CLINICAL STUDY

GH secretagogue receptor gene polymorphisms are associated with stature throughout childhood

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Abstract

Context: Ghrelin plays a major role in GH physiology and energy metabolism. Polymorphisms of its receptor (GH secretagogue receptor (GHSR)) may influence childhood growth and weight regulation. **Objective:** To correlate *GHSR* polymorphisms with auxological parameters throughout childhood in a healthy cohort.

Study design: Longitudinal retrospective population-based genetic association study.

Subjects and methods: *GHSR* genotypes were evaluated in 1362 children and compared with height/length, weight, and body mass index (BMI) data across an observation span of 10 years (0, 1, 3, 5, 8, and 10 years). Five different *GHSR* SNPs (rs2922126, rs2981464, rs482204, rs562416, and rs572169), minor allele frequency >0.1, were genotyped. Identification of potential genetic associations with height, weight, and BMI, using additive and dominant/recessive models, was optimized by comparing allele or genotype frequencies between the tallest and the shortest 27% of subjects for each auxological variable. Significance of association was evaluated by χ^2 test.

Results: The rs482204 TT genotype, vs TC/CC, was associated with greater stature across the entire observation period ($P < 0.05$). Similarly, the rs562416 TT genotype, vs TG/GG, correlated positively with tall stature at 3, 8, and 10 years. Other SNPs and genotypes showed no association with height at any age. No association was found between any tested SNPs and weight or BMI.

Conclusions: Longitudinal investigation between birth and 10 years in a population-based cohort revealed a significant association of the rs482204 and rs562416 *GHSR* polymorphisms on height, whereas no association between *GHSR* polymorphisms and weight or BMI was ascertainable.

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Introduction

Diversity of adult human height within a population is largely explained (>80%) by genetic variation and hence represents an ideal model for studies elucidating common complex traits with a moderate to high heritability. The GH/insulin-like growth factor (IGF) axis represents the most important pathway determining growth, with additive effects on many different novel genes continuing to be discovered (1). Ghrelin, the endogenous ligand for the GH secretagogue receptor (GHSR), stimulates GH release in addition to causing weight gain through increased food intake and reduced fat utilization (2, 3). Common ghrelin gene variants have been investigated for a potential influence on

stature (4) and body weight (5, 6) without significant associations being detected.

GHSR, located on chromosome 3q26.3 (MIM* 601898), encodes a G-protein-coupled receptor that was discovered in 1996 before its natural ligand, ghrelin (7). It gives rise to two splice variant mRNAs encoding *GHSR1a* and *GHSR1b*. *GHSR1a* consists of 366 amino acids, with seven transmembrane domains. *GHSR1b* consists of 289 amino acids with five transmembrane domains. *GHSR1a* has a high affinity and specificity for binding of GH secretagogues (GHS), whereas *GHSR1b* does not bind GHS.

Importantly, GHSR shows high constitutive activation, and even in the absence of ghrelin, the receptor signals at 50% of its maximal capacity *in vitro* (8).

In contrast to many other systems regulated by two ligands acting as agonists on two distinct receptors with opposite signaling effects, ghrelin seems to serve as a unique agonist that fine-tunes the action of GHSR with no endogenous inverse agonist yet discovered. Its peculiar nature makes *GHSR* an interesting candidate gene to look for the effects of genetic alterations of GHSR on stature and body weight.

Indeed, Pantel *et al.* (9) described a loss of constitutive activity of the GHSR caused by a dominant disruptive mutation (Ala204Glu), which was associated with idiopathic short stature in one family and with isolated GH deficiency in another. Later, this group also reported a patient with isolated GH deficiency due to compound heterozygous *GHSR* mutations (10). Similarly, a significantly higher occurrence of four novel heterozygous *GHSR* variants was observed in 127 Japanese patients with either GH deficiency or short stature compared with controls (4.72 vs 0.53%) (11).

Several large studies in various populations have investigated the effects of common *GHSR* variants on stature, weight, and body mass index (BMI) (4, 5, 12, 13, 14, 15). Baessler *et al.* (12) reported an association between *GHSR* variants and obesity; however, other studies have yielded negative results (5, 13, 14, 15).

