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**BRIEF REPORT: Ghrelin receptor gene polymorphisms and body size in children and adults**

Abbreviated title: **GHSR polymorphisms and body size**

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***Abstract***

**Background:** The growth hormone secretagogue receptor type 1a gene (*GHSR*) encodes the cognate receptor of ghrelin, a gut hormone that regulates food intake and pituitary growth hormone secretion. Previous studies in US families and in a German population suggested *GHSR* to be a candidate quantitative locus for association with human obesity and growth.

**Aim:** To test common genetic variation in *GHSR* for association with body size in children and adults.

**Methods:** Sequencing was performed to systematically identify novel single nucleotide polymorphisms (SNPs) in *GHSR*. A set of three haplotype-tagging (ht)SNPs was identified which captured all the genetic variation in *GHSR*. These three htSNPs were then genotyped in three large population-based UK cohort studies (two adult and one childhood cohort) comprising 5,807 adults and 843 children.

**Results:** No significant genotype or haplotype associations were found with adult or childhood height, weight or BMI.

**Conclusion:** Common variation in *GHSR* is not associated with body size in UK adults or children.

## Introduction

The growth hormone secretagogue receptor type 1a gene (*GHSR*) encodes the cognate receptor of ghrelin, a gut hormone that regulates energy homeostasis, food intake and the release of growth hormone by the anterior pituitary (1). The full-length receptor (type 1a) contains 366 amino-acids encoded by two exons on chromosome 3q25. A splice variant (type 1b) comprises only exon 1 and a short region of the intron. It is not clear if this variant is transcribed to protein *in vivo* but theoretically it would code for a 289 amino-acid protein, representing 5 transmembrane domains. The type 1b receptor is widely distributed (as opposed to the type 1a receptor), but has no biological activity in GH-releasing or calcium-related functional assays (2). In animal models genomic regions syntenic with the human *GHSR* gene are candidate quantitative trait loci for energy expenditure and body temperature regulation (3). In addition, *Ghsr*-null mice have a lean phenotype (4) and improvement of glycemc profiles in leptin deficient mice with concomitant suppression of *Ghsr* suggest a possible role of this gene in obesity and related co-morbidities (5). In humans the *GHSR* locus is in physical proximity to genomic linkage peaks for obesity (6, 7).

Rare deleterious mutations in *GHSR* have been associated with short stature in humans (8, 9). Carriers of an alanine 204 to glutamate amino-acid change were three standard deviations smaller than average for height and weight (8). The 204 glutamate variant lowers cell membrane receptor density, but does not alter receptor affinity for the agonist and increased signal transduction was recorded (8). Another non-synonymous *GHSR* variation at amino-acid 279 was detected in one heterozygous obese child with short normal stature, but its function is yet unknown (9).

Common polymorphisms in *GHSR* have been associated with obesity in both a cross-sectional and a family-based association study (10). Five SNPs in a single strong linkage disequilibrium (LD) block covering *GHSR* exon 1 and its 5' adjacent region were all associated with BMI and obesity in the German population-based MONICA cohort (10). In a large US family study, the same susceptibility haplotype was more commonly transmitted to obese offspring (10).

The SNPs genotyped in the above study (10) were selected from a public database (dbSNP). In order to confirm those observations and to systemically test for association with common genetic variations we re-sequenced *GHSR* to identify the common haplotype tagging (ht)SNPs, and then examined these SNPs for association with BMI in three population-based UK cohort studies comprising 5,807 adults and 843 children.

## **Methods**

### **Populations and measurements**

The adult populations came from two distinct cohort studies: the MRC Ely Study (11) and EPIC-Norfolk (6, 12). The children were derived from one population, the Avon Longitudinal Study of Parents and Children (ALSPAC) (13, 14).

The Ely Study is a population-based study of the aetiology and pathogenesis of type 2 diabetes and related metabolic disorders (11). The study consists of an ethnically homogeneous Caucasian population, aged between 40 and 65 years at baseline. Body weight and height were measured at clinic visits using standard methods. This cohort was recruited from a population-sampling frame with a high response rate (74%), and is therefore representative of the eastern England population. Body weight was known for 811 Ely Study participants.

The EPIC-Norfolk cohort (6, 12) is a prospective population-based study of 25,639 men and women aged between 40 and 79 years, resident in Norfolk, UK. Participants were recruited from age–sex registers of general practices in Norfolk as part of the 10-country collaborative EPIC study designed to investigate dietary and other determinants of cancer. The sub-cohort used for this study is a random sample of 5,000 participants (EPIC5000) who were free of disease (cancer, coronary heart disease and diabetes) at baseline, who had arrayed DNA samples available, and had height and weight measured at clinic visits using standard methods (15). Body weight was known for 4996 EPIC5000 participants.

