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Tests of linkage and/or association of the LEPR gene polymorphisms with obesity phenotypes in Caucasian nuclear families

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Liu, Yong-Jun, Sonia M. S. Rocha-Sanchez, Peng-Yuan Liu, Ji-Rong Long, Yan Lu, Leo Elze, Robert R. Recker, and Hong-Wen Deng. Tests of linkage and/or association of the LEPR gene polymorphisms with obesity phenotypes in Caucasian nuclear families. *Physiol Genomics* 17: 101–106, 2004. First published February 17, 2004; 10.1152/physiolgenomics.00213.2003.—Genetic variations in the leptin receptor (LEPR) gene have been conceived to affect body weight in general populations. In this study, using the tests implemented in the statistical package QTDT, we evaluated association and/or linkage of the LEPR gene with obesity phenotypes in a large sample comprising 1,873 subjects from 405 Caucasian nuclear families. Obesity phenotypes tested include body mass index (BMI), fat mass, percentage fat mass (PFM), and lean mass, with the latter three measured by dual-energy X-ray absorptiometry (DXA). Three single nucleotide polymorphisms (SNPs), namely *Lys109Arg* (A/G), *Lys656Asn* (G/C), *Pro1019Pro* (G/A), in the LEPR gene were analyzed. Significant linkage disequilibrium ($0.394 \leq |D'| \leq 0.688$, $P < 0.001$) was observed between pairs of the three SNPs. No significant population stratification was found for any SNP/phenotype. In single-locus analyses, evidence of association was observed for *Lys656Asn* with lean mass ($P = 0.002$) and fat mass ($P = 0.015$). The contribution of this polymorphism to the phenotypic variation of lean mass and fat mass was 2.63% and 1.15%, respectively. Subjects carrying allele *G* at the *Lys656Asn* site had, on average, 3.16% higher lean mass and 2.71% higher fat mass than those without it. In the analyses for haplotypes defined by the three SNPs, significant associations were detected between haplotype *GCA* ($P = 0.005$) and lean mass. In addition, marginally significant evidence of association was observed for this haplotype with fat mass ($P = 0.012$). No statistically significant linkage was found, largely due to the limited power of the linkage approach to detect small genetic effects in our data sets. Our results suggest that the LEPR gene polymorphisms contribute to variation in obesity phenotypes.

leptin receptor; quantitative transmission disequilibrium test

THE DISCOVERY OF LEPTIN and its receptor, LEPR, has defined a novel molecular pathway for energy metabolism and regulation of body weight (14, 35, 42). Leptin is a fat tissue-derived cytokine that reports nutritional information and regulates energy expenditure by activating LEPR in the hypothalamus (14). LEPR is a single transmembrane protein signaling through the JAK-signal transducer and activator of transcription (STAT) pathway (7, 8, 35). Functional mutation in the LEPR gene in animal models and humans could cause extreme obesity (9, 20, 24). However, such mutations are extremely rare and are not likely to be responsible for common obesity. It is conceivable that common polymorphisms in the LEPR gene could modify the function of LEPR and lead to variation in leptin levels and body weight in the general population.

A number of common polymorphisms and rare variants of the human LEPR gene have been identified. Their potential associations with obesity were evaluated in different populations, largely using the population-based association approach (reviewed in Ref. 5). Up to the present, consistent associations have been difficult to demonstrate. For example, association between a polymorphism *Gln223Arg* and obesity phenotypes was observed in middle-aged Caucasian males (6), postmenopausal Caucasian women (29), a Mediterranean population (41), and young Dutch adults (38), but not in American Caucasians (33), Pima Indians (36), blacks (6), and Japanese (22). Linkage between LEPR gene locus and adiposity was reported in French Caucasians (4), whereas no linkage was detected in Pima Indians (26) and Mexican Americans (3). Our whole genome linkage scan in 53 Caucasian pedigrees (11) suggested a major quantitative trait locus (QTL) on chromosome 1p, a region harboring the LEPR gene. Our follow-up linkage study by genotyping denser markers in an expanded sample provided further support to this finding (21).

