Population-specific coding variant underlies genome-wide association with adiponectin level

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Adiponectin is a protein hormone that can affect major metabolic processes including glucose regulation and fat metabolism. Our previous genome-wide association (GWA) study of circulating plasma adiponectin levels in Filipino women from the Cebu Longitudinal Health and Nutrition Survey (CLHNS) detected a 100 kb two-SNP haplotype at KNG1–ADIPOQ associated with reduced adiponectin (frequency = 0.050, \( P = 1.8 \times 10^{-25} \)). Subsequent genotyping of CLHNS young adult offspring detected an uncommon variant [minor allele frequency (MAF) = 0.025] located \( \sim 800 \) kb from ADIPOQ that showed strong association with lower adiponectin levels \( P = 2.7 \times 10^{-15}, n = 1695 \) and tagged a subset of KNG1–ADIPOQ haplotype carriers with even lower adiponectin levels. Sequencing of the ADIPOQ-coding region detected variant R221S (MAF = 0.015, \( P = 2.9 \times 10^{-69} \)), which explained 17.1% of the variance in adiponectin levels and largely accounted for the initial GWA signal in Filipinos. R221S was not present in 12,514 Europeans with previously sequenced exons. To explore the mechanism of this substitution, we re-measured adiponectin level in 20 R221S offspring carriers and 20 non-carriers using two alternative antibodies and determined that the presence of R221S resulted in artificially low quantification of adiponectin level using the original immunoassay. These data provide an example of an uncommon variant responsible for a GWA signal and demonstrate that genetic associations with phenotypes measured by antibody-based quantification methods can be affected by uncommon coding SNPs residing in the antibody target region.

INTRODUCTION

Secreted almost exclusively from adipocytes into the bloodstream, adiponectin is an abundant protein hormone that affects metabolic processes including glucose regulation and fat metabolism (1). Circulating plasma adiponectin level is substantially heritable (2–4), and low levels are associated with increased body mass index (BMI) and risk of cardiovascular disease, type 2 diabetes, atherosclerosis and metabolic syndrome (1,5–8). Understanding the genetic basis of adiponectin level may help unravel the etiology of these complex diseases.

The gene ADIPOQ, which encodes adiponectin, is an obvious candidate for influencing adiponectin level and its effects on target tissues. Candidate gene association studies (9–12) as well as genome-wide association (GWA) studies (13–16) of common SNPs [minor allele frequency (MAF) \( \geq 0.05 \)] have identified several potentially functional ADIPOQ variants. In vitro studies have shown that rare (MAF \( < 0.005 \)) and uncommon (0.005 \( \leq \) MAF \( < 0.05 \)) missense variants can disrupt the multimerization necessary for adiponectin secretion (17) and that SNPs in the ADIPOQ promoter may alter its transcription (18). Nonetheless, the true causal variants responsible for the previously reported GWA signals at ADIPOQ have not yet been established.

We previously reported a 100 kb two-SNP haplotype spanning the KNG1–ADIPOQ gene region that is associated with reduced adiponectin level in 1776 Filipino women from the Cebu Longitudinal Health and Nutrition Survey (CLHNS)
(19). Compared with the strongest single SNP associated with adiponectin in the GWA study (rs864265, MAF = 0.124, β = −0.123, $P = 3.8 \times 10^{-9}$), the C-T rs11924390-rs864265 haplotype showed substantially stronger evidence of association with a larger effect size (frequency = 0.050, β = −0.385, $P = 1.8 \times 10^{-25}$). Imputation of SNPs from the 1000 Genomes Pilot Project (June 2010) failed to identify any single SNP with association evidence at this level of significance, suggesting either that the true causal variant was poorly imputed, thus weakening its evidence of association, or that a lower frequency variant or more than one variant may be responsible for the GWA signal (19).

In the current study, we further characterized this KNG1–ADIPOQ haplotype association by identifying an uncommon missense variant responsible for much of the signal. In young adult offspring from the CLHNS, in whom the haplotype showed a similar effect (frequency = 0.052, β = −0.386, $P = 8.7 \times 10^{-32}$) (19), the observation of an additional putative association signal in the region led serendipitously, although indirectly, to an explanation for the original GWA signal at KNG1–ADIPOQ.

