

Genome-wide association study identifies polymorphisms in *LEPR* as determinants of plasma soluble leptin receptor levels

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Plasma soluble leptin receptor (sOB-R) levels were inversely associated with diabetes risk factors, including adiposity and insulin resistance, and highly correlated with the expression levels of leptin receptor, which is ubiquitously expressed in most tissues. We conducted a genome-wide association study of sOB-R in 1504 women of European ancestry from the Nurses' Health Study. The initial scan yielded 26 single nucleotide polymorphisms (SNPs) significantly associated with sOB-R levels ($P < 5 \times 10^{-8}$); all mapping to the leptin receptor gene (*LEPR*). Analysis of imputed genotypes on autosomal chromosomes revealed an additional 106 SNPs in and adjacent to this gene that reached genome-wide significance level. Of these 132 SNPs (including two non-synonymous SNPs, rs1137100 and rs1137101), rs2767485, rs1751492 and rs4655555 remained associated with sOB-R levels at the 0.05 level ($P = 9.1 \times 10^{-9}$, 0.0105 and 0.0267, respectively) after adjustment for other univariately associated SNPs in a forward selection procedure. Significant associations with these SNPs were replicated in an independent sample of young males ($n = 875$) residing in Cyprus ($P < 1 \times 10^{-4}$). These data provide novel evidence revealing the role of polymorphisms in *LEPR* in modulating plasma levels of sOB-R and may further our understanding of the complex relationships among leptin, leptin receptor and diabetes-related traits.

INTRODUCTION

Leptin receptor (OB-R) plays an essential role in the pleiotropic effects of leptin on body weight regulation, reproduction

and metabolic pathways (1–3). In *db/db* mice and *fa/fa* rats that express truncated OB-R lacking a functional intracellular domain, a phenotype characteristic of early onset of obesity

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and extreme resistance to insulin develops (4). Likewise, in humans, a homozygous mutation in the human leptin receptor gene (*LEPR*) located on chromosome 1p31 results in truncated OB-R and, subsequently, early onset of severe obesity, as well as lack of pubertal development and decreased secretion of growth hormone and thyroid-stimulating hormone (5). In humans with normal *LEPR* gene expression, several isoforms of membrane-bound OB-R with identical extracellular and transmembrane domains but a variable intracellular domain are expressed on the surface of a wide spectrum of cells in almost all tissues through posttranscriptional alternative RNA splicing (6). According to the length of the intracellular domain, these isoforms can be classified into a long form (OB-R_L) primarily expressed in the hypothalamus and several short forms (OB-R_S) primarily expressed in peripheral tissues (6,7). The OB-R_L mediates leptin's effects on the central neural system, such as body weight control and appetite regulation, whereas the function of OB-R_S is not entirely clear (6,7). Soluble OB-R (sOB-R) is a special isoform of leptin receptor in the circulation that lacks both transmembrane and intracellular domains (6). sOB-R is formed by ectodomain shedding of OB-R expressed in peripheral tissues (8,9). sOB-R levels are highly correlated with the cell surface expression of OB-R ($r = 0.80$) (10), suggesting sOB-R levels may serve as a surrogate of the cell surface expression levels of OB-R in peripheral tissues. Studies have documented inverse correlations of sOB-R levels with adiposity and insulin resistance (11–14). Consistently, we recently found a striking inverse association between sOB-R levels and risk of developing type 2 diabetes (15). While evidence regarding the role of sOB-R in leptin metabolism and diabetes etiology is accumulating, thus far, little data existed regarding the genetic determinants of sOB-R levels. Although a few functional variants in *LEPR* region have been hypothesized to be such determinants, in a Japanese population two non-synonymous single nucleotide polymorphisms (SNPs) in *LEPR* region were not associated with variations of sOB-R levels (14). We, therefore, performed a genome-wide association (GWA) study of sOB-R levels in 1504 women from the Nurses' Health Study (NHS) to elucidate on the genetic variants predicting sOB-R levels.

RESULTS

The study population consisted of 684 type 2 diabetes cases and 820 diabetes-free controls from NHS that had both sOB-R measurements and genotype data. Distribution of age, body mass index (BMI), and other diabetes lifestyle and dietary risk factors is shown in Supplementary Material, Table S1. All participants had European ancestry as defined using the principal component of genetic variation based on the full NHS type 2 diabetes GWA study (which includes subjects with self-described African American and Asian ancestry) and the HapMap CEU, YRI and CHB/JPT samples. The mean age of this study sample was 56.2 years (SD = 6.9 years) and the mean level of sOB-R was 31.4 ng/ml (SD = 10.5 ng/ml). The majority (77.7%) of our study participants were postmenopausal. We used Affymetrix Genome-Wide Human 6.0 array to measure genotypes. In the initial scan, 704 409 SNPs passing quality control filters were tested for association with log-

transformed sOB-R levels using linear regression. Since sOB-R levels are strongly correlated with BMI and diabetes status (12,13,16) and further influenced by fasting status (11), we adjusted for these covariates together with age, menopausal status and postmenopausal hormone use in the regression analysis. In the initial GWA scan, we identified 26 SNPs mapping to the *LEPR* region on chromosome 1p31 that accounted for the excess P -values $< 5 \times 10^{-8}$ (Table 1). All are intronic SNPs with the exception of rs1137100 (Lys109Arg). SNP rs12062820 was most strongly associated with sOB-R levels ($P = 1.56 \times 10^{-14}$) and has a minor allele frequency (MAF) of 17%. Each copy of the minor allele (C, non-ancestral) was associated with an increase in covariate-adjusted sOB-R levels.

