Influence the development of obesity.

...signals from feedback loops that regulate food intake, energy...

...mental components manifesting in imbalances in energy intake...

...disorder, obesity encompasses various genetic and environ-

...mally affects health (25). Obesity increases the risk of mul-

...conditions, including cardiovascular disease, Type 2 dia-

...diabetes, cancer, and premature death (25). As a multifactorial disorder, obesity encompasses various genetic and environ-

...mental components manifesting in imbalances in energy intake and expenditure (25). Energy homeostasis is maintained by signals from feedback loops that regulate food intake, energy expenditure, lipid metabolism, and glucose metabolism (43). Hence, variation in the genes involved in these pathways may influence the development of obesity.

Uncoupling proteins (UCPs) are a family of inner mitochondrial membrane transporters that dissipate the proton gradient, releasing stored energy as heat, and are considered potentially important determinant of defense against obesity (9). Three distinct UCPs (UCP1, UCP2, and UCP3) have been identified. UCP1, existing only in brown adipose tissue, is unlikely to be a major gene involved in body weight regulation in humans (24). UCP2 has a wide tissue distribution at varying levels (15). UCP3 is expressed predominantly in skeletal muscle, a major site of thermogenesis in humans, making UCP3 an attractive target for studies toward body weight regulation (42).

The purpose of this study was to investigate whether the UCP3 gene contributes to obesity risk in the general population. Using the tests implemented in the statistical package quantitative transmission disequilibrium test (QTDT), we examined association and linkage between UCP3 gene polymorphisms and obesity-related phenotypes in a large sample of 405 Caucasian nuclear families comprising 1,873 subjects.

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 obesity at the Osteoporosis Research Center of Creighton University. The study was approved by the Creighton University Institutional Review Board. All subjects were United States Caucasians of northern European origin. Only healthy people were included in the study with the inclusion/exclusion criteria detailed elsewhere (30). In brief, individuals having serious chronic diseases/conditions that may have potential influence on bone mass were excluded. We excluded individuals with 1) serious residuals from cerebral vascular disease; 2)
uncontrolled diabetes mellitus (including Type 1 and Type 2 diabetes); 3) chronic renal disease; 4) serious chronic liver disease or alcoholism; 5) significant chronic lung disease; 6) therapy at pharmacological levels for >6-mo duration with corticosteroid or anticonvulsant; 7) hyper- or hypoparathyroidism, hyperthyroidism, Paget’s disease, osteomalacia, osteogenesis imperfecta; and 8) recent major gastrointestinal diseases (within the past year) such as peptic ulcer, malabsorption, chronic ulcerative colitis, regional enteritis, or any significant chronic diarrhea state. No restrictions were imposed in terms of body weight or diet history. Individuals who were overweight [body mass index (BMI) > 25 kg/m²] or obese (BMI > 30 kg/m²) were not excluded from our study provided they qualified under the inclusion/exclusion criteria. 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 was recruited, including 740 parents, 744 daughters, and 389 sons. Among these, 341 families were composed of both parents and at least 1 offspring. In the remaining 64 families, there were at least 2 children with either 1 or no parent. The average family size was 4.62 ± 1.78 (mean ± SD), ranging from 3 to 12, and there were a total of 1,512 sib pairs. According to their roles in nuclear families, the age range of the study subjects was as follows: father, 43–87 yr; mother, 40–85 yr; son, 19–57 yr; and daughter, 19–59 yr. The descriptive characteristics of the subjects are presented in Table 1.

**Genotyping**

We searched public single nucleotide polymorphism (SNP) databases (e.g., dbSNP) and the literature for candidate SNP markers within the UCP3 gene and its flanking region. To ensure that the selected SNPs are polymorphic in our population, they were first genotyped in a subsample of 190 unrelated subjects. We only genotyped those SNPs with a minor allele frequency of >10% for the remaining subjects. The genotyping procedure for all SNPs was similar, which involved 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, 1.5 mM MgCl₂, 0.4 µM each of the primers, and 0.35 units Taq polymerase (ABI, Applied Biosystems; Foster City, CA). We used the following procedure: 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 solution (Third Wave Technology). The reaction mix was overlaid with 3.75 l mineral oil, denatured at 95°C for 5 min, and then incubated at 63°C for 20 min. All the PCR and invader assay reactions were performed on 9700 Thermal Cyclers (ABI). After the invader reaction, the fluorescence intensity for both colors (FAM dye and red dye) was read using a Cytoflour 4000 multiwell plate reader (ABI). The data were then loaded to the software Invader Analyzer (Third Wave Technology), and the genotype for each sample was called according to the ratio of the fluorescence intensity of the two dyes. We used PedCheck software (36) to verify Mendelian inheritance of the alleles within each family. The overall genotyping error and missing rate was ~1%.