**RESULTS**

To study the genetic basis of circulating plasma adiponectin level and other metabolic traits, we genotyped using the MetaboChip a set of young adult offspring from the CLHNS. The offspring did not show evidence of population substructure (Supplementary Material, Fig. S1), and an assessment of global ancestry showed them clustering with other Asian individuals as expected (Supplementary Material, Fig. S2). Of 140 696 polymorphic MetaboChip SNPs tested for association with natural log-transformed adiponectin level in 1695 offspring, the two most strongly associated SNPs ($P < 5.0 \times 10^{-8}$) were located ~800 kb apart on chromosome 3 (Fig. 1). Located upstream of the ADIPOQ gene, the first SNP, rs864265 ($P = 1.0 \times 10^{-12}$, Table 1), had been detected by our previous GWA study (19). The second SNP, rs117016164, showed stronger evidence of association ($P = 2.7 \times 10^{-15}$, Table 1), and is located downstream of the ETV5 gene. Originally identified by the 1000 Genomes Project as a singleton in a CHB HapMap sample (NA18593), rs117016164 had not been detected in CEU, JPT or YRI samples. This SNP was uncommon in the CLHNS offspring (MAF = 0.025), and exhibited very low linkage disequilibrium (LD) with all other SNPs within the densely typed ETV5 gene region ($r^2 < 0.1$, chr3:187.2–187.4 Mb in Fig. 1). These data suggested a possible second and novel association signal 800 kb away from the original GWA signal.

To validate the evidence of association of rs117016164 with adiponectin in the CLHNS offspring, we re-genotyped the SNP and, observing concordant genotypes, then performed a permutation test, which generated strong and consistent evidence of association ($P_{\text{perm}} < 10^{-6}$). In addition, the 1764 CLHNS mothers showed directionally consistent association ($P = 3.2 \times 10^{-7}$, Table 1). Using a general linear mixed model accounting for sample relatedness, the combined set of offspring and mothers strengthened the evidence of association of rs117016164 with adiponectin levels ($P_{\text{combined}} = 7.0 \times 10^{-17}$).

We next examined the relationship between rs117016164 and the two SNPs from the previously identified adiponectin-associated KNG1–ADIPOQ haplotype (rs119243940 and rs864265). The SNP rs117016164 was in low LD with both rs119243940 ($D' = 0.38, r^2 = 0.004$) and rs864265 ($D' = 0.37, r^2 = 0.024$). Carrying the C-T haplotype at KNG1–ADIPOQ was associated with lower adiponectin levels in CLHNS offspring ($P = 1.0 \times 10^{-37}$, Fig. 2A). When rs117016164 was included to form three-SNP haplotypes, all carriers of the original two-SNP C-T haplotype still had significantly lower adiponectin levels than non-carriers; they carried haplotype H4 (C-C-T, $P = 2.9 \times 10^{-18}$) and/or haplotype H6 (T-C-T, $P = 2.7 \times 10^{-39}$) (Table 2). A post hoc comparison of these two haplotypes showed that their strengths of association were significantly different ($P = 2.9 \times 10^{-18}$), suggesting that the minor allele (T) of rs117016164 tagged a subset of KNG1–ADIPOQ haplotype carriers with even lower adiponectin levels. A consistent pattern of strong haplotype association was also observed in the mothers (Supplementary Material, Table S1). These data suggested that the H4 and H6 haplotypes together likely harbored two or more SNPs that might account for the observed evidence of association at the KNG1–ADIPOQ locus.

Three pieces of evidence led us to hypothesize that coding variants in ADIPOQ could explain the observed haplotype associations. First, although ETV5 was a plausible candidate gene based on a nearby SNP previously reported to be associated with BMI (20) and the inverse relationship between adiponectin level and BMI, rs117016164 was not located in a conserved region or a regulatory element predicted based on chromatin state. Second, the evidence of

![Figure 1.](image-url)
long haplotypes linking rs117016164 to the two adiponectin-associated SNPs in the \textit{KNG1-ADIPOQ} region reinforced the possibility that causal variants could be located in other genes in the region; among these, \textit{ADIPOQ} was the strongest biological candidate. Third, although decreased gene expression by a non-coding regulatory variant is a more likely scenario at many GWA loci, amino acid substitutions in \textit{ADIPOQ} have been shown to disrupt the multimerization of the adiponectin protein necessary for its secretion into the bloodstream (17). We hypothesized then that one or more low-frequency-coding variants in \textit{ADIPOQ} could have large effects on adiponectin levels consistent with the observed associations.