To assess whether any stronger genetic markers of sOB-R levels exist in the *LEPR* region, we expanded our genome scan by imputing 2 543 887 SNPs spanning chromosomes 1–22. One hundred and six additional SNPs achieved GWA significance ($P < 5 \times 10^{-8}$). Each was imputed with high accuracy (MACH $r^2 \geq 0.70$) and all mapped to *LEPR* or regions adjacent to *LEPR* (Fig. 1 and Supplementary Material, Table S2). The strongest association was found for rs2767485 ($P = 5.77 \times 10^{-15}$); an intronic SNP with a MAF of 17%. A second non-synonymous SNP, rs1137101 (Gln223Arg; $P = 1.91 \times 10^{-10}$), in the gene was also identified. These genotyped and imputed SNPs with GWA significance span six haplotype blocks in *LEPR* (CEU HapMap Release 22). We searched for *LEPR* expression levels for SNPs in *LEPR* region in a database (mRNA by SNP Browser V1.0.1) (17,18). Five SNPs in *LEPR* region (rs10158579, rs1171278, rs2025805, rs970467 and rs7602) were significantly associated with average *LEPR* expression levels (all P -values were $\leq 1.0 \times 10^{-4}$).

In secondary analyses that were restricted to non-diabetic women produced similar results, although P -values were somewhat augmented probably due to decreased statistical power (Supplementary Material, Table S3). When we conducted GWA scan in cases and controls separately and pooled the results of these two groups using a fixed-effects linear regression model, we observed a largely similar set of SNPs in *LEPR* region or regions adjacent to *LEPR* (Supplementary Material, Table S4). In addition, the directions of the associations for these SNPs were remarkably consistent between cases and controls. The quantile–quantile plot suggests no evidence of systematic bias in the distribution of P -values for the analysis; the genomic inflation factor $\lambda = 1.006$ (Supplementary Fig. S1A). Similar results were observed with further adjustment for residual population structure within this European-ancestry sample: $\lambda = 1.010$ (Supplementary Fig. S1B).

To identify SNPs independently associated with sOB-R levels, we performed a forward selection procedure of all genome-wide significant SNPs. We forced age at blood draw, BMI, diabetes case-control and fasting status, menopausal status, postmenopausal hormone use, as well as the two non-synonymous SNPs (rs1137100 and rs1137101) into the regression model. This procedure yielded three SNPs that were independently associated with sOB-R levels at the 0.05 significance level: rs2767485 ($P = 9.08 \times 10^{-9}$), rs1751492 ($P = 0.0105$) and rs4655555 ($P = 0.0267$) (Table 2). We calculated the percent of variation of sOB-R independently explained by the adjusted covariates and SNPs in the final model. This percent was 0.51% for age at blood draw,

Table 1. Top genotyped SNPs ($P < 5 \times 10^{-8}$) for plasma sOB-R levels^a in the Nurses' Health Study

SNP ^b	<i>n</i>	Position (bp) ^c	Alleles (+/-)	MAF ^d	Geometric LS mean (95% CI), ng/ml ^e			Beta ^f	SE	<i>P</i> -value ^f
					0	1	2			
rs12062820	1504	65743083	T/C	0.17	29.0 (28.5–29.5)	31.7 (30.9–32.5)	36.1 (33.2–39.1)	0.096	0.012	1.56E–14
rs11208656	1504	65743375	A/G	0.17	29.0 (28.5–29.5)	31.7 (30.9–32.5)	36.1 (33.2–39.1)	0.096	0.012	1.56E–14
rs1782755	1504	65759392	C/A	0.17	29.0 (28.5–29.5)	31.6 (30.9–32.4)	36.1 (33.4–39.0)	0.095	0.012	1.91E–14
rs1751492	1504	65765213	T/C	0.30	31.3 (30.7–31.8)	29.0 (28.4–29.6)	26.9 (25.8–28.1)	–0.076	0.010	6.42E–13
rs2154381	1504	65768289	A/G	0.30	31.3 (30.7–31.8)	29.0 (28.4–29.6)	26.9 (25.7–28.1)	–0.075	0.010	7.41E–13
rs1186403	1503	65773121	T/C	0.30	31.3 (30.7–31.8)	29.0 (28.4–29.6)	26.9 (25.7–28.1)	–0.075	0.010	8.51E–13
rs6697315	1504	65766144	T/C	0.36	31.4 (30.8–32.1)	29.3 (28.7–29.9)	27.4 (26.4–28.4)	–0.069	0.010	7.14E–12
rs10889556	1504	65742140	A/G	0.27	31.1 (30.6–31.7)	28.9 (28.3–29.5)	26.7 (25.3–28.1)	–0.075	0.011	7.23E–12
rs11208674	1504	65811905	T/C	0.29	31.2 (30.6–31.7)	29.0 (28.4–29.7)	27.0 (25.8–28.2)	–0.072	0.010	9.27E–12
rs4655518	1503	65812446	T/G	0.29	31.2 (30.6–31.7)	29.0 (28.4–29.7)	27.0 (25.8–28.2)	–0.072	0.010	1.16E–11
rs1475398	1495	65755845	G/C	0.27	31.1 (30.5–31.7)	28.9 (28.3–29.5)	26.7 (25.4–28.1)	–0.075	0.011	1.62E–11
rs10493379	1504	65818515	G/A	0.28	31.1 (30.5–31.7)	29.0 (28.4–29.6)	26.9 (25.7–28.2)	–0.071	0.011	3.09E–11
rs3790425	1504	65815700	A/G	0.28	31.1 (30.5–31.6)	29.0 (28.4–29.6)	26.9 (25.7–28.2)	–0.071	0.011	3.23E–11
rs11208679	1502	65822326	G/A	0.28	31.1 (30.5–31.7)	29.0 (28.4–29.6)	26.9 (25.7–28.2)	–0.071	0.011	3.78E–11
rs1171261	1504	65773990	C/T	0.27	31.1 (30.5–31.7)	28.9 (28.3–29.5)	26.9 (25.6–28.4)	–0.073	0.011	3.83E–11
rs2104564	1495	65742018	T/C	0.27	31.1 (30.6–31.7)	28.9 (28.3–29.5)	26.7 (25.4–28.2)	–0.073	0.011	4.52E–11
rs10158279	1504	65806284	G/T	0.50	28.2 (27.4–28.9)	29.9 (29.4–30.5)	31.8 (31.0–32.6)	0.061	0.009	1.12E–10
rs1137100	1504	65809029	A/G	0.27	31.0 (30.5–31.6)	29.0 (28.4–29.6)	26.8 (25.4–28.2)	–0.071	0.011	1.71E–10
rs10789184	1504	65809550	G/A	0.27	31.0 (30.5–31.6)	29.0 (28.4–29.6)	26.8 (25.4–28.2)	–0.071	0.011	1.71E–10
rs12405556	1504	65835705	G/T	0.26	31.0 (30.5–31.6)	28.9 (28.3–29.5)	27.0 (25.7–28.5)	–0.069	0.011	2.88E–10
rs10889567	1504	65829638	T/C	0.45	31.4 (30.7–32.2)	29.9 (29.4–30.5)	27.9 (27.0–28.7)	–0.059	0.009	5.54E–10
rs10789190	1504	65836168	G/A	0.45	31.4 (30.7–32.2)	29.9 (29.4–30.5)	27.9 (27.0–28.7)	–0.059	0.009	5.54E–10
rs10128072	1504	65729684	A/C	0.13	29.3 (28.8–29.7)	31.8 (31.0–32.7)	34.9 (31.6–38.5)	0.084	0.014	1.43E–9
rs1475397	1456	65755746	C/T	0.26	29.1 (28.6–29.6)	30.4 (29.8–31.1)	34.6 (32.9–36.4)	0.067	0.011	1.64E–9
rs7602	1480	65670539	G/A	0.20	29.0 (28.5–29.5)	31.4 (30.7–32.1)	33.1 (31.2–35.1)	0.071	0.012	1.93E–9
rs17127673	1478	65728313	A/G	0.13	29.3 (28.8–29.7)	31.8 (31.0–32.7)	34.7 (31.6–38.2)	0.084	0.014	2.42E–9