Motivated by the potential importance of LEPR gene in energy homeostasis and body weight regulation, yet with rather inconsistent results so far, we performed a study to test the LEPR gene as a QTL for obesity. We surveyed the literature and searched the public database for single-nucleotide polymorphisms (SNPs) in the LEPR gene. SNPs were selected based on a comprehensive consideration of *1*) functional relevance and importance; *2*) degree of heterozygosity, i.e., allele frequencies (we included those with minor allele frequencies >0.15 in the analyses); and *3*) their use in previous genetic studies. We examined three highly polymorphic SNPs [i.e., *Lys109Arg* (exon 4), *Lys656Asn* (exon 14), and *Pro1019Pro* (exon 20)] and haplotypes defined by these for linkage and/or association with obesity phenotypes in a large sample with 1,873 subjects from 405 Caucasian nuclear families. Important to this study is that we employed a robust approach, the family-based transmission disequilibrium test (TDT), which is immune to population stratification, a potential confounding factor to population-based association studies (1, 34).

MATERIALS AND METHODS

Subjects

The study subjects came from an expanding database being created for studies to search for genes underlying the risk to osteoporosis and

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obesity at the Osteoporosis Research Center of Creighton University. The study was approved by the Creighton University Institutional Review Board. All subjects were Caucasians of European origin. Only healthy people were included in the study with the exclusion criteria that were detailed elsewhere (12). In brief, individuals having serious chronic diseases/conditions that may have potential influence on bone mass were excluded. These diseases/conditions have general influence on human endocrine and metabolism. Application of the exclusion criteria may help minimize, empirically, nongenetic influence on obesity phenotypes. No restrictions were imposed in terms of body weight or diet history. Individuals who were overweight [body mass index (BMI) $>$ 25 kg/m^2] or obese (BMI $>$ 30 kg/m^2) but permitted under the exclusion criteria entered the study. For each study subject, the information on age, sex, medical history, and family history was acquired. A total of 405 nuclear families comprising 1,873 subjects were recruited, including 740 parents, 744 daughters, and 389 sons. Among these, 341 families were composed of both parents and at least one offspring. In the remaining 64 families, there were at least two children with either one or no parent. The average family size was 4.62 $\pm$ 1.78 (mean $\pm$ SD), ranging from 3 to 12, and there were a total of 1,512 sib pairs. The descriptive characteristics of the study subjects are presented in Table 1.

Genotyping

DNA was extracted from whole blood using a commercial isolation kit (Gentra Systems, Minneapolis, MN) following the procedure detailed in the kit. The genotyping procedure for all SNPs was similar, involving polymerase chain reaction (PCR) and invader assay reaction (Third Wave Technology, Madison, WI). PCR was performed in a 10-μl reaction volume with 35 ng genomic DNA, 0.2 mM each dNTP, 1× PCR buffer and 1.5 mM $MgCl_2$, 0.4 μM of each the primers, and 0.35 U *Taq* polymerase [Applied Biosystems (ABI), Foster City, CA]. The following procedure was used: 95°C for 5 min, 30 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, and then 72°C for 5 min. After amplification, an invader reaction was performed in a 7.5-μl reaction volume, with 3.75 μl diluted PCR product (1:20), 1.5 μl probe mix, 1.75 μl Cleavase FRET mix, and 0.5 μl Cleavase enzyme/$MgCl_2$ solution (Third Wave Technology). The reaction mix was overlaid with 15 μl mineral oil and denatured at 95°C for 5 min, then incubated at 63°C for 20 min. All the PCR and invader assay reactions were performed on thermal cyclers (ABI model 9700). After the invader reaction, the fluorescence intensity for both colors (FAM dye and Red dye) was read using a multi-well plate reader (Cytofluor model 4000, ABI). The data were then loaded to the software Invader Analyzer (Third Wave Technology), and the genotype for every sample was called according to the ratio of the fluorescence intensity of the two dyes. PedCheck software (28) was used to verify Mendelian inheritance of the alleles within each family. The overall genotyping error and missing rate was ~1%.