To identify rare or uncommon coding variants in \textit{ADIPOQ}, we sequenced the translated exons of the gene in 47 CLHNS offspring, a sample set enriched for carriers of the H6 and H4 haplotypes. We observed three variants in the coding region, each in at least one sample: G15G (rs2241766), a common variant that previously showed modest association with adiponectin in the CLHNS mothers ($P = 0.0077$) (19); G48D, a novel missense variant in one individual with average adiponectin level (2.15 $\mu$g/ml); and R221S, a missense variant first reported in Japanese (21). To help predict their potential impact on protein structure and function, we performed bioinformatic annotation of the two observed missense variants. G48D was predicted by polymorphism pheno- typing (PolyPhen) to be ‘likely damaging’ with a score of 0.997 and by sorting intolerant from tolerant (SIFT) to ‘affect protein function’ with a score of 0.00. R221S was predicted by PolyPhen to be ‘likely benign’ with a score of 0.000 and by SIFT to be ‘tolerated’ with a score of 0.41. The difference in the predicted effects of the two variants reflects that \textit{ADIPOQ} amino acid 48 is more highly conserved across related protein sequences, and therefore more sensitive to substitution by an unlike residue. Eight other previously reported missense variants (9,10,21–24) were not found in these 47 offspring samples, including G45R, G84R, G90S, R92X, Y111H, R112C, I164T and H241P. However, as the I164T substitution was uncommon (MAF $= 0.009$) and strongly associated with lower adiponectin in Japanese (12), we included this SNP in follow-up genotyping.

Table 1. Association of three SNPs with plasma adiponectin level in CLHNS offspring and mothers

<table>
<thead>
<tr>
<th>SNP (gene)</th>
<th>Allele 1/2</th>
<th>CLHNS offspring ($n = 1695$)</th>
<th>CLHNS mothers ($n = 1764$)</th>
<th>Combined samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MAF</td>
<td>$\beta$ (SE)</td>
<td>$P$-values</td>
<td>$R^2$</td>
</tr>
<tr>
<td>rs864265 (\textit{ADIPOQ})</td>
<td>T/G</td>
<td>0.129 $-0.134$ (0.018)</td>
<td>$1.0 \times 10^{-13}$</td>
<td>0.032</td>
</tr>
<tr>
<td>rs117016164 (\textit{ETV5})</td>
<td>T/C</td>
<td>0.025 $-0.304$ (0.038)</td>
<td>$2.7 \times 10^{-15}$</td>
<td>0.036</td>
</tr>
<tr>
<td>R221S (\textit{ADIPOQ})</td>
<td>A/C</td>
<td>0.015 $-0.853$ (0.046)</td>
<td>$2.9 \times 10^{-69}$</td>
<td>0.171</td>
</tr>
</tbody>
</table>

Effect betas ($\beta$), standard errors (SE), $P$-values and squared type-II partial correlations ($R^2$) are reported in terms of the minor allele (underlined) and for natural log-transformed values of adiponectin. MAFs are reported for all 1774 unrelated offspring and 1798 mothers, regardless of covariate completeness. In the analyses of offspring, models were adjusted for sex and BMI. In the analyses of mothers, models were adjusted for age, age$^2$, household assets, natural log-transformed income, menopausal status and BMI. In the combined analyses, models accounting for sample relatedness were adjusted for sex, age, age$^2$, household assets, natural log-transformed income and BMI. All covariates were from the 2005 survey.

Figure 2. Association of \textit{KNG1–ADIPOQ} haplotype in CLHNS offspring is attenuated by \textit{ADIPOQ} missense variant R221S. Adiponectin residuals plotted by number of copies of (A) the associated C-T \textit{KNG1–ADIPOQ} haplotype, (B) the \textit{ADIPOQ} missense variant R221S and (C) the \textit{KNG1–ADIPOQ} haplotype when conditioned on R221S.
We directly genotyped three candidate coding variants (G48D, I164T and R221S) in the complete sets of offspring and mothers, and tested each for association with plasma adiponectin level. No additional G48D carriers were identified, and I164T was monomorphic in both the mothers and offspring. In contrast, R221S was present in the offspring with MAF = 0.015, and carriers had significantly lower adiponectin levels than non-carriers (P = 2.9 × 10^{-6}).