**Measurements**

BMI was calculated as the ratio of total body weight to the square of height (in units of kg/m²). Body weight was measured in light indoor clothing, using a calibrated balance beam scale, and height was measured using a calibrated stadiometer. Fat mass and lean mass were measured by dual-energy X-ray absorptiometry using a Hologic 2000+ or 4500 scanner (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. Percent fat mass (PFM) is the ratio of fat mass to the sum of fat mass, lean mass, and bone mass.

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, respectively, 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 the tested SNPs were estimated in all the subjects using a maximum-likelihood method implemented in the program SOLAR (4). Using a variance component analysis for quantitative traits (which is implemented in SOLAR), we estimated heritability of the four obesity phenotypes. Age and sex were adjusted as covariates in the analyses.

For quantitative traits, the essence of the transmission disequilibrium test is to examine the difference between average phenotypic values of children with different alleles transmitted from a heterozygous parent (3). Under a flexible variance-component framework, tests of population stratification, linkage, total association, and within-family association between each of the SNPs and obesity phenotypes are implemented in the statistical software package QTDT (1). The permutation procedure built in the QTDT may yield significance levels (P values) of the tests that are not biased. 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 $β_b = β_w$. Linkage tests are based on the standard variance-component methods and the identity by descent among relatives. Total association tests use all information including $β_b$ and $β_w$.

**Table 1. Descriptive characteristics of the study subjects**

<table>
<thead>
<tr>
<th></th>
<th>Mother</th>
<th>Father</th>
<th>Daughter</th>
<th>Son</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>380</td>
<td>360</td>
<td>744</td>
<td>389</td>
</tr>
<tr>
<td>Age, yr</td>
<td>62.40±10.40</td>
<td>62.86±10.70</td>
<td>37.73±10.33</td>
<td>36.06±10.92</td>
</tr>
<tr>
<td>Height, m</td>
<td>1.62±0.06</td>
<td>1.77±0.07</td>
<td>1.65±0.06</td>
<td>1.80±0.07</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>73.81±15.21</td>
<td>90.30±15.26</td>
<td>70.40±16.42</td>
<td>87.60±15.94</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>28.04±5.57</td>
<td>28.85±4.42</td>
<td>25.77±5.86</td>
<td>27.02±4.39</td>
</tr>
<tr>
<td>Fat mass, kg</td>
<td>28.70±9.87</td>
<td>24.73±7.90</td>
<td>24.44±10.45</td>
<td>20.13±8.11</td>
</tr>
<tr>
<td>PFM, %</td>
<td>37.54±5.96</td>
<td>26.94±5.00</td>
<td>33.46±7.00</td>
<td>22.50±6.30</td>
</tr>
<tr>
<td>Lean mass, kg</td>
<td>45.13±6.76</td>
<td>65.08±8.63</td>
<td>46.13±6.89</td>
<td>67.28±9.08</td>
</tr>
</tbody>
</table>

All data are presented as means ± SD of the raw phenotypic values without adjustment for covariates; n = 1,873 subjects. BMI, body mass index; PFM, percent fat mass.

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β, components and may yield false positive/negative results due to population stratification. Within-family association (via the transmission disequilibrium test), however, is significant only if there is linkage disequilibrium (LD), and it is robust to population stratification. When significant association is observed, the approximate phenotypic variation due to the detected marker is calculated as 2p(1 − p)2V_p, where V_p is the total phenotypic variance (V_p = V_e + V_g + V_a), p is the allele frequency of the marker, and a is the estimate of additive effect, i.e., E(β_a) = a (1). V_e is the residual component of variance, V_g is the polygenic component of variance, and V_a is the additive component of variance.