Table 1. *Descriptive characteristics of the study subjects*

	Mother (n = 380)	Father (n = 360)	Daughter (n = 744)	Son (n = 389)
Age, yr	62.40±10.40	62.86±10.70	37.73±10.33	36.00±10.92
Height, m	1.62±0.06	1.77±0.07	1.65±0.06	1.80±0.07
Weight, kg	73.81±15.21	90.30±15.26	70.40±16.42	87.60±15.94
BMI, kg/m^2	28.04±5.57	28.85±4.42	25.77±5.86	27.02±4.39
Fat mass, kg	28.70±9.87	24.73±7.90	24.44±10.45	20.13±8.11
PFM, %	37.54±5.96	26.94±5.00	33.46±7.00	22.50±6.30
Lean mass, kg	45.13±6.76	65.08±8.63	46.13±6.89	67.28±9.08

Values are the raw data, as means ± SD; n = 1,873 subjects. BMI, body mass index; PFM, percentage fat mass.

Measurement

Fat mass and lean mass were measured by dual-energy X-ray absorptiometry (DXA) with a scanner (model 2000+ or 4500; Hologic, Bedford, MA). Both machines were calibrated daily. The body composition bar was used on every whole body scan on the Hologic 2000+. On the Hologic 4500, the bar was not needed for the body scans; instead, it was scanned every week. Percentage fat mass (PFM) is the ratio of fat mass to body weight (i.e., the sum of fat mass + lean mass + bone mass). At the same visit as body composition scan, weight was measured in light indoor clothing, using a calibrated balance beam scale, and height was measured using a calibrated stadiometer. The measurement precision of BMI as reflected by the coefficient of variation was 0.2%. The coefficients of variation for fat mass, PFM, and lean mass were 2.2%, 2.2%, and 1.0%, respectively, for measurements obtained on the Hologic 2000+, and were 1.2%, 1.1%, and 0.7%, respectively, for measurements on the Hologic 4500. Members of the same nuclear family were usually measured on the same type of machine. Except for lean mass and fat mass, the phenotypes are correlated significantly. The phenotypic correlations were 0.88 (between BMI and fat mass), 0.49 (between BMI and lean mass), 0.54 (between BMI and PFM), 0.12 (between fat mass and lean mass), 0.81 (between fat mass and PFM), and −0.34 (between lean mass and PFM).

Statistical Analyses

Single-locus analyses. The allele frequencies of each SNP were estimated in all the subjects of the nuclear families via a maximum-likelihood method implemented in the program SOLAR (available at **http://www.sfbr.org/sfbr/public/software/solar**).

Under the flexible variance-component framework, tests of population stratification, linkage, total association, and within-family association between each of the SNPs and obesity phenotypes were implemented in the statistical software package QTDT ("quantitative trait disequilibrium tests") (available at **http://www.sph.umich.edu/csg/abecasis/QTDT/**). The orthogonal model of Abecasis et al. (1) was adopted in the analyses, where the genotype score is decomposed into orthogonal between-family (β_b) and within-family (β_w) components. Population stratification is examined by testing whether $\beta_b = \beta_w$ (15). Linkage tests are based on the standard variance-component methods and the identity by descent (IBD) among relatives. Total association tests use all information including β_b and β_w components and may yield false-positive/false-negative results due to population stratification. Within-family association (via TDT), however, is significant only if there is linkage disequilibrium (LD), and it is robust to population stratification. When both linkage and association are detected, Fulker et al. (15) suggested testing linkage while simultaneously modeling association to evaluate whether the tested marker is the functional mutation underlying the trait. When significant association is observed, the approximate phenotypic variation due to the detected marker is calculated as $2p(1 - p)a^2$, where p is the allele frequency of the marker, and a is the estimate of additive effect, i.e., $E(\beta_w) = a$ (Ref. 1). It is noteworthy that the trade-off for conducting the within-family association test is a reduction in power to detect allelic association (37). In the absence of population stratification, total association confers more power and can be more sensitive than within-family association to detect correlation between a marker and a specific trait (1). In light of such power consideration, we first tested population stratification. If no evidence of population stratification was observed, then the more powerful test of total association was utilized. Alternatively, an orthogonal model robust to population stratification was used.