Here, we present an investigation of possible associations between common *GHSR* variants and stature, growth, weight, and BMI. In contrast to the cross-sectional design of most previous studies, we had the unique opportunity to perform a population-based longitudinal study, which followed 1362 healthy children from birth to 10 years of age.

Subjects and methods

Subjects

The Western Australian Pregnancy Cohort (Raine) Study (www.rainestudy.org.au) was designed to measure the relationships between early life events and subsequent health and behavior. This is one of the largest and most closely followed prospective cohorts in the world. Recruitment has previously been described in detail (16). In brief, 2900 pregnant women were enrolled between 1989 and 1991 through the state maternity hospital, King Edward Memorial Hospital (KEMH), and nearby private clinics in Perth, Western Australia, into a randomized controlled trial to evaluate the effects of repeated ultrasound exposure in pregnancy. These women were recruited between 16 and 20 weeks of gestation (mean 18 weeks), with sufficient English language skills and an intention to deliver their baby at KEMH and to reside in Western Australia. They delivered 2868 live-born children. Mothers provided data regarding pregnancy, maternal physical health, and psychosocial and demographic characteristics at enrollment and at 34 weeks of gestation.

The analyses in this study were based on 1362 (660 females and 702 males) Raine Study children who met the criteria of live, unrelated, singleton births, with no congenital abnormalities who attended for regular follow-up. No other exclusion criteria such as preterm birth, low birth weight, or chronic illness were applied. These children were genotyped for the five SNPs across the *GHSR* gene described. Anthropometric data at birth and 1, 3, 5, 8, and 10 years were available. Among the 586 girls evaluated for 10 years, menarche data could be obtained in 546 (93.2%), and 14 (2.6%) had already experienced menarche at the time of their measurements. The protocols for the study were approved by the Human Research Ethics Committees at KEMH and/or Princess Margaret Hospital for Children in Perth, Western Australia.

Sequencing and SNP selection

Sequencing of *GHSR* SNPs A salting out method was used to isolate genomic DNA from leukocytes. Sequencing of the promoter region, exons 1 and 2 in *GHSR*, plus the 3'-UTR was performed using Ensembl Transcript ENST00000241256 as reference sequence. SNPs were genotyped using TaqMan (ABI Prism 7900HT Sequence Detection System; Applied Biosystems, Foster City, CA, USA) except for rs2981464, which was genotyped using Amplification Refractory Mutation System-PCR (17). For confirmation of results, samples of each genotype were sequenced and subsequently used as controls in each experiment. To ensure that the data were reproducible, 10% of the samples were genotyped in duplicate. Details on genotyping (primers, probes, and PCR conditions) are available on request.

To discover common genetic variants in our population group, we selected five SNPs (rs2922126, rs2981464, rs482204, rs562416, and rs572169) based on linkage disequilibrium ($r^2 < 0.8$) and minor allele frequency > 0.1 (range 0.11–0.46) covering the whole gene including the 5'-UTR promoter and 3'-UTR (<http://manticore.niehs.nih.gov>).

Analyses and statistics

Lengths/heights, weights, and BMIs of individuals within each year cohort were converted to SDS. As these cohorts were random population samples, height SDSs were calculated with respect to the sample population. All heights, weights, and BMIs were first, for girls and boys separately, normalized for age within the age group by regressing the actual value to an estimated value at the beginning of the age group, e.g. to 2 years of age for the 2- to 3-year olds. The mean and s.d. of these values were then used to calculate the SDS ($\text{SDS} = (\text{regressed value} - \text{mean})/\text{s.d.}$). Birth data were normalized with respect to length of gestation to 40 weeks. Actual weights and lengths were used for babies

born after 40 weeks, as mean values for these babies were not significantly different to those born at 40 weeks.

Association analysis of SNP alleles with height, weight, and BMI was based on a comparison of the top and bottom 27% of the population according to trait SDS. This percentage has been shown to be the theoretical optimum proportion of a distribution to detect allelic association with variation in a quantitative trait (18, 19).

Additive and dominant/recessive models for each allele were tested. For the additive model, allele frequency differences were compared between groups by a χ^2 test. For each dominant/recessive model, the putative recessive-homozygote genotype frequency and the combined heterozygote and dominant homozygote frequencies were compared between groups by χ^2 test. Hardy-Weinberg equilibrium was tested using a χ^2 test by comparing observed genotype frequencies with those predicted.