A trade off for conducting the within-family association test is the reduction in power to detect allelic association (45). In the absence of population stratification, total association confers more power and is more sensitive than within-family association to detect correlation between a marker and an underlying trait (45). Thus, in light of such power consideration, we first tested population stratification. If no evidence of population stratification was observed, the more powerful test of total association would be utilized.

Variance-component methods implemented in the QTDT make a critical assumption that the underlying trait follows a multivariate normal distribution. In the present study, all four obesity phenotypes showed a marked departure from normal distribution (P < 0.01) using the Anderson-Darling test and, therefore, were transformed to approach normality using the Box-Cox procedure implemented in the statistical software MINITAB (Minitab; State College, PA). Before these analyses, we performed the allele-wise test on each marker. Haplotype frequencies were estimated in unrelated subjects (parents from each nuclear family) and we excluded from the statistical analyses.

To avoid potential bias, we characterized the pair-wise LD and constructed haplotypes for the three highly polymorphic SNPs (i.e., −55 C/T, Tyr99Tyr, and Tyr210Tyr), which were genotyped in the total sample of 1,873 subjects. The three SNPs were arranged in the order of −55 C/T − Tyr99Tyr − Tyr210Tyr in haplotype construction. All eight possible haplotypes were observed. The three common haplotypes were CTC, CTT, and TCT, with allele frequencies of 40.6%, 31.2%, and 23.5%, respectively. These three haplotypes accounted for >95% of the total allele frequencies. The allele frequencies of the selected five SNPs and haplotypes defined by −55 C/T − Tyr99Tyr − Tyr210Tyr are shown in Table 2.

Significant pair-wise LDs (0.393 ≤ D’ ≤ 0.940, P < 0.0001) were found between pairs of SNPs. We did not find significant deviation from the Hardy-Weinberg equilibrium for any SNP/phenotype.

Linkage and Association Between Individual SNPs and Obesity Phenotypes

Table 3 summarizes the results of population stratification, association, and linkage tests. In our sample, the heritability of BMI, fat mass, PFM, and lean mass was 42%, 33%, 58%, and 51%, respectively. We did not find significant evidence of population stratification for any of the phenotypes. Because no population stratification was observed, we utilized the more powerful test of total association instead of within-family association. In single locus analyses, evidence of association was observed between −55 C/T and BMI (P = 0.034). This polymorphism contributes to 2.29% of BMI variation. In the children’s group, BMI values of the subjects with CC, CT, and

### Table 2. Frequencies of the alleles and haplotypes

<table>
<thead>
<tr>
<th>SNPs (Minor Allele)</th>
<th>Frequency, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>−55 C/T (T)</td>
<td>26.5</td>
</tr>
<tr>
<td>Val9Met (A)</td>
<td>2.8</td>
</tr>
<tr>
<td>Tyr99Tyr (C)</td>
<td>25.8</td>
</tr>
<tr>
<td>Tyr210Tyr (C)</td>
<td>44.7</td>
</tr>
<tr>
<td>IVS6 +1G &gt; A (A)</td>
<td>7.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>Frequency, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTC</td>
<td>40.6</td>
</tr>
<tr>
<td>CTT</td>
<td>31.2</td>
</tr>
<tr>
<td>TCT</td>
<td>23.5</td>
</tr>
<tr>
<td>TCC</td>
<td>2.5</td>
</tr>
<tr>
<td>CCT</td>
<td>0.8</td>
</tr>
<tr>
<td>TTT</td>
<td>0.7</td>
</tr>
<tr>
<td>CCC</td>
<td>0.4</td>
</tr>
<tr>
<td>TTC</td>
<td>0.3</td>
</tr>
</tbody>
</table>

For single nucleotide polymorphisms (SNPs), two rare alleles, Val9Met and IVS6 +1G > A, were eliminated from statistical analyses and haplotype construction. Haplotypes were constructed by the three highly informative SNPs: −55 C/T - Tyr99Tyr - Tyr210Tyr.
TT genotypes were 27.32 ± 5.33, 26.74 ± 5.21, and 25.96 ± 5.18, respectively. Subjects carrying the T allele had an average of 3.5% lower BMI than those without it (P = 0.003). We also found evidence of linkage between −55 C/T and BMI (P = 0.031). Evidence of linkage was also observed for both Tyr99Tyr (P = 0.032) and Tyr210Tyr (P = 0.027) with BMI, suggesting that the UCP3 gene locus is linked to BMI in this study population. However, we did not find a significant association between these two SNPs and BMI. For three other phenotypes, fat mass, PFM, and lean mass, we did not find evidence of linkage or association for any of the SNPs.