Variance-component methods implemented in the QTDT make a critical assumption that the underlying trait follows a multivariate normal distribution. In this study, the four obesity phenotypes demonstrated departure from normal distribution and were transformed to

Table 2. *Frequencies of minor alleles and the haplotypes*

No.	SNPs	Frequency, %
1	*Lys109Arg* (A***A***G-A***G***G)	29.1
2	*Lys656Asn* (AA***G***-AA***C***)	18.2
3	*Pro1019Pro* (CC***G***-CC***A***)	35.3
No.	**Haplotypes**	**Frequency, %**
1	*AGG*	46.2
2	*GCA*	17.1
3	*ACA*	12.4
4	*GCG*	11.9
5	*AGA*	6.3
6	*ACG*	4.8
7	*GCA*	1.0
8	*GCG*	0.3

SNP, single-nucleotide polymorphism. Bold, underscore, and italic fonts indicate allelic changes.

approach normality via the Box-Cox procedure implemented in the statistical software Minitab (Minitab, State College, PA).

Haplotype analyses. Haplotype reconstruction in the nuclear families was carried out using the program Genehunter version 2.1 (**http://www.hgmp.mrc.ac.uk/Registered/Option/genehunter.html**). Genehunter extracts complete multipoint inheritance information to infer maximum-likelihood haplotypes for all individuals in nuclear families (19). The SNPs were arranged in the order of *Lys109Arg-Lys656Asn-Pro1019Pro* in haplotype reconstruction. Haplotype frequencies were estimated using unrelated subjects only (parents from each nuclear family). Pair-wise LD between the SNPs was calculated by the normalized measure, D' (13). The statistical significance of the observed LD was examined by Monte-Carlo approximation of Fisher's exact test (31). For QTDT haplotype analyses, we first utilized the QTDT multi-allelic tests option which include alleles in one test as a categorical variable with allele frequencies of <5% being pooled to give a global significance. Then we performed the allele-wise test on each allele separately, with all other alleles being pooled into another category. Employing the aforementioned sequential tests within QTDT, χ^2 and P values were computed for those haplotypes present in at least 30 informative offspring.

Correction for multiple testing. Since multiple markers and phenotypes were analyzed, a correction for multiple testing is required. Bonferroni correction seems to be too conservative and potentially damaging to statistical power (27). This is because statistical tests here are highly correlated in that *1*) SNPs in the LEPR gene are in significant LD (shown in the results below) and *2*) the tested phenotypes are significantly correlated. As a less stringent yet suitable adjustment, Monte-Carlo permutation was performed to circumvent multiple testing (23). This method was proposed by McIntyre et al. (23) and has been incorporated in the QTDT. After 1,000 times of permutations, an empirical threshold, $P \leq 0.01$, was established for an individual test to achieve a global significance level of 0.05. All the statistical analyses were performed on the transformed data with age and sex as covariates.

RESULTS

The frequencies of the alleles and haplotypes for the LEPR gene are presented in Table 2. The minor allele frequencies of *Lys109Arg*, *Lys656Asn*, and *Pro1019Pro* are 29.1%, 18.2%, and 35.3%, respectively. The heterozygosity of the three SNPs is 0.40, 0.26, and 0.47, respectively. No deviation from Hardy-Weinberg equilibrium was observed in the parents' group. Significant LD ($0.394 \leq |D'| \leq 0.688$, $P < 0.001$) exists between pairs of three SNPs.

Table 3 presents a summary of the results of QTDT analyses. For single-locus analyses, since no population stratification was detected, we then performed total association tests. Evidence of total association, even after permutation tests, was observed for *Lys656Asn* with lean mass ($P = 0.002$) and fat mass ($P = 0.015$). The contribution of this polymorphism to the phenotypic variation of lean mass and fat mass was 2.63% and 1.15%, respectively. Subjects carrying allele *G* had, on average, 3.16% higher lean mass and 2.71% higher fat mass than those without it. We did not find significant evidence of linkage or within-family association at individual SNPs for

Table 3. *P values of tests of population stratification, total association, within-family association, and linkage for individual SNPs and the haplotypes*