Results

Anthropometric data

Mean (s.d.) height, weight, and BMI values regressed to the starting age of each cohort as well as percentages of overweight/obese subjects are presented in Table 1.

Sequencing of SNPs

There was a 0% error rate detected for all SNPs analyzed. All SNPs for each year were in Hardy-Weinberg equilibrium except for rs562416 at year 1 ($P=0.046$).

Association of GHSR SNPs with length/height in the tallest 27% and shortest 27%

Additive model and dominant/recessive model

Applying the additive model, the T allele of rs482204 was significantly more common in the tallest 27% at birth, 1, 3, and 5 years compared with the shortest

27%. Using the dominant/recessive model, the TT genotype significantly predominated in the tall cohort at all time points. The rs562416 TT genotype was observed significantly more frequently in the tallest 27% at 3, 8, and 10 years (Table 2). Both SNPs (rs482204 and rs562416) were located within the 3'-UTR region. Nonassociations were observed for SNPs rs2922126, rs2981464, and rs572169.

Association of rs482204 and rs562416 with length/height in the whole population

SNPs that had shown significant associations with height/length in the tallest/shortest 27% (rs482204 and rs562416) were subsequently tested in the whole study population. In accordance, the TT genotype of rs482204 was positively associated with height/length compared with TC/CC ($P<0.05$) across the observation span in the whole study population, except for year 1. No difference between genders was ascertainable (Fig. 1). The TT genotype of rs562416 also correlated positively with height at 3, 5, 8, and 10 years in the whole population. Interestingly, we observed a significant gender difference in height within the rs562416-GG individuals in years 1 ($P=0.02$), 5 ($P=0.03$), 8 ($P=0.01$), and 10 ($P=0.03$) with males taller and females shorter (Fig. 1). However, the number of individuals with this genotype (4–6 females and 6–10 males) was very small ($\approx 1.2\%$). It should also be noted that although the GG individuals, as a group, appear to be taller than the TG and TT genotype individuals, this difference was not significant at any age.

Association of GHSR SNPs with weight and BMI

Additive model and dominant/recessive model

No associations between five tested SNPs and weight or BMI were observed, either in the additive or in the dominant/recessive model.

Table 1 Anthropometric data of age cohorts.

	Birth	Year 1	Year 3	Year 5	Year 8	Year 10
Height (cm) mean (s.d.) ^a						
Male	50.3 (2.1)	76.3 (2.9)	96.6 (3.8)	112.1 (5.0)	129.0 (5.9)	141.8 (6.8)
Female	49.6 (2.3)	74.8 (2.5)	95.0 (3.4)	110.9 (4.5)	128.1 (5.4)	141.6 (6.6)
Weight (kg) mean (s.d.) ^a						
Male	3.66 (0.4)	10.2 (1.2)	15.2 (1.8)	19.8 (3.4)	28.3 (5.8)	37.9 (9.1)
Female	3.57 (0.5)	9.5 (1.1)	14.6 (1.8)	20.3 (3.3)	27.7 (5.6)	39.0 (8.8)
BMI (kg/m ²) mean (s.d.) ^a						
Male	—	17.5 (1.4)	16.2 (1.2)	15.7 (1.7)	16.9 (2.5)	18.6 (3.4)
Female	—	16.6 (1.3)	16.1 (1.3)	16.4 (1.8)	16.8 (2.6)	18.8 (3.3)
Overweight ((obese) %) ^b						
Male	—	—	11.9 (6.2)	10.5 (7.2)	11.9 (9.7)	15.1 (11.9)
Female	—	—	13.9 (4.8)	13.1 (5.4)	14.0 (6.7)	16.0 (7.8)

^aMean (s.d.) values regressed to starting age of cohort.

^bBased on the BMI-SDS (CDC-2000) at actual age. Overweight: >85th centile but ≤95th centile for BMI-SDS. Obese: >95th centile for BMI-SDS.

Table 2 Relative frequencies of GH secretagogue receptor (*GHSR*) SNP alleles and genotypes found to be associated with height.