ALSPAC is a geographically based birth cohort (14). The initial ALSPAC sample consists of 14,541 pregnancies. Children in the present study derived from a 10% sub-cohort (“Children in Focus”) who were chosen at random from the last 6 months of ALSPAC births and attended research clinics at various time intervals between 4 to 61 months of age (1432 families attended at least one clinic) (13). At age 7 years, body weight was measured using electronic scales, and standing height by stadiometer (Leicester height measure; Child Growth Foundation, London, UK). Using a topical anaesthetic, a non-fasting venous blood sample was collected. Samples were centrifuged and stored at  $-70^{\circ}\text{C}$ . IGF-I levels were measured by direct ELISA (Diagnostic Systems Laboratories, Sinsheim, Germany) (16). Body weight at age 7 years was known for 843 of these children. Lymphocyte DNA was prepared as described previously (17).

All the studies were approved by the local research ethics committees, and informed consent was obtained from each participant or their parent.

### **SNP selection**

The selection of SNPs to cover the genetic variation of the *GHSR* gene in the European population was based on DNA resequencing performed in 70 obese French

children (BMI >97 percentile for age) with a strong component of early onset obesity in their family background (18). The SNPs rs495225 (T171C), rs2232169 (C447G) and rs572169 (G477A) were selected following haplotype-tagging identification methods suggested by Johnson et al (19).

### **Genotyping**

Genotyping was performed sequentially; initially in the Ely Study and ALSPAC populations using the MassEXTEND (hME) assay on the SEQUENOM platform (Sequenom<sup>TM</sup>, Sandiego, CA). Genotyping calling rate was 96% (95% CI: 0.95-0.99).

Subsequently EPIC5000 samples were genotyped using Custom TaqMan assays (Applied Biosystems, Warrington, UK) on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Genotyping call rate was 98% and repeated assays in 75 DNA samples showed 100% concordance. Details for all genotyping primers, probes and PCR conditions are available upon request from the corresponding author.

### **Statistical analysis**

Each SNP was tested for Hardy–Weinberg equilibrium using the  $\chi^2$  test. We used linear regression to test the association between each SNP (unilocus tests) and the continuous anthropometric outcomes (dependent variables) adjusting for age and sex. All SNPs were considered as linear factors (i.e. additive or co-dominant models). Results are displayed as p-value for the Wald test of the coefficients in the linear model, separately in each study, and then jointly for adult populations. These analyses were performed with SAS version 8.2 (SAS Institute Inc, Cary, NC) and SPSS v11 (SPSS, Chicago, IL).

Common haplotypes were inferred from the three htSNPs. As phase was unknown, assignment of haplotype probabilities was performed using the SNP HAP program (19).



Tests for main haplotype effects were performed using a linear model weighted by haplotype probability, and clustered by the individual identification to obtain robust standard errors (STATA regression command `xi:regres`) (20). Results are displayed as p-value for main haplotype effects. Significance was taken at  $p < 0.05$ .

## Results

Details of the *GHSR* SNPs identified are shown in Figure 1. Genotype frequencies of the three *GHSR* htSNPs genotyped were in Hardy-Weinberg equilibrium ( $p > 0.2$ ) in each study population (see supplementary data Table 1). Genotype frequencies of T171C differed slightly between the EPIC5000 and Ely Study populations ( $p = 0.044$ ).

The phenotypic characteristics of the study populations are summarised in Supplementary data Table 2. In ALSPAC children none of the three htSNPs were associated with height, weight, BMI or IGF-I levels (Table 1; all  $p > 0.16$ ). In adults from the Ely Study, borderline associations were seen between T171C and BMI ( $p = 0.041$ , positive association with minor allele), and between G477A and IGF-I levels ( $p = 0.015$ , positive association with minor allele). In contrast, in EPIC5000 adults there was only a weak inverse association between the G477A minor allele and height ( $p = 0.035$ ). None of the associations were concordant between the Ely and EPIC5000 studies, and in a combined analysis of these two adult populations no htSNP showed an association with height, weight or BMI (all  $p > 0.11$ ; Table 1).

Three major haplotypes (frequency  $> 0.1$ ) could be inferred from the three *GHSR* htSNPs in the adult populations (supplementary data Table 3). Similar to the individual SNP analyses, there was no association between common *GHSR* haplotypes and body size in adults from Ely and EPIC5000 combined (supplementary data Table 3; all  $p > 0.20$ ).

## Discussion

In three large UK cohort studies we failed to identify any consistent association between common haplotypic variation in *GHSR* and body weight, height or BMI in adults or children.