				Haplotype	
Tests	*Lys109Arg*	*Lys656Asn*	*Pro1019Pro*	Multi-allelic	Allele-wise
Population stratification					
BMI	0.887	0.235	0.488	0.219	
Fat mass	0.740	0.162	0.680	0.475	
PFM	0.548	0.235	0.646	0.628	
Lean mass	0.862	0.357	0.920	0.434	
Total association					
BMI	0.764	0.069	0.698	0.109	
Fat mass	0.322	0.015*	0.442	0.020*	0.012* (*GCA*)
PFM	0.647	0.099	0.424	0.332	
Lean mass	0.281	0.002*	0.610	0.006*	0.005* (*GCA*)
Within-family association					
BMI	0.920	0.806	0.438	0.289	
Fat mass	0.380	0.689	0.841	0.339	0.061 (*GCA*)
PFM	0.647	0.099	0.424	0.332	
Lean mass	0.548	0.170	0.777	0.248	0.085 (*GCA*)
Linkage					
BMI	0.450	0.532	0.862	0.521	
Fat mass	0.371	0.416	0.603	0.438	
PFM	1.000	1.000	1.000	0.298	
Lean mass	0.238	0.241	0.380	0.625	

All tests were conducted by employing the program QTDT. All data were adjusted for age and sex prior to analysis. *Statistically significant.

each phenotype. Since no evidence of linkage between any of the SNPs and the phenotypes was detected, as was expected, no significant results for the tests of linkage while modeling association were found (data not shown).

Haplotype analyses yielded interesting and compatible results (Table 3). All the eight possible haplotypes were observed, with the most common form of haplotype *AGG* accounting for almost half of the frequencies. In multi-allelic tests, no significant results were obtained for population stratification, linkage, or within-family association. We observed significant total associations for lean mass ($P = 0.006$) and marginally significant association for fat mass ($P = 0.020$). In allele-wise tests, consistent with multi-allelic tests, we did not find significant population stratification or linkage for any individual haplotypes. Significant total associations were detected between lean mass and haplotype *GCA* ($P = 0.005$). In addition, for fat mass, marginally significant evidence of total associations was observed with this haplotype ($P = 0.012$). For within-family association tests, although the significance for these haplotypes with obesity phenotypes was not observed, some evidence was still found for haplotype *GCA* ($P = 0.061$) with fat mass and lean mass ($P = 0.085$). The contribution of haplotype *GCA* to the phenotypic variation of lean mass and fat mass was 2.23% and 1.74%, respectively. Individuals carrying haplotype *GCA* showed, on average, 4.41% higher lean mass and 3.50% higher fat mass than those without it. For those unmentioned haplotypes, either no significant results were detected, or these were not tested because there were not sufficient informative subjects.

DISCUSSION

This is a nuclear family-based study using the TDT approach. The TDT directly tests for LD between a trait and a marker locus and is not susceptible to false-positive results due to population stratification (1, 34). The commonly used population-based association approach or case-control study, while valuable, are limited in that spurious associations may be yielded due to very recent admixture or population samples that are stratified with respect to genetically differentiated groups (10). The linkage approach, which may reveal genomic regions harboring QTLs without prior knowledge for underlying traits, is often of limited statistical power to detect genes of small effects (30, 32). The TDT is much more powerful compared with the traditional linkage approach in testing linkage of specific candidate genes to complex traits (2). Although the ideal study design for detecting SNP/phenotype association is currently debatable, the present study represents our efforts to test the importance of the LEPR gene on variation of obesity phenotypes by employing a robust approach.