The ADIPOQ R221S variant was in low LD with the ETV5 variant (rs117016164) (D' = 0.54, r^2 = 0.17). We observed similar evidence of R221S association in the mothers (P = 2.0 × 10^{-53}) (Supplementary Material, Fig. S3, Table S1) and in the combined mothers and offspring (P_{combined} = 4.3 × 10^{-99}) (Table 1). R221S explained 17.1 and 13.2% of phenotypic variation in the offspring and mothers, respectively.

To determine the relationship between the uncommon coding variant R221S and the associated haplotypes, we performed conditional analyses. Conditioning on R221S, offspring carriers of the two-SNP C-T KNG1–ADIPOQ haplotype still had significantly lower adiponectin levels, but the association was substantially attenuated (P = 5.6 × 10^{-10}, Fig. 2C). Carriers of the three-SNP H6 haplotype showed greatly attenuated association (P = 2.9 × 10^{-14}). The mothers showed similar attenuations (Supplementary Material, Fig. S4). To examine the associations of additional variants in the 187.2–188.2 Mb region of chromosome 3, we re-evaluated the evidence of adiponectin association after conditioning on R221S. Overall, there were no substantial changes in any SNP associations at loci other than KNG1–ADIPOQ in the offspring (data not shown) or mothers (Supplementary Material, Fig. S4), including at CDH13, a previously reported GWA signal in the CHLNS (19); similar results were observed after removing R221S carriers from analysis (data not shown and Supplementary Material, Fig. S4).

Table 2. Association of three-SNP haplotypes with plasma adiponectin level in CLHNS offspring

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>rs117016164</th>
<th>rs11924390</th>
<th>rs864265</th>
<th>Haplotype frequency</th>
<th>Number of carriers</th>
<th>β</th>
<th>SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>C</td>
<td>C</td>
<td>G</td>
<td>0.435</td>
<td>1196</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>H2</td>
<td>C</td>
<td>T</td>
<td>G</td>
<td>0.421</td>
<td>1166</td>
<td>0.017</td>
<td>0.012</td>
<td>0.15</td>
</tr>
<tr>
<td>H3</td>
<td>C</td>
<td>T</td>
<td>T</td>
<td>0.075</td>
<td>256</td>
<td>0.022</td>
<td>0.022</td>
<td>0.31</td>
</tr>
<tr>
<td>H4</td>
<td>C</td>
<td>C</td>
<td>T</td>
<td>0.044</td>
<td>154</td>
<td>-0.237</td>
<td>0.029</td>
<td>2.9 × 10^{-14}</td>
</tr>
<tr>
<td>H5</td>
<td>T</td>
<td>C</td>
<td>G</td>
<td>0.010</td>
<td>33</td>
<td>-0.060</td>
<td>0.058</td>
<td>0.30</td>
</tr>
<tr>
<td>H6</td>
<td>T</td>
<td>T</td>
<td>G</td>
<td>0.005</td>
<td>19</td>
<td>0.035</td>
<td>0.076</td>
<td>0.64</td>
</tr>
<tr>
<td>H7</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>0.001</td>
<td>5</td>
<td>-0.199</td>
<td>0.164</td>
<td>0.23</td>
</tr>
</tbody>
</table>