^asOB-R levels were log-transformed.^bAll SNPs are in *LEPR* gene. Except rs1137100, which is a missense SNP (Lys109Arg) in *LEPR*, all SNPs are in introns of *LEPR*.^cPosition based on NCBI build 36.3.^dMinor allele frequency among study participants.^eLeast square geometric means according to the number of minor allele copies were adjusted for age at blood draw, diabetes case–control status, fasting status, BMI, menopausal status and postmenopausal hormone use.^fRegression coefficients and *P*-values for every one copy of minor allele were estimated from linear regression models adjusted for the same set of covariates.

15.60% for BMI, 3.34% for diabetes status, 0.02% for fasting status, 1.25% for menopausal status, 0.55% for postmenopausal hormone use, 0.14% for rs1137100, 0.17% for rs1137101, 2.19% for rs2767485, 0.44% for rs1751492 and 0.33% for rs4655555. Jointly, these five SNPs explained 4.59% of the total variation of sOB-R levels.

We estimated pairwise LD of each SNP with rs2767485 in the NHS sample (Fig. 2). All SNPs with $P < 1 \times 10^{-13}$ are in strong to perfect LD with rs2767485 ($r^2 \geq 0.79$), whereas rs1751492, rs4655555 and the two functional non-synonymous SNPs are only in weak LD with rs2767485 ($r^2 < 0.20$). With respect to pairwise LD among rs2767485, rs1751492, rs4655555 and the two non-synonymous SNPs, with exception of the strong LD between rs1751492 and rs1137100 ($r^2 = 0.84$), these SNPs are only in weak to moderate LD with each other ($r^2 \leq 0.52$). Based on these five SNPs, we were able to construct six haplotypes. None of these haplotypes provided additional predictive value for sOB-R levels beyond these SNPs (data not shown).

We further examined associations between these SNPs in relation to other diabetes-related traits, including BMI, waist-to-hip ratio, high-molecular-weight adiponectin and inflammatory biomarkers (Supplementary Material, Table S5). We found a significant inverse association between rs2767485 and fasting insulin levels ($P = 0.008$)

and a significant positive associate between rs4655555 and CRP levels ($P = 0.005$). *P*-values for other associations were not significant or with borderline significance.

In an independent sample of 875 adolescent males (mean age = 18.4 years; SD = 0.6 years) studied during an initial health screening in preparation for their 2-year mandatory service in the Cyprus Army, SNPs rs1137100, rs1137101, rs2767485, rs1751492 and rs4655555 were each significantly associated with sOB-R levels after adjustment for age and BMI (Table 3). The direction of these associations was also consistent with those observed in the NHS sample.

In addition to the top SNPs with GWA significance, we also identified a number of genotyped and imputed SNPs on various chromosomes that reached suggestive significance level ($P < 1 \times 10^{-5}$) (Supplementary Tables S6 and S7). After excluding SNPs that are in or adjacent to *LEPR* region (65000 and 67000 kb on chromosome 1), we re-plotted the quantile–quantile plot before (Supplementary Fig. S2A) and after (Supplementary Fig. S2B) adjustment for the residual population structure and we observed genomic inflation factors ($\lambda = 1.006$ and 1.009, respectively) that were similar to those based on the complete list of SNPs.

