypic categories. We also included the stage of puberty in the model of ANCOVA to adjust for this factor. All tests were two-sided. Analysis was performed with SAS version 6.11. To safeguard against false-positive results, we performed randomization tests of the hypothesis of equal fat mass/leptin regressions between genotypic categories (21). A total of 1,000 randomizations were used to estimate the relevant statistical distributions from each test.

RESULTS

The prevalence of the alleles was slightly different (+ allele 0.61 vs. 0.50, $P < 0.05$) in the two cohorts of obese girls. Overall, the distribution of the three genotypes in the obese girls of the pooled cohorts (+/+ 30%, +/- 50%, and -/- 20%) was comparable with the distribution found in related subjects of normal weight (+/+ 25%, +/- 52%, and -/- 23%) (Table 1), with that found in 116 lean Caucasian adults recruited as

control subjects (+/+ 26%, +/- 47%, and -/- 27%), and with that reported in the literature (19). This finding suggests that in our sample, there was no major gene or regulatory sequence primarily predisposing to early-onset obesity that was in close linkage disequilibrium with this polymorphism.

As expected, we found that serum leptin correlated with fat mass (Fig. 1). However, the degree of hyperleptinemia for a comparable fat mass varied among patients, indicating that individual factors other than fatness itself influence leptin levels during the period of active fat deposition. The aim of the current study was to search for such factors using multivariate analysis. Sex is a factor already identified in obese adults (22) and children (9,22,23). In our sample, age, puberty, and duration of obesity did not have detectable effects on the relationship between leptin and fat mass.

Our results support the hypothesis that the Lep -2,549 genotype is associated with changes in leptin production. In both cohorts, leptin levels (absolute values and after normalization to body fat) were ~20–25% lower in the -/- Lep -2,549 obese girls (Table 1). To more precisely document genotype-dependent differences in the relationship between leptin and fat mass, we examined this relationship in the genotype subgroups. We used a model that included fat mass, genotype, and an interaction term involving fat mass and genotype as explanatory variables and leptinemia as a dependent variable. Serum leptin correlated with fat mass in both cohorts ($r = 0.65$ and 0.74 , respectively, $P < 0.0001$). After adjustment to fat mass, we found a significant effect of genotype on serum leptin as well as a significant interaction of fat mass and genotype (Table 2). The regression of leptin to fat mass in the different subgroups of cohort I, as classified by genotype, is presented in Fig. 3. For the sake of simplicity, we limited our attention to comparisons involving the homozy-

TABLE 2

Results of the ANCOVA performed to test the effect of Lep-2,549 genotype on serum leptin levels adjusted to fat mass (or BMI) and stage of puberty

	Cohort I	Cohort II
Fat mass	<0.001	<0.001
Lep-2,549 genotype	<0.05	<0.05
Fat mass × Lep-2,549 genotype	<0.02	<0.025

Data are P values.