Effect betas (β), standard errors (SE) and P-values were calculated for each haplotype relative to the most common reference haplotype. Frequencies are reported for all 1774 unrelated offspring, regardless of covariate completeness. Significant associations (P < 0.05) are in boldface. Models were adjusted for sex and for BMI from the 2005 survey. A post hoc comparison of H4 and H6 showed that their strengths of association were significantly different (P = 2.9 × 10^{-14}).
(P = 0.076) and the most significantly associated SNP was rs58575091 (P = 3.7 × 10⁻⁷). We re-evaluated eight ADIPOQ SNPs that were identified as candidate functional variants in the literature (19), and of those, only rs266729 and rs182052 still showed nominal association (P < 0.05) after conditioning on R221S (Supplementary Material, Table S2). These associations remained significant after conditioning on both R221S and copies of the C-T haplotype (rs862465, MAF = 0.129) explained 3.2 and 2.2% of variation, respectively. We discovered R221S by detecting in the offspring a long three-SNP haplotype created by another uncommon SNP nearly 800 kb away from ADIPOQ. This SNP (rs117016164) was genotyped fortuitously, as it was included on the MetaboChip to fine-map the ETV5 gene region previously associated with BMI (20). The location of rs117016164 downstream of ETV5 and its lack of overlap with predicted regulatory elements in a relevant cell type, such as adipocytes, made ETV5 a less compelling candidate gene for regulating adiponectin level. Furthermore, rs117016164 was the only variant in the ETV5 region to show evidence of association comparable with that of rs862465 near ADIPOQ, suggesting the presence of a long haplotype spanning the entire region. Prior biochemical experiments have shown that ADIPOQ amino acid substitutions can disrupt the multimer aggregation required for adiponectin secretion (17). Targeted sequencing of ADIPOQ-coding regions in carriers of the associated haplotypes then revealed R221S, which segregated primarily on the two long haplotypes associated with lower adiponectin level in the entire cohort. Therefore, the causal variant was not only detectable 800 kb away, but was also nearly 10 times less frequent and explained a far greater proportion of phenotypic variance than the GWA index SNP, in line with a previously proposed association causation scenario (25). The proportion of reported GWAS signals explained by uncommon variants remains to be determined, but R221S is a clear example of one.

Because R221S has been reported previously only in Japanese (21), Korean (26,27) and Japanese–Brazilian (28) cohorts, the variant may be specific to Asian-ancestry populations. This R221S variant (CGT to AGT) was also observed to be monomorphic in 12,514 individuals of European ancestry with sequenced exons (M.R. Nelson et al., manuscript in preparation). A different ADIPOQ missense variant (I164T) with low frequency in Japanese (MAF = 0.009) has been shown to be associated with lower adiponectin levels (12), consistent with biochemical evidence that the I164T-containing adiponectin does not form trimers or high-molecular-weight multimers and impairs secretion from the adipocyte (17). However, we did not observe I164T or any other of seven known missense variants among the CLHNS samples.

The observed data did not support a biological role for R221S in lowering adiponectin level. Despite explaining ~13–17% of variation in adiponectin levels in the CLHNS, we observed no evidence of association of R221S with metabolic traits or with waist circumference, which is a well-known predictor of adiponectin levels. This result could reflect limited power. R221S also did not show any deficiency in formation of adiponectin trimers, hexamers or high-molecular-weight multimers in vitro (17), and bioinformatic annotations that suggested R221S would not substantially alter the protein’s conformation. Among the previous reports of R221S, adiponectin levels in carriers were significantly lower (28), indistinguishable from non-carriers (21), or not reported due to low MAF (26,27). We hypothesized that the R221S variant is located on the surface of the protein at a position that may alter the epitope recognized by the adiponectin antibody. Two previous reports support this scenario. The first describes an African-American-specific K29M amino acid substitution in the soluble circulating...
they illustrate an unusual combination of association SNPs databases. Although these findings as a whole do not elu-
the value of targeted sequencing for discovering a variant residing in the antibody target region. We also demonstrated

tions with phenotypes measured by antibody-based quantifica-
common variants. Our work highlights how genetic associa-
tion measurements in a subset of samples and thus was largely
adiponectin-coding gene resulted in artificially low adiponec-
that a population-specific uncommon missense variant in the
plasma adiponectin level in a Filipino cohort and showed

in vitro
factor-binding site (32). It is also part of a three-SNP haplo-
type shown to have an in vitro effect on ADIPOQ promoter ac-
tivity and on DNA-binding activity of nuclear proteins (18).
Other putative regulatory regions near ADIPOQ (33–35) may also be promising targets for additional variants. Ultim-
ately, both more comprehensive sequencing of the ADIPOQ gene region and improved imputation of SNPs from the
1000 Genomes Project may be needed to systematically identify additional candidate causal variants, especially of
low frequency.