Lastly, since the two non-synonymous SNPs (rs1137100 and rs1137101) may potentially influence the affinity of antibody used in ELISA assay to detect sOB-R levels, we

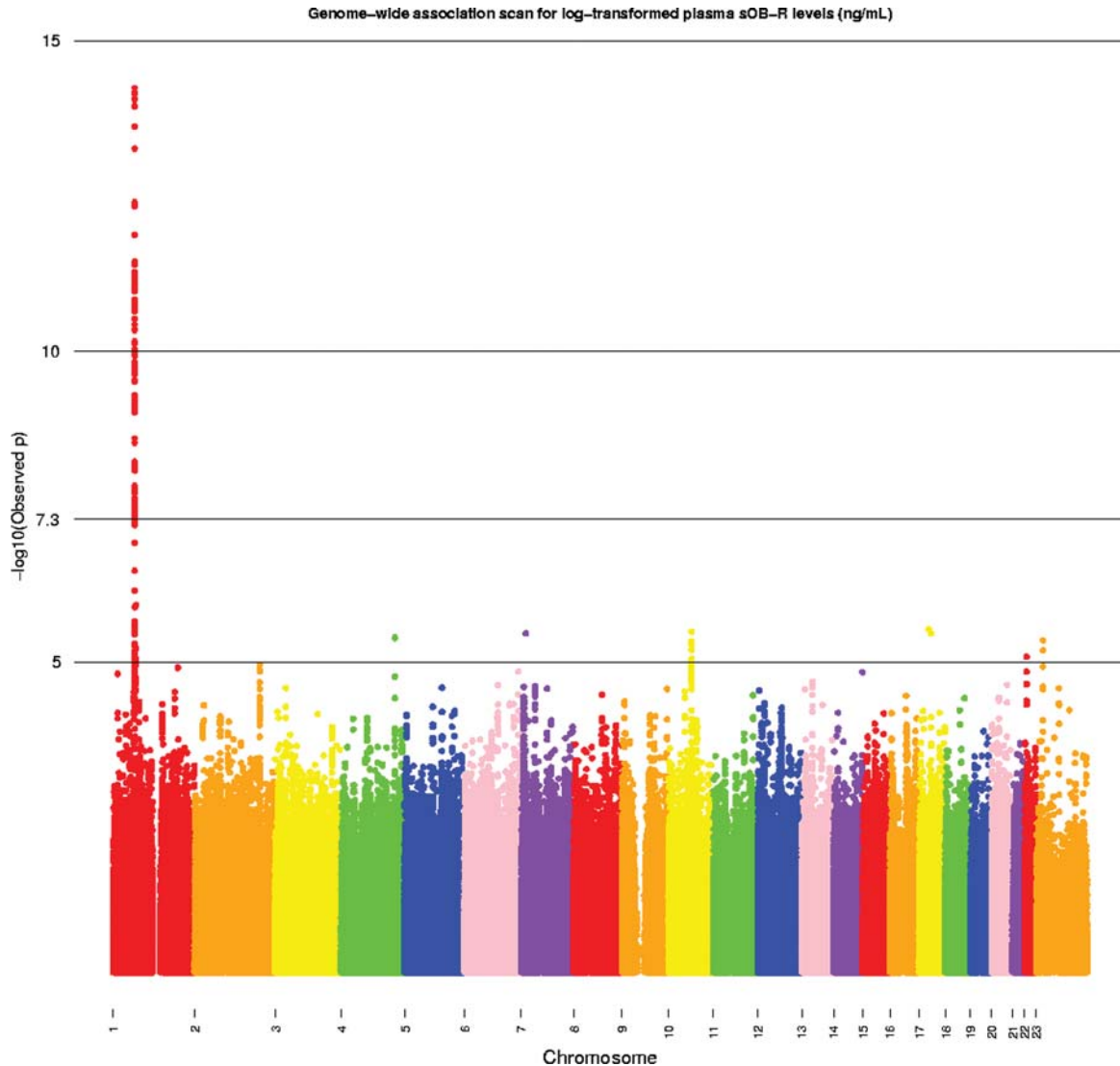


Figure 1. Genome-wide scan of plasma sOB-R levels in the Nurses' Health Study. *P*-values were adjusted for age at blood draw, fasting and diabetes case-control status, BMI, menopausal status and postmenopausal hormone use. The Y-axis was minus log-transformed observed *P*-values; the value of 7.3 corresponded to a *P*-value of 5×10^{-8} . *P*-values for loci on chromosome X (i.e. chromosome 23) were based on genotyped SNPs only.

conducted several sensitivity analyses to evaluate whether the strong association between *LEPR* region and sOB-R levels can be explained by these two SNPs. First, we searched PolyPhen online database (<http://genetics.bwh.harvard.edu/pph/>) for predicted functional consequences associated with the two non-synonymous SNPs (19–21), and the results indicated that these two non-synonymous SNPs would unlikely lead to significant structural or functional changes. Second, we examined LD between these two functional SNPs and the remaining significant SNPs in *LEPR* region. Of the remaining 131 significant SNPs, 46 (35.1%) was in strong LD ($r^2 \geq 0.8$), 23 (17.6%) were in moderate LD ($r^2, 0.5-0.7$), 37 (28.2%) were in weak LD ($r^2, 0.2-0.4$), and 25 (19.1%) were not in LD ($r^2 < 0.2$) with rs1137100. With respect to LD with rs1137101, these figures were 10 (7.6%), 11 (8.4%), 85 (64.9%) and 25 (19.1%), respectively. In addition, we examined the association between SNPs in *LEPR* region and sOB-R levels within participants who were homozygotes of

the ancestral allele of both or either SNPs, respectively. Although *P*-values were increased due to reduced power, the top SNP, rs2767485, remained highly significant. The *P*-values were 2.86×10^{-5} for participants who were homozygotes of the ancestral allele of both SNPs, 8.14×10^{-7} for participants who were homozygotes of the ancestral allele of rs1137100, and 1.63×10^{-5} for participants who were homozygotes of the ancestral allele of rs1137101. Several SNPs that were in strong LD with rs2767485 (i.e. rs12079231, rs12062820, rs11208656, rs1782755, rs1627238, rs1171275, rs1180445, rs1171278 and rs1171276) were associated with sOB-R levels at a similar significance level in these analyses.