**Linkage and Association Between Haplotypes and Obesity Phenotypes**

Because the three SNPs (−55 C/T, Tyr99Tyr, and Tyr210Tyr) were in significant LD, we performed analyses for haplotypes constructed by them. Haplotype analyses yielded compatible results (Table 3). In multiallelic tests, we found evidence of linkage (P = 0.002) and association (P = 0.035) with BMI. In allele-wise tests, marginal evidence of association was found for haplotype CTC with BMI (P = 0.063). This haplotype CTC, accounting for about 40% of the allele frequencies, is the most common form of haplotype. In linkage analyses allele-wise tests treat all haplotypes as one locus, so the results were the same as that of the multiallelic tests. For those unmentioned haplotypes, either no significant results were detected or they were not tested due to insufficient informative subjects. For fat mass, PFM, and lean mass, we did not obtain any significant results for linkage or association.

**DISCUSSION**

In this study, we found significant linkage and association between the UCP3 −55 C/T polymorphism and BMI. Subjects carrying the T allele had, on average, 3.5% lower BMI than those without it. Our results are in agreement with the study in a British Caucasian population (19), wherein carriers of the −55 T allele had a lower BMI. The UCP3 −55 C/T polymorphism was also associated with BMI in French Caucasians in interaction with physical activity (37) and dietary intake and body composition in Hispanic and non-Hispanic white females (11). Association between −55 C/T and obesity-related phenotypes in various populations raises a hypothesis that this polymorphism in the 5′ sequence of the UCP3 gene could modify UCP3 gene expression and therefore modulate energy homeostasis and body weight regulation.

The UCP3 −55C/T transition is placed 55 bp upstream of the most commonly used transcription initiation site of skeletal muscle (14). This polymorphism is potentially interesting because it is located only 6 bp from the TATA box and 4 bp downstream of a putative peroxisome proliferator-activated receptor (PPAR) responsive element and could modify the PPAR responsiveness of the UCP3 gene (2, 41). Thus the UCP3 gene could be one of the PPAR-γ targets involved in the modulation of lipid metabolism and insulin sensitivity. In French Caucasians, the subjects bearing the TT genotype of the UCP3 −55C/T polymorphism had a lower risk for developing Type 2 diabetes than others did (34). In Pima Indians, subjects who carried the −55 T allele had significantly higher UCP3 mRNA concentration than −55 T homozygotes (40), suggesting that the −55 T allele could increase UCP3 mRNA expression compared with the C allele. In addition, the mRNA expression of UCP3 was positively correlated with the sleeping metabolic rate in Pima Indians, and a decrease in the full-length UCP3 message was associated with a increase in BMI, which is conceived to be due to an observed reduction in fat oxidation rates (39). Therefore, a possible explanation for our results is that the −55 T allele is associated with an increase in the UCP3 message, which, if similar to the situation in Pimas, is associated with increased fat oxidation and reduced BMI. Further studies are needed to assess the functional consequence of the −55 C/T polymorphism by examining its direct effects on expression of UCP3.

We also tested the UCP3 Tyr99Tyr and Tyr210Tyr polymorphisms for linkage and association with obesity phenotypes. These two SNPs were selected because they were highly polymorphic in our sample and may render relatively high statistical power, as the statistical power of a family-based association study depends directly on the degree of allelic heterozygosity. Although we did not find any signif-
icant association for Tyr99Tyr and Tyr210Tyr with obesity phenotypes, there was some evidence of linkage, suggesting that they might be close to a functional variant. Haplotype-based analysis, which considers the variants segregating at multiple loci, can provide additional information in studies of complex traits. This has been reflected in our linkage tests for haplotypes, in which the evidence of significance has increased markedly. Haplotype analyses can also provide more information for the transmission disequilibrium test analyses. However, one trade off is the increase in the degrees of freedom of the test. This may partially explain why in allele-wise haplotype tests the significance of association between haplotype CTC and BMI \( (P = 0.063) \) has decreased a bit compared with that between −55 C/T and BMI \( (P = 0.034) \) in single locus analyses.