In summary, we characterized a GWA signal for lower plasma adiponectin level in a Filipino cohort and showed
that a population-specific uncommon missense variant in the adiponectin-coding gene resulted in artificially low adiponec-
tin measurements in a subset of samples and thus was largely responsible for the observed association signal detected with
common variants. Our work highlights how genetic associa-
tions with phenotypes measured by antibody-based quantifica-
tion methods can be affected by uncommon coding SNPs residing in the antibody target region. We also demonstrated
the value of targeted sequencing for discovering a variant with potential functional relevance not yet present in public
SNP databases. Although these findings as a whole do not elu-
cidate the biology underlying the genetics of adiponectin level, they illustrate an unusual combination of association
pheno

MATERIALS AND METHODS

Subjects
The CLHNS participants available for this study included 1798 mothers and 1779 male and female young adult offspring from a 1983–1984 Filipino birth cohort (36). Trained field
staff conducted in-home interviews and collected quantitative anthropometric measurements, blood samples and comprehensive
environmental data (available on-line at http://www.cpc.
unc.edu/projects/cebu/). Outcome and covariate measures were taken from the 2005 survey. Informed consent was
obtained from all CLHNS subjects, and the study protocol
was approved by the University of North Carolina Institutional Review Board for the Protection of Human Subjects. Basic
descriptive characteristics of the mothers and offspring are summarized in Supplementary Material, Table S3.

Genotyping and imputation
The 1779 CLHNS offspring were genotyped for 196 725 SNPs on the MetaboChip (Illumina, San Diego, CA, USA), a custom
array of SNPs designed to replicate and fine-map loci associated with metabolic traits. Genotyping was performed by the
Mammalian Genotyping Core at the University of North Carolina at Chapel Hill (UNC-CH) using the protocol recom-
manded by the manufacturer. Individual sample success rates exceeded 98.6%. Due to poor genotype clustering, 8610 SNPs
were removed from analysis. SNP quality control filtering was then performed using PLINK v1.07 (37). We removed 1652
SNPs for success rates ≤97%, 126 SNPs for deviation from Hardy–Weinberg equilibrium (P < 10−6), 228 SNPs for Mendel-
ian inheritance errors (combined ≥3 discrepancies among 79 duplicate pairs and 4 HapMap CEPH trios genotyped on the
MetaboChip), 21 SNPs for ≥3 genotype discrepancies with available HapMap genotypes for the 4 CEPH trios and
45 397 SNPs that were monomorphic. Five SNPs did not pass more than one of these filters, so the final set of high-
quality MetaboChip data included 140 696 SNPs.

Using MACH version 1.0 (38), additional genotypes of
SNPs in the 187.2–188.2 Mb region of chromosome 3 from the
November 2010 release (23 November 2010) of the
1000 Genomes Project were imputed in the mothers based on reference haplotypes from CEU, CHB and JPT samples. MACH
was also used to generate best-guess phased haplo-
types (those with highest posterior probabilities) of 351 directly

gotyped SNPs comprising the ETV5-KNG1-ADIPOQ gene region in the mothers (19) and of 1148 SNPs in the off-
spring. To infer high-quality haplotypes, we specified 200 rounds of Markov sampling considering 500 haplotype states
when updating each individual.

Additional SNPs were genotyped using TaqMan allelic
discrimination (Applied Biosystems, Foster City, CA, USA), in-
cluding ADIPOQ missense SNPs G48D (rs469105329), I164T
(rs469105330) and R221S (rs469105331), and MetaboChip
ETV5 SNP rs117016164 (chr3: 187 239 040, hg18). Primer
sequences are provided in Supplementary Material,
The two KNG1-ADIPOQ SNPs rs119243940 and rs864265 were previously genotyped (19). Success rates were >95% and all SNP genotypes were consistent with Hardy–Weinberg equilibrium ($P > 0.05$). The non-missing TaqMan genotypes of rs117016164 had 99.8% concordance with the original MetaboChip genotypes. Bioinformatic annotations were assigned to missense variants in the adiponectin protein (Uniprot ID: Q15848) using PolyPhen (version 2) (39) and SIFT (40), each using default parameters.