DISCUSSION

According to the length of intracellular domain, isoforms of OB-R can be classified into short forms, long form and

Table 2. Covariates and selected SNPs in *LEPR* region in relation to plasma sOB-R levels

	Model 1 ^a			Model 2 ^b			Model 3 ^c			Model 4 ^d		
	Beta (SE)	P-value	r ² (%) ^e	Beta (SE)	P-value	r ² (%) ^e	Beta (SE)	P-value	r ² (%) ^e	Beta (SE)	P-value	r ² (%) ^e
Age (year)	0.004 (0.001)	0.0044	0.54	0.004 (0.001)	0.0048	0.53	0.004 (0.001)	0.0046	0.54	0.003 (0.001)	0.0057	0.51
BMI (kg/m ²)	-0.02 (0.001)	4.79 × 10 ⁻⁵⁵	15.08	-0.02 (0.001)	5.15 × 10 ⁻⁵⁶	15.33	-0.02 (0.001)	1.27 × 10 ⁻⁵⁶	15.50	-0.02 (0.001)	5.46 × 10 ⁻⁵⁷	15.60
Diabetes status	0.11 (0.01)	2.81 × 10 ⁻¹³	3.50	0.10 (0.01)	8.24 × 10 ⁻¹³	3.38	0.10 (0.01)	1.09 × 10 ⁻¹²	3.34	0.10 (0.01)	1.09 × 10 ⁻¹²	3.34
Fasting status	0.005 (0.01)	0.7394	0.0001	0.008 (0.01)	0.6492	0.0002	0.008 (0.01)	0.5740	0.02	0.008 (0.01)	0.5548	0.02
Menopausal status	0.03 (0.007)	1.99 × 10 ⁻⁵	1.21	0.03 (0.007)	9.66 × 10 ⁻⁶	1.30	0.03 (0.007)	1.21 × 10 ⁻⁵	1.28	0.03 (0.007)	1.45 × 10 ⁻⁵	1.25
Postmenopausal hormone use	-0.06 (0.02)	0.0053	0.52	-0.06 (0.02)	0.0052	0.52	-0.06 (0.02)	0.0046	0.54	-0.06 (0.02)	0.0041	0.55
rs1137100	-0.04 (0.01)	0.0032	0.58	-0.04 (0.01)	0.0046	0.54	0.02 (0.03)	0.3998	0.05	0.04 (0.03)	0.1428	0.14
rs1137101	-0.04 (0.01)	0.0038	0.56	-0.02 (0.01)	0.1909	0.11	-0.02 (0.01)	0.0922	0.19	-0.02 (0.01)	0.1093	0.17
rs2767485	-	-	-	-0.08 (0.01)	1.72 × 10 ⁻⁹	2.40	-0.07 (0.01)	6.57 × 10 ⁻⁸	2.02	-0.07 (0.01)	9.08 × 10 ⁻⁹	2.19
rs1751492	-	-	-	-	-	-	-0.07 (0.02)	0.0082	0.47	-0.06 (0.02)	0.0105	0.44
rs4655555	-	-	-	-	-	-	-	-	-	0.04 (0.02)	0.0267	0.33

^aIndependent variables included age, BMI, diabetes case-control status (yes/no), fasting status (≥8 or <8 h since last meal), rs1137100 and rs1137101.
^bBased on model 1, rs2767485 was further included in the model as an independent variable.
^cBased on model 2, rs1751492 was further included.
^dBased on model 3, rs4655555 was further included.
^er² was calculated as model sum of squares divided by corrected total sum of squares in the linear regression and represents the proportion of variability of sOB-R independently explained by each variable.

soluble OB-R (7). The long form OB-R (OB-R_L) with the full length of intracellular domain is primarily expressed in the hypothalamus, whereas multiple short forms of OB-R (OB-R_S) with various length of intracellular domain are primarily expressed in peripheral tissues (7). Although, in comparison to OB-R_L, isoforms of OB-R_S lack the motif regulating energy homeostasis, increasing evidence suggests that OB-R_S may be involved in the modulation of insulin sensitivity and other peripheral effects through the insulin receptor substrate/phosphatidylinositol-3-OH kinase (IRS/PI3K) pathway (22–26). In humans, circulating sOB-R is formed by ectodomain shedding of membrane-anchored OB-R (primarily OB-R_S) (8,9), which are expressed on the surface of a wide variety of cells in almost all peripheral tissues (6). The strong correlation between sOB-R levels and the cell surface expression levels of OB-R (10) suggests that sOB-R may reflect the total amount or activity of OB-R_S in peripheral tissues. sOB-R levels are inversely associated with diabetes risk or diabetes-related environmental risk factors (12,13,15,16). The genetic determinants of sOB-R, however, are unclear. To our knowledge, only one study has investigated the role that common genetic variation plays in modulating sOB-R levels: in a Japanese population consisting of 127 men and 90 women, the two functional *LEPR* SNPs (rs1137100 and rs1137101) identified in the current study were not associated with sOB-R levels (14). However, homozygotes of the minor allele of these two SNPs are much rarer in Japanese than western populations; according to the HapMap data, less than 1% of Japanese are homozygous of the minor allele of either SNPs, whereas in populations with European ancestry this proportion is ~40% for rs1137100 and 20% for rs1137101 (27). Together with a modest sample size, this study had a fairly limited statistical power to detect any significant differences in sOB-R levels by the genotypes.