Baessler et al. previously studied 2 SNPs within *GHSR* and a further 8 SNPs within the 40 to 50 kb up- and downstream adjacent regions (10) and found that an LD block of 5 SNPs was associated with BMI in 1,095 individuals from 178 pedigrees with multiple obese members. That LD block included the synonymous SNP G477A (rs572169); the minor allele (A) was transmitted more frequently than expected to obese cases, was more prevalent in obese cases than controls, and an additive genetic association with higher BMI was confirmed in a further 1,418 Caucasians (10). Of the 5 SNPs in that study, we included only G477A (rs572169), however this SNP would be expected to closely represent the other four obesity risk-associated SNPs due to high LD ( $r^2 > 0.75$ ). The relatively large size of our study provided >98% power to replicate the association with obesity risk reported by Baessler et al. (10).

The reason for lack of consistency between the two reports is unknown. Baessler et al. observed a consistent association in Caucasian populations from US and Germany (10), and our three UK populations were also largely Caucasian in origin, with a minor allele frequency of the G477A SNP in our populations (MAF = 0.31) being similar to the other study. It is possible that the genetic association might be dependent on, or modified by some further environment, lifestyle or other genetic factor. However, the lack of further confirmatory studies published since that original report might suggest that the original association was a false positive finding. It is

possible that genetic variations in the ghrelin gene, the ligand for the GHSR, may contribute to obesity risk directly, or by interaction with GHSR variants.

In addition to G477A (rs572169), we genotyped two other SNPs to further cover the common variation in *GHSR* exons as indicated by our resequencing of 70 obese children. Inspection of the HapMap Build 35 CEU population data (<http://www.hapmap.org/>), which were released following the genotyping of this study, showed that in addition to the three SNPs that we genotyped, only one further intronic SNP (A216G, rs2948694) would be needed to fully cover the *GHSR* gene including the introns and 1 kbp up- and downstream; however that additional SNP is relatively rare (MAF = 0.07).

A limitation of our study is the slight discrepancies we obtained between the different study populations. Slight differences in genotype frequencies between the Ely Study and EPIC5000 populations could reflect differences in selection of these cohorts. Furthermore, borderline associations in the Ely Study with adult body weight and BMI were not confirmed in larger EPIC5000 study. We checked that the first results were not driven by a few individuals (data not shown), and conclude that they might have arisen by chance. Associations with circulating IGF-1 levels in the Ely Study could not be tested in EPIC5000 due to lack of data, but were not supported by associations with body size.

In conclusion, in a systematic study of common *GHSR* variation in three large population-based UK cohort studies comprising 5807 adults and 843 children, we found no association with body weight, height or BMI.

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**Table 1:** P-values for the associations between each *GHSR* htSNP and body size in children (ALSPAC) and adults (Ely and EPIC5000 studies).

		<b>GHSR htSNPs</b>		
		<b>G477A rs572169</b>	<b>T171C rs495225</b>	<b>C447G rs2232169</b>
<b>Children</b>		P-value	P-value	P-value
ALSPAC (N=843)	Height	0.66	0.22	0.62
	Weight	0.29	0.88	0.62
	BMI	0.20	0.64	0.94
	IGF-I	0.16	0.64	0.40
<b>Adult cohorts</b>		P-value	P-value	P-value
Ely Study (N=811)	Height	0.36	0.20	0.51
	Weight	0.52	0.014 (+ve)	0.83
	BMI	0.67	0.041(+ve)	0.16
	IGF-I	0.015 (+ve)	0.70	0.11
EPIC5000 (N=4996)	Height	0.035 (-ve)	0.97	0.47
	Weight	0.15	0.83	0.51
	BMI	0.54	0.69	0.18
<b>Combined adult cohorts</b>		P-value	P-value	P-value
EPIC5000 + Ely* (N=5,807)	Height	0.11	0.69	0.63
	Weight	0.30	0.26	0.83
	BMI	0.72	0.25	0.47

Analyses are adjusted for age and gender.

\*Additionally adjusted for study population (EPIC5000 or Ely Study)

p-values are from co-dominant (additive) models.

Where  $p < 0.05$ , “+ve” or “-ve” indicate the direction of association with minor allele copy number



**Figure 1:** Schematic diagram of the *GHSR* gene demonstrating the common SNPs identified by sequencing of 70 French obese children. The three haplotype-tagging SNPs genotyped in the UK population studies are indicated by solid triangles and bold type. Only SNP C231G (ttccgcgagctgctgcaccacc/gaccaacctctacctgtccag) is not annotated in HAPMAP Build 35.