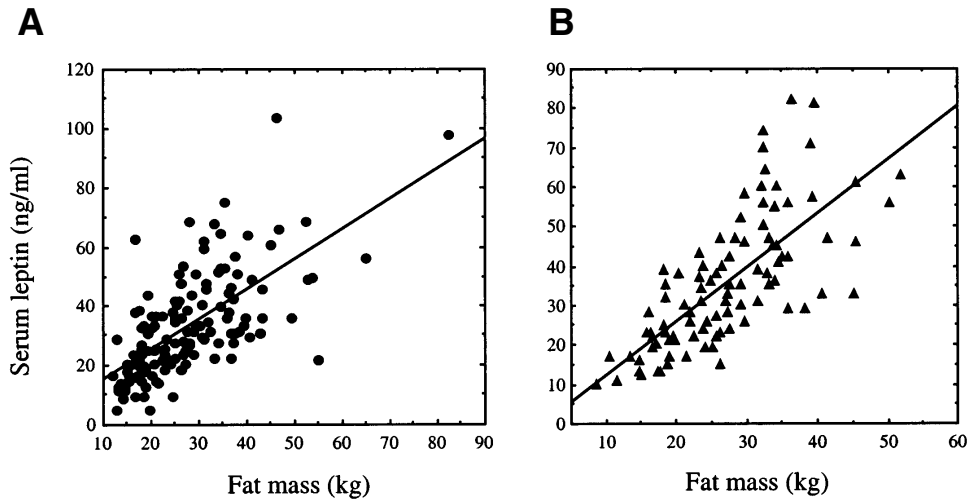


FIG. 1. The linear relationship between leptin and fat mass in cohorts I (A) and II (B) is respectively described by the equations $y = 1.0x + 7.1$ ($r = 0.65$, $P < 0.0001$) and $y = 1.4x - 2.9$ ($r = 0.74$, $P < 0.0001$).

gous genotypes (+/+ and -/-). We note, however, that results that included heterozygotes resulted in the same conclusions: allele '+' showed an apparent dominant effect. Approximately 56% of leptin variance was explained by fat mass and genotype. Homozygous obese girls with genotype -/- had lower leptin values for a given fat mass than those of other genotypes (Fig. 3). To strengthen our observations, we assessed the significance of the differences in slopes via permutation tests. The two homozygous categories were different ($P < 0.01$) based on 1,000 permutation tests.

Previous observations and results of statistical analyses were replicated in cohort II (Tables 1 and 2 and Fig. 4). In particular, the equations describing leptin regression to fat mass had slopes of closely comparable values with those in cohort 1, which is again consistent with the fact that obese girls with the -/- Lep -2,549 genotype produce less leptin for a given adiposity than those of other genotypes. The results

are similar when fat mass is substituted for BMI in the statistical analyses.

DISCUSSION

The present data suggest the existence of genotype-specific effects in the regulation of circulating leptin levels in response to active fat deposition. Depending on their sequence at the polymorphic locus -2,549 bp of the leptin gene, obese individuals of comparable adiposity appear to secrete more or less leptin. We were able to demonstrate this effect in very obese children, a situation that magnified (or clarified) the phenotypic differences between genotypes, as leptin levels became increasingly divergent as more fat was accumulated. In situations of normal weight to mild obesity, these genotypic effects may not be present or detectable. Allelic variation at the -2,549 locus, however, does not seem to be associated with the causation of early-onset obesity itself, because the preva-