To our knowledge, the current study is the first GWA investigation on plasma sOB-R levels. We replicated two functional and three model-selected SNPs in an independent sample with characteristics quite different from the NHS population. In addition, several observations strongly support that associations between *LEPR* variants and sOB-R levels are likely to be causal. First, biological plausibility for our findings is strongly supported by *a priori* evidence generated from basic biological research. Secondly, all identified genome-wide significant SNPs are localized to *LEPR*, two of which have been previously examined in candidate studies. In addition, a few SNPs in *LEPR* region were significantly associated the *LEPR* expression levels (17,18) and, thus, likely lead to a significant change in the levels of sOB-R. Thirdly, although all identified SNPs are in *LEPR* region, not all of them are in strong LD, indicating that the current finding is unlikely a pure play of chance. Lastly, when we restricted our GWA scan within controls only, although the diminished power made the top SNPs less significant, we essentially obtained the same set of SNPs. Furthermore, results independently generated from cases and controls were remarkably consistent.

Several caveats in the current study deserve discussion as well. First, although we cannot entirely exclude the possibility that the associations for the two functional SNPs were due to the potentially altered affinity of antibody induced by the

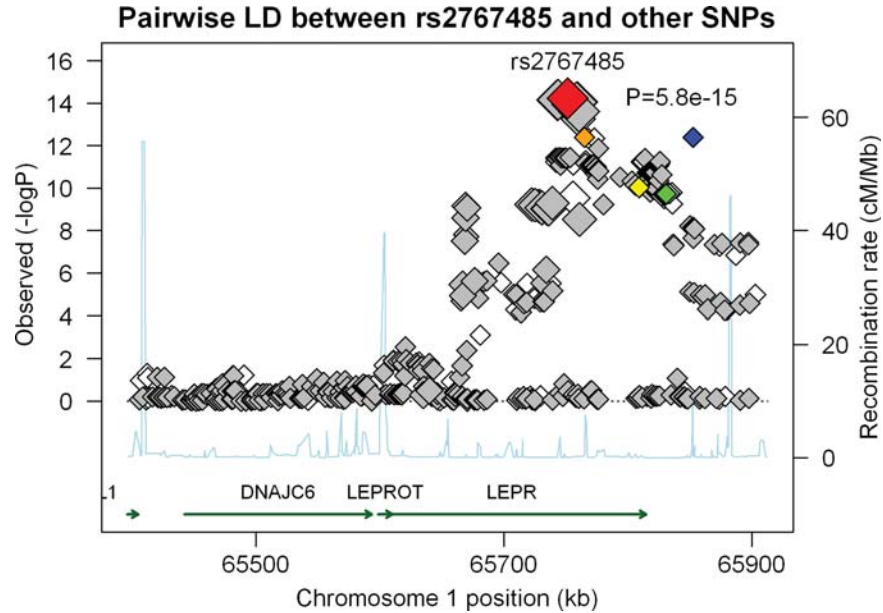


Figure 2. *P*-values and pairwise LDs with rs2767485 for SNPs in and adjacent to *LEPR* region between position 65400 kb and 65900 kb on chromosome 1. SNPs included in this panel included all SNPs [108 genotyped (◇) and 384 imputed (◆) SNPs, including rs2767485 (◆), rs1751492 (◆), rs1137100 (◆), rs1137101 (◆), and rs4655555 (◆)] within the region. Recombination rates in this region were plotted in the background in light blue. Pairwise LDs between rs2767485 and other SNPs were estimated using HapMap LD data. For 12 SNPs that are covered only in Affymetrix 6.0, we calculated the pairwise LDs using our observed data. The size of the diamonds represents the strength of LD; the largest diamonds represent a LD (r^2) ≥ 0.80 , whereas other sizes (from large to small) represent a LD of ≥ 0.5 , ≥ 0.2 and < 0.2 , respectively.

non-synonymous SNPs, the structural and functional change of protein associated with these SNPs is estimated to be small. Furthermore, when we restricted the analysis within homozygotes of the ancestral alleles of the non-synonymous SNPs, the top SNPs were still highly significantly associated with sOB-R levels. Nevertheless, further investigation is warranted to shed light on this issue. Second, we only measured sOB-R levels once. Measurement error due to random assay variability and/or within-person variation of sOB-R levels is inevitable. Although the long-term variation of sOB-R is not well documented, data show that the 24 h within-person variation of sOB-R closely resembles that of high-molecular-weight adiponectin (28), for which the long-term within-person variation is small (29). In addition, sOB-R levels were measured in a blinded way that genotype information had no influence on the measurements. Therefore, the measurement error of sOB-R is more likely to be non-differential and, therefore, more likely to cause a bias to the null in our results. Third, to minimize genotyping error, we only utilized SNPs that passed standard quality control criteria which were developed for our GWA study. The major reasons for the exclusion were rare SNPs (MAF < 0.02) and technical error (call rate $< 98\%$). Although we cannot exclude the possibility that some of the excluded SNPs may actually correlate with sOB-R levels, the identified genome-wide significant associations are unlikely the consequence of genotyping error. Fourth, our study population exclusively consisted of women with genetically determined European ancestry and the replication sample was exclusively composed of young men with European ancestry. Whether the results in the current study can be generalized to other ethnicities deserves further investigation. Lastly, our moderate sample size restrained our ability to detect loci in other

gene regions that regulate sOB-R levels as well. The loci found in the current study that reached suggestive GWA significance level warrant further investigation in future studies. The strengths of the current GWA study included relatively large sample size, high quality genotype data, careful quality control and minimal population stratification.