Recently, there are some debates on whether the primary physiological function of UCP3 is the regulation of energy expenditure (21). For instance, fasting, an energy expenditure attenuating condition, unregulated UCP3 expression (35), and UCP3 knockout mice had a normal metabolic rate and normal body weight, even though their mitochondria showed improved coupling (18, 46). Although the exact function of UCP3 remains to be elucidated, there has been accumulating evidence showing that UCP3 is involved in the regulation of the production of reactive oxygen species, mitochondrial fatty acid transport, and the regulation of glucose metabolism in skeletal muscle (21). Thus an important function of UCP3 might be influencing, if not necessarily regulating, energy metabolism and body weight (21). As such, UCP3 remains an interesting target for the intervention of obesity.

Earlier studies on associations between the UCP3 gene and obesity have yielded inconsistent results. For example, association was reported in a French cohort of morbidly obese subjects, in which the −55 T/T genotype was associated with increased BMI (37). However, in Danish Caucasian subjects, no association was observed between −55 C/T and BMI or long-term body weight change (10). In African American women, no significant variation in resting energy expenditure (REE) was seen for −55 C/T; but for Tyr210Tyr, REE was significantly lower in subjects with the CC genotype than in those with the TT genotype (23).

There are several possible reasons for this inconsistency. First, there might be true variability in association among different populations, particularly of different ethnic groups (31). Even when a causal variant is under investigation, it might be more or less important in different populations, especially if the variant has low genetic effects, variable penetrance, and variable allele frequencies in different populations. In terms of allele frequencies, differences exist for the UCP3 −55 C/T polymorphism between our United States Caucasian nuclear families and French cohort (37) and African American women (23), with the minor allele frequencies being 26.5%, 22%, and 21%, respectively. For Tyr210Tyr, the minor allele frequency was 44.7% in our United States Caucasian families but was 22.7% in African American women (23). Second, diversity in study designs may also contribute to the situation. For example, population stratification, if not checked and controlled, could lead to spurious association outcomes (12). Recent empirical studies demonstrated that modest amount of stratification can exist even in well-designed studies (16, 32). In this regard, our results should be robust because we adopted the transmission disequilibrium test (a family-based disequilibrium test), which obviates concerns about population stratification by examining the transmission of alleles from parents to offspring.

False positive associations may exist in the literature, but there are also many real associations lurking among the data (31). These true associations probably confound modest but real effects on common disease risk. Studies using large samples may convincingly identify such variants, whereas studies of small samples may miss the chance of detection. Our large sample containing 1,873 subjects from 405 nuclear families confirms a relatively high statistical power. Assuming a marker is in strong LD with a functional mutation \( (D' = 0.9) \) that explains 2% of phenotypic variation, this sample has more than 80% power to detect the association via the transmission disequilibrium test.

In this study, the effect of UCP3 −55 C/T on BMI was not reflected on variation in three other phenotypes (fat mass, PFM, and lean mass), although BMI is phenotypically correlated with them. The lack of association may be partially due to insufficient power to detect changes in these phenotypes. On the other hand, a significant and high phenotypic correlation does not necessarily imply a significant and high genetic correlation (13). A limitation with this study is that the quantitative traits studied here were focused on BMI, fat mass, PFM, and lean mass. Some other phenotypes may also be valuable in studying obesity risk. For example, the waist-to-hip ratio is a better index than BMI in reflecting central obesity, which is associated with increased cardiovascular risk. Earlier studies (e.g., Refs. 7 and 20) have found that the UCP3 −55 C/T polymorphism was associated with the waist-to-hip ratio in Caucasian and Asian populations.

In summary, our results suggest that the UCP3 −55 C/T polymorphism may contribute to BMI variation in the general population. Confirmation of the association observed herein awaits further studies such as meta-analysis and/or independent replication studies. Further in vitro functional analyses and clinical studies are also necessary to define the precise contribution of this variant to human obesity.

GRANTS

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