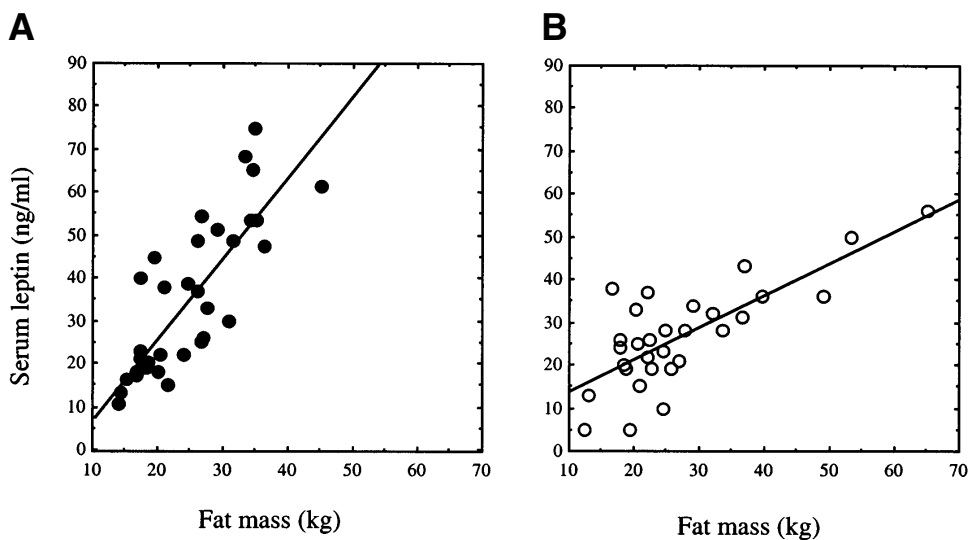


FIG. 2. In cohort I, the relationship between serum leptin and fat mass was different, depending on the Lep -2,549 genotype. Here we show and compare the +/+ (A) and -/- (B) homozygotes at this locus. The equation describing the leptin-to-fat mass relationship in obese girls ($n = 30$) with the -/- genotype is $y = 0.7x + 6$ ($r = 0.72$, $P < 0.0001$) versus $y = 1.9x - 8.3$ ($r = 0.78$, $P < 0.0001$) in those with the +/+ genotype. The value of the slope in -/- girls was 0.7 ± 0.2 (SD) (with a ± 2 SD CI of 0.3-1.1). The value was 1.9 ± 0.2 in those ($n = 32$) with the +/+ genotype (CI 1.5-2.3). For comparison, the slope was 1.1 ± 0.2 ($r = 0.74$, $P < 0.0001$, CI 0.7-1.5) in heterozygous +/- obese girls ($n = 78$).

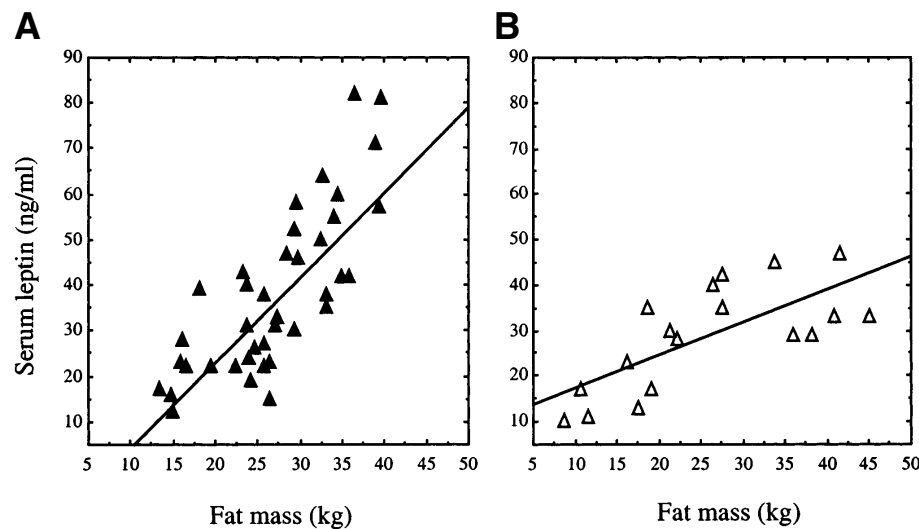


FIG. 3. In cohort II, the relationship between serum leptin and fat mass was different depending on the *Lep* -2,549 genotype. The equation describing the leptin-to-fat mass relationship in obese girls with the +/+ genotype (A) ($n = 40$) was $y = 1.8x - 11.8$ ($r = 0.75$, $P < 0.0001$) versus $y = 0.9x + 7$ ($r = 0.75$, $P < 0.0001$) versus in those with the -/- genotype (B) ($n = 18$). The value of the slope in -/- girls was 0.9 ± 0.2 (SD) (with a ± 2 SD CI of 0.4–1.3). The value was 1.9 ± 0.2 in those ($n = 39$) with the +/+ genotype (CI 1.5–2.3). For comparison, the slope was 1.4 ± 0.2 ($r = 0.75$, $P < 0.0001$, CI 1.0–1.8) in heterozygous +/- obese girls ($n = 36$).

lence of genotypes was essentially similar in our cohort and in Caucasians of normal weight. However, our data do not exclude an effect of this genotype during the later evolution of obesity in adulthood. The discordance of our observation versus that of higher leptin values in -/- *Lep* -2,549 obese adults (19) could be attributable to major differences in age-related modifications of leptin physiology, history of obesity (adult patients were not studied during weight gain), and 32% of the studied adult population consisting of males (our unpublished results suggest a possible sex-specific opposite effect of the -2,549 polymorphism).