In the current study, two non-synonymous SNPs in *LEPR* were associated with plasma sOB-R levels at the genome-wide significance level. Several candidate gene studies have examined these functional SNPs in relation to body weight and type 2 diabetes-related traits. Mixed associations were documented for these SNPs in relation to insulin sensitivity or type 2 diabetes (30–34). Similarly, the association between these SNPs and body weight or BMI was also inconclusive (35–37). Likewise, in our study, these two functional SNPs were not associated with type 2 diabetes status or diabetes-related traits. Since these two SNPs can only explain a minimal amount of variation of sOB-R levels, this minor change of OB-R activities associated with these variants may not translate into a dramatic effect on insulin sensitivity or other diabetes-related risk factors. However, it is likely that OB-R expression levels are determined by many other common functional SNPs which jointly may explain a substantial amount of sOB-R, and subsequently affect diabetes-related traits. In the current study, in addition to the significant SNPs in *LEPR* region, we also found a few SNPs in other regions that were associated with sOB-R levels at suggestive genome-wide significance level. These regions certainly warrant more detailed investigation in future studies with larger sample size. As in many other GWA studies, the identified top SNP (rs2767485) with the best *P*-value in our study is located in the intron region of the *LEPR* gene. Interestingly, we

Table 3. Relation of selected SNPs in *LEPR* region in relation to plasma sOB-R levels in a replication population^a

SNP	<i>n</i>	Position (bp) ^b	Alleles (+/−)	MAF ^c	Geometric LS mean (95% CI), ng/ml ^d			Beta ^e	SE	<i>P</i> -value ^e
					0	1	2			
rs1137100	875	65809029	A/G	0.11	23.0 (22.5–23.4)	20.7 (20.2–21.3)	21.6 (19.2–24.3)	−0.089	0.019	3.83E−6
rs1137101	875	65831101	A/G	0.33	23.7 (23.3–24.2)	21.7 (21.1–22.4)	21.0 (20.2–21.8)	−0.071	0.012	2.05E−8
rs2767485	875	65751505	T/C	0.30	21.6 (21.0–22.2)	23.2 (22.8–23.7)	24.3 (23.3–25.5)	0.065	0.013	6.27E−7
rs1751492	875	65765213	T/C	0.14	23.1 (22.6–23.6)	20.9 (20.4–21.4)	21.3 (19.6–23.1)	−0.086	0.017	9.37E−7
rs4655555 ^f	875	65852857	T/A	0.06	22.9 (19.9–26.6)	20.7 (17.9–23.7)	21.9 (21.9–22.0)	−0.100	0.025	6.32E−5

^asOB-R levels were log-transformed.

^bPosition based on NCBI build 36.3.

^cMinor allele frequency among study participants in the replication sample.

^dLeast square geometric means according to the number of minor allele copies were adjusted for age at blood draw and BMI.

^eRegression coefficients and *P*-values for every one copy of minor allele were estimated from linear regression models adjusted for the same set of covariates.

^fLeast square means could not be obtained due to rare homozygous minor alleles. Median (interquartile range) was provided instead.

documented a significant inverse association between this SNP and fasting insulin levels, probably because this SNP explained larger amount of variation of sOB-R levels than other SNPs. Whether the observed associations for this SNP are causal or not warrants further investigation.

In summary, we identified multiple SNPs in the *LEPR* gene associated with plasma sOB-R levels and confirmed the role of *LEPR* as a candidate gene for regulating sOB-R levels. Although further investigation is warranted, we provided first evidence for genetic determinants for sOB-R levels that may play an important role in insulin metabolism. These novel findings will likely shed light on the complex relationship among leptin, sOB-R and metabolic traits.

MATERIALS AND METHODS

Study population

The NHS was established in 1976 when 121 700 female registered nurses aged 30–55 years and residing in 11 large US states completed a mailed questionnaire on their medical history and lifestyle characteristics. Every 2 years since baseline, follow-up questionnaires have been sent to update information on exposures and newly diagnosed illnesses. Beginning in 1980, on a 2–4 year cycle, dietary information has been updated using validated semi-quantitative food frequency questionnaires. Blood was collected from 32 826 NHS members between 1989 and 1990. Demographics and health status of participants who provided blood samples were generally similar to those who did not. Participants for the current study were a subset of those included in a nested case–control study of sOB-R in relation to type 2 diabetes in the NHS (38). Briefly, between blood draw and June 2004, we identified 1038 incident type 2 diabetes cases and randomly selected 1136 healthy controls using risk-set sampling. Cases and controls were matched for age at blood draw, date of blood collection, fasting status (≥ 8 versus < 8 h since last meal) and ethnicity. We restricted the current analysis to unrelated, genetically defined women of primarily European ancestry who had high quality genotyping data. After excluding participants with missing phenotype and covariate data, 1504 participants were included in the final

analysis. All study participants have signed and returned consent forms. The study protocol was approved by the institutional review board of the Brigham and Women's Hospital and the Human Subjects Committee Review Board of Harvard School of Public Health.

Replication population

The replication population was comprised of 18-year-old males enrolled during a health screening prior to performing their 2-year mandatory service in the Cyprus Army. Participants were randomly selected from the July 2006 and July 2007 enrollment cycles. Exclusion criteria included health reasons for which a person was unable to participate in the military service. In total, 1056 volunteers were recruited and were representative of the 18-year-old male population of Cyprus. Overnight fasting blood samples were collected among these participants. In the current study, we excluded participants with missing genotype and sOB-R data, leaving 875 participants for the analysis. All participants completed a written consent form and the study protocol was approved by the Cyprus National Bioethics Committee and the Human Subjects Committee Review Board of Harvard School of Public Health.