Sequencing

We sequenced the two translated exons of ADIPOQ in 47 offspring samples by PCR amplification and Sanger sequencing. We chose all samples with adiponectin residuals >2.5 standard deviations above or below the population mean. Primer sequences were previously published (21). Sequencing was performed by the UNC-CH Genome Analysis Facility on a 3730xl DNA Analyzer (Applied Biosystems).

Statistical analyses

Adiponectin levels were natural log-transformed to approximate normality and SNPs were tested for association using multivariable linear regression models in PLINK. We assumed an additive mode of inheritance reporting $β$ coefficients representing the estimated change in mean transformed trait value due to each additional copy of the minor allele. In the offspring, 1 member of each of 5 twin pairs were removed and 79 additional samples were excluded because they lacked non-pregnant measures of BMI in 2005. The final sample sets consisted of 1695 non-sibling offspring and 1764 mothers with complete sets of outcomes and covariates. Sex and BMI covariates were significantly associated ($P < 0.05$) with plasma adiponectin level in the offspring (Supplementary Material, Table S5). In the mothers, age, median-centered age, household assets, natural log-transformed household income and menopausal status during the 2005 survey were included as covariates, as previously described (19). For combined analysis, age, mean-centered age, household assets, natural log-transformed household income and BMI during the 2005 survey were used as covariates. Combined analysis of selected SNPs in the offspring and mothers together was performed in SAS version 9.3 (SAS, Inc., Cary, NC, USA) using a general linear mixed model that accounted for the relatedness between mother–child pairs. Conditional analyses of directly genotyped SNPs in the offspring and mothers were performed in SAS using linear regression. Conditional analyses of imputed SNPs in the mothers adjusting for R221S were performed using MACH2QTL (38). Haplotype association analyses in the offspring and mothers were performed in SAS. All conditional and haplotype analyses used the corresponding outcome and covariates from the main association analyses.

To capture population substructure in the offspring, we constructed 10 principal components (PCs) using the software EIGENSOFT (41,42). We first identified a set of 40 239 independent MetaboChip SNPs (estimated $r^2 < 0.001$ between all pairs of SNPs within 1 Mb windows) with observed MAF >0.05. PCs were then constructed in 1670 CHLNS offspring who had pair-wise identity-by-descent ($\hat{r}$) <0.1 with all other samples, as estimated using PLINK. The remaining 104 offspring who had $\hat{r} \geq 0.1$ with any other sample(s) were then projected onto these PCs. Supplementary Material, Figure S1 shows the first two PCs in all 1774 offspring samples. We also performed a complementary analysis of global ancestry in these samples (Supplementary Material, Fig. S2) by re-generating PCs using a subset of 24 033 SNPs shared with 165 CEU, 84 CHB, 86 JPT and 167 YRI HapMap Phase III samples (genotypes available at ftp://ftp.ncbi.nlm.nih.gov/hapmap/genotypes/hapmap3/plink_format/draft_2/). The construction of PCs in the CLHNS mothers has been described previously (43). No PCs were associated with adiponectin level in either the offspring (Supplementary Material, Table S5) or mothers (19).

Quantification of adiponectin level

Adiponectin level in plasma was originally measured in the offspring and mothers with a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA, #DY1065) consisting of two monoclonal antibodies that recognize epitopes within amino acids 104–244. Selected offspring samples ($n = 40$) re-measured by western blot included 20 R221S carriers and 20 sex- and BMI-matched non-carriers. The distributions of adiponectin and BMI were comparable between these two subsets and the respective groups from which they were drawn (data not shown). Plasma proteins were resolved by polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Invitrogen, Carlsbad, CA, USA). Duplicate membranes were separately probed with rabbit polyclonal antisera generated by peptide antigens DQETTTQGPVG (44) and DGRDTGPEKEKGSGD (Sigma, St Louis, MO, USA), corresponding to amino acids 17–27 and 56–70, respectively. Alexa Fluor 680-conjugated goat anti-rabbit IgG was then used as a secondary antibody (Invitrogen). Proteins were detected using an Odyssey imaging system (LI-COR, Lincoln, NE, USA) and the adiponectin bands (30 kDa) were quantified by densitometry using the LI-COR software. The adiponectin standards used for each assay did not allow direct comparison of the original and alternative measurements. Thus, all densitometry readings using a given antibody were natural log-transformed and zero-centered on the mean value of R221S non-carriers from that group.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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