The relationship between circulating leptin levels and body fat is known to be of physiological and possibly pathological relevance (16,17,24). Clinical studies suggest that body weight is regulated by a set-point mechanism (3,16,17,25). With the cloning of the leptin gene and the identification of leptin (2), it appeared that leptin production by adipocytes and hypothalamic sensitivity to leptin had major roles in determining this set-point equilibrium (16,17,24). According to our observations, genetic factors related to the leptin gene that are possibly involved in the regulation of leptin production could be important in defining this set point in obese individuals, and, therefore, it may influence therapeutic interventions. This idea is consistent with studies in *ob/ob* mice expressing a leptin transgene that sets serum leptin at one-half the wild-type value (18). These mice have threefold more fat than wild-type mice (18), and their treatment by low doses of leptin results in significant weight loss. These data suggest that leptin administration could be more efficient in individuals with low-leptin obesity.

Our study provides no insight into the mechanisms responsible for the observed genotypic effects. The 5' flanking regulatory region of the human leptin gene contains dozens of putative binding sites for known transcription factors (20), including Sp-1 sites, cAMP response element, glucocorticoid response element, and CCAAT/enhancer-binding protein sites. Transfection of sense reporter constructs with different lengths of genomic fragments of the 5' flanking region into

F442A adipocytes showed that the 3-kb 5' flanking region contains all of the necessary elements to support basal transcription and serve as a promoter. Moreover, the 300-bp fragment was apparently more active than the 3-kb fragment, indicating that the remote region might be inhibitory for transcription in the F442A adipocytes. The -2,549 *Lep* polymorphism does not correspond to a known binding sequence for transcription factors. It may be that the *Lep* -2,549 polymorphism is in linkage disequilibrium with regulatory sequences modulating transcription of the leptin gene. However, the existence of the regulatory sequence(s) that mediates sex steroid effects on the leptin gene and their a fortiori relationship with the -2,549 polymorphism remain entirely speculative. More generally, cautious interpretation of a phenotypic association with a single SNP is warranted, at least until it is known whether this polymorphism acts as a simple marker through linkage disequilibrium or a functional variant.

Linkage analyses of the leptin gene with common obesity phenotypes have produced conflicting results. Several studies found evidence for linkage (26,27) but only in superobese adults or adults with obesity-related traits (28). The failure to detect linkage in other studies (28) might suggest heterogeneity in the degree of obesity and/or ethnic background of study subjects. More than 300 obese individuals of various ethnicities have been screened for mutations in the coding region of the leptin gene (28). Few missense mutations were found, and these were equally distributed among obese and nonobese subjects (4,28). In the promoter region of the leptin gene, a polymorphism (C-188A) initially described in 29 morbidly obese patients (29) subsequently showed similar frequency in 249 obese and 141 lean subjects (19). In Hispanic-Americans, a major quantitative trait locus (QTL) for serum leptin level and fat mass was located on chromosome 2 (30). Our data suggest that another QTL for serum leptin level in Caucasian girls may be located in the proximity of the leptin gene.

In summary, although most humans develop hyperleptinemia when they become obese, the factors responsible for individual variation of leptin levels, other than sex, remain

largely unknown (22,31). In obese girls, however, leptin secretion was known to show large variations for comparable degrees of adiposity (7–9,22). The –2,549 locus polymorphism located in the promoter region of the leptin gene appears to be associated with these changes. Although we have only documented this effect in obese humans (given their wide range of adiposity), potential genotypic influence on the allometric relationship between body fat and serum leptin will have to be systematically studied in the general population.

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