Here, we report association for *Lys656Asn* with lean mass and fat mass. The variant *Lys656Asn* is located in the extracellular region of the LEPR gene. It causes a change in charge (lysine to asparagine at codon 656) and may have potential effects on the signaling capacity of LEPR. In this study, subjects carrying allele *G* had, on average, 3.16% higher lean mass and 2.71% higher fat mass than those without it. Our results are in agreement with that of the Quebec Family Study (4), in which female carriers of the *C* allele had 3 kg less of fat-free mass and 5 kg less of fat mass than noncarriers. In a group of overweight and obese postmenopausal Caucasian women, carriers of the *C* allele had increased hip circumference, total abdominal fat, and subcutaneous fat measured by computed tomography (CT) scan (39). Moreover, *Lys656Asn* was associated with fasting insulin and fasting glucose as well as in response to the oral glucose tolerance test in postmenopausal women with impaired glucose tolerance (40). These studies suggest that *Lys656Asn* may play a role in body weight regulation and may exert peripheral effects on regulation of glucose and insulin levels. It is noteworthy that the total association detected in this study should be robust and reliable, although we did not find correspondingly within-family association (via TDT). This is because total association confers more power than within-family association given the absence of population stratification. Our simulation shows that, assuming strong LD ($|D'| = 0.9$) between a marker and a functional mutation having 2% effect, we have more than 80% power to detect association via the TDT. However, this power is estimated under ideal conditions without taking into account of genetic interaction, heterogeneity, etc. Hence, significant total association could be detected without within-family association. In this study, further analyses in subsamples stratified by age or gender were not pursued, because of the consideration that results might be affected with the limited number of subjects remaining in subgroups.

Some studies, however, did not detect this allelic association. Negative results were reported for *Lys656Asn* in Caucasian populations (6, 33, 37, 41) and some other racial populations such as blacks (6) and Japanese (22). A meta-analysis suggested that there was no compelling evidence that any of the three polymorphisms (i.e., *Lys109Arg*, *Gln223Arg*, and *Lys656Asn*) are associated with BMI or waist circumference (17). The conflicting findings might arise from the inadequate power of small sample sizes, population structure, varying effects of disease-predisposing variants, or gene-environment interactions. For example, in a Mediterranean Caucasian population (41), the minor allele frequency of *Lys656Asn* (23.7%) was higher than that of our study population (18.2%). As we know, genetic variance due to a functional mutation is highly related with its allele frequency, and, generally, the more frequent the allele, the higher the heritability attributable to this functional mutation. In the HERITAGE Family Study (6), although the minor allele frequencies of *Lys656Asn* in Caucasians (18%) and blacks (17%) were comparable to ours, the analytical approach, sample sizes, and tested phenotypes were quite different. Finally, given the relatively small effects of a variant contributing to a complex trait, as reflected in our study ($\sim$2%), a potential association may be detected in some studies but missing in others.

Haplotype analysis, considering all of the variants segregating at multiple loci, can provide additional precision in genetic dissection of complex traits (25). However, in this study a potential limitation of the haplotype analyses is that only three SNPs were analyzed across a large gene spanning $>$200 kb. Nevertheless, as the three SNPs were in significant LD, haplotype analyses may still offer increased information. Indeed, in both multi-allelic and allele-wise tests, we detected significant total association, confirming the findings of single-locus analyses. Even for within-family association tests, suggestive evidence was found for haplotype *GCA* with fat mass ($P = 0.061$) and lean mass ($P = 0.085$).

We did not find linkage, which seems to be unexpected given the significant association detected. There are several possible reasons. First, the linkage approach is usually of limited statistical power with currently used sample sizes, especially in the case of small genetic effects. Second, SNPs as used in this study generally have lower heterozygosity and are less informative than microsatellite markers for linkage analyses. Assuming the overall heritability of a trait is 0.6 and the QTL effect is 2%, the power to detect linkage is only ~20% with this study sample. Since the association approach is generally more powerful and sensitive than the linkage approach, association could be detected without linkage.

The effect of *Lys656Asn* on lean mass and fat mass in our study is not reflected on BMI and PFM, although these traits are phenotypically correlated. This may be partially due to the insufficient power to detect a specific genetic effect. On the other hand, a significant and high phenotypic correlation does not necessarily imply a significant and high genetic correlation (16), which indicates the degree of shared genetic determination of two phenotypes.

In summary, using a large sample and a robust analytical approach, this study provides support that the LEPR gene polymorphisms are associated with obesity phenotypes. Further studies by examining denser markers and determining LD patterns throughout the gene region are warranted to fully elucidate the role of the LEPR gene in predisposition to obesity (16, 18).

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