Laboratory procedures

In the original case–control study, each diabetes case was matched with one to two controls for age, blood draw date, ethnicity, and fasting status. Each diabetes case–control set was shipped in the same batch to the laboratory and analyzed in the same run. Within each batch, samples were assayed by the same technicians in random sequence under identical conditions. sOB-R was measured by ELISA technique (R&D Systems, Minneapolis, MN, USA) with a sensitivity of 0.06 ng/ml. This assay utilizes a monoclonal antibody specific to human sOB-R (amino acid position 20 to 839). Laboratory control samples ($n = 20$) were run along with the case–control samples. Based on the measurements of these control samples, the average intra-assay coefficient of variation (CV%) was 7.3% for the sOB-R assay. In the replication sample, sOB-R levels were measured using the same assay with a similar

accuracy (CV% = 6.1%). In both samples, assessment of sOB-R levels was independent of genotyping procedures.

GWAS genotyping, imputation and quality control

For the GWA study samples, DNA was extracted from white blood cells using the QIAmp™ (QIAGEN Inc., Chatsworth, CA, USA) blood protocol. Genotyping was done using the Affymetrix Genome-Wide Human 6.0 array and the Birdseed calling algorithm (39). Genotypic data for a total of 3429 NHS samples passed laboratory technical quality control criteria which included SNP fingerprints for sample tracking and early detection of sample misidentification, missing call rate (MCR), the use of HapMap controls to check genotype quality independent of study samples and the tracking of reagent and instrumental performance.

Relatedness was evaluated using pairwise identity-by descent using 80 k SNPs in a method of moments approach implemented in PLINK software (40). Five pairs of duplicate samples were identified and removed. One pair of full siblings and eight sets (six pairs and two triplets) of possible first cousins were also identified. Gender was confirmed by examining the mean of the intensities of SNP probes on the X and Y chromosomes. One male sample was mis-identified as a female sample and was excluded. Twenty-seven subjects with gross chromosomal anomalies, determined by analyzing relative intensity ('Log-RRatio') and allelic imbalance ('BAlleleFreq', BAF) (41), and 22 samples having a MCR $\geq 2\%$ were also removed.

More than 96% (879 071) of the 909 622 SNP probes on the array passed the quality control standards of the genotyping center (Broad Institute of MIT and Harvard) for NHS samples. We further excluded SNPs which were monomorphic, had a MCR $\geq 2\%$, more than one discordance, a HWE P -value $< 1 \times 10^{-4}$ or a MAF < 0.02 . Duplicate SNPs (assayed with different probes) were also removed. A total of 704 409 SNPs passed quality control and were included for the analysis.

We used MACH (<http://www.sph.umich.edu/csg/abecasis/MACH>) to impute 2 543 887 SNPs on chromosome 1 to 22 with NCBI build 36 of Phase II HapMap CEU data (release 22) as the reference panel. Imputation results were expressed as 'allele dosages' (fractional values between 0 and 2). Imputed SNPs with MAF < 0.02 and/or with poor imputation quality scores (MACH $r^2 \leq 0.30$) were filtered from the analysis.

Replication sample genotyping

In the replication sample, genotyping was carried out using the TaqMan SNP Genotyping Assay (Applied Biosystems, Foster City, CA, USA). The primers and probes were ordered from Applied Biosystems. The fluorescence of PCR products was detected by the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). A random 10% samples were included as duplicates for quality control. The overall genotyping success rate was 98.7% and the concordance rate for duplicate samples was 99.5%.

Determination of population structure

Population structure was investigated by principal component analysis (42). We used a set of 12 021 SNPs selected to have

very low levels of LD and to have MAF > 0.05 in populations with European ancestry (43). These SNPs were chosen using a three-step algorithm out of a pool of 40 817 SNPs on autosomal chromosomes that (i) are common to Affymetrix 500k, Illumina HumanHap300 and Illumina HumanHap550 platforms; (ii) have a MAF $> 5\%$; (iii) have Hardy–Weinberg proportion exact test $P > 10^{-3}$ in two independent control samples (43). Study subjects passing quality control ($n = 3369$) were analyzed together with a set of 209 HapMap II founders (59 CEU, 60 YRI, 45 JPT and 45 CHB) (Supplementary Material, Fig. S2). Subjects with first and second principal components in the box defined by the means of the first and second principal components among self-described whites ± 3 SD were classified as having primarily European ancestry. We found a high concordant rate of self-described European ancestry with genetically identified European ancestry: 99.2% of participants with self-described European ancestry were confirmed using these principal components.

Statistical analysis

The associations between each SNP and log-transformed plasma sOB-R levels were evaluated using linear regression adjusting for age, BMI, diabetes and fasting status, menopausal status and postmenopausal hormone use. We assumed an additive model for each SNP. Observed genotypes were analyzed using PLINK software (40), while imputed data (expressed as allele dosage) were analyzed using ProbABEL software (44).

We used a forward-selection regression procedure to identify genome-wide significant SNPs that were associated with sOB-R levels independent of other SNPs. We forced age, BMI, diabetes and fasting status, menopausal status and postmenopausal hormone use, as well as two missense SNPs (rs1137100 and rs1137101) into the linear regression model. The selection criterion was P less than 0.05 for a SNP to enter the final model. We further estimated the proportion of variation of sOB-R that was explained by selected SNPs in the linear regression analyses. This proportion was measured by r^2 , which is the difference of model sum of squares between models with and without the SNP(s) of interest divided by the corrected total sum of squares of the full model. These analyses were performed in SAS 9.1 (SAS Institute, Inc., Cary, NC, USA).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. C.J.G. is an employee of the Merck & Co., Inc. Other authors had no financial or personal conflict of interest to disclose.

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