SNP/model	Birth		Year 1		Year 3		Year 5		Year 8		Year 10	
rs482204	n=1330		n=1240		n=927		n=1217		n=1217		n=1198	
Additive												
Allele	T	C	T	C	T	C	T	C	T	C	T	C
Population	0.74	0.26	0.74	0.26	0.74	0.26	0.74	0.26	0.74	0.26	0.74	0.26
Shortest 27%	0.71	0.29	0.72	0.28	0.70	0.30	0.71	0.29	0.72	0.28	0.71	0.29
Tallest 27%	0.77	0.23	0.77	0.23	0.80	0.20	0.77	0.23	0.75	0.25	0.76	0.24
P value	0.020		0.033		0.001		0.014		0.119		0.068	
C-dominant												
Genotype	TT	TC/CC	TT	TC/CC	TT	TC/CC	TT	TC/CC	TT	TC/CC	TT	TC/CC
Population	0.55	0.45	0.54	0.46	0.54	0.46	0.54	0.46	0.55	0.45	0.54	0.46
Shortest 27%	0.50	0.50	0.51	0.49	0.50	0.50	0.49	0.51	0.49	0.51	0.49	0.51
Tallest 27%	0.59	0.41	0.60	0.40	0.62	0.38	0.59	0.41	0.57	0.43	0.58	0.42
P value	0.020		0.020		0.005		0.010		0.042		0.018	
T-dominant												
Genotype	TT/TC	CC	TT/TC	CC	TT/TC	CC	TT/TC	CC	TT/TC	CC	TT/TC	CC
Population	0.94	0.06	0.94	0.06	0.93	0.07	0.94	0.06	0.94	0.06	0.94	0.06
Shortest 27%	0.92	0.08	0.93	0.07	0.91	0.09	0.93	0.07	0.94	0.06	0.93	0.07
Tallest 27%	0.95	0.05	0.94	0.06	0.97	0.03	0.95	0.05	0.94	0.06	0.93	0.07
P value	0.223		0.533		0.005		0.328		0.872		0.875	
rs562416	n=1338		n=1247		n=922		n=1223		n=1222		n=1204	
Additive												
Allele	T	G	T	G	T	G	T	G	T	G	T	G
Population	0.86	0.14	0.86	0.14	0.86	0.14	0.86	0.14	0.86	0.14	0.86	0.14
Shortest 27%	0.86	0.14	0.85	0.15	0.84	0.16	0.85	0.15	0.84	0.16	0.85	0.15
Tallest 27%	0.85	0.15	0.87	0.13	0.87	0.13	0.87	0.13	0.88	0.12	0.88	0.12
P value	0.706		0.434		0.088		0.180		0.059		0.062	
G-dominant												
Genotype	TT	TG/GG	TT	TG/GG	TT	TG/GG	TT	TG/GG	TT	TG/GG	TT	TG/GG
Population	0.74	0.26	0.73	0.27	0.73	0.27	0.74	0.26	0.73	0.27	0.74	0.26
Shortest 27%	0.73	0.27	0.71	0.29	0.68	0.32	0.70	0.30	0.68	0.32	0.70	0.30
Tallest 27%	0.73	0.27	0.75	0.25	0.76	0.24	0.76	0.24	0.77	0.23	0.78	0.22
P value	0.933		0.223		0.028		0.081		0.018		0.032	
T-dominant												
Genotype	TT/TG	GG	TT/TG	GG	TT/TG	GG	TT/TG	GG	TT/TG	GG	TT/TG	GG
Population	0.99	0.01	0.99	0.01	0.99	0.01	0.99	0.01	0.99	0.01	0.99	0.01
Shortest 27%	0.99	0.01	0.99	0.01	1.00	0.00	0.99	0.01	0.99	0.01	0.99	0.01
Tallest 27%	0.98	0.02	0.98	0.02	0.98	0.02	0.98	0.02	0.98	0.02	0.99	0.01
P value	0.203		0.203		0.178		0.254		0.254		0.704	

Figures in bold represent $P < 0.05$.

Discussion

We performed a longitudinal genetic association study of five different *GHSR* SNPs in a population of healthy children at six different time points from birth until 10 years of age. A significant association between rs482204 and stature was observed across the entire time span, including at birth. Similarly, rs562416 correlated with height at ages 3, 8, and 10 years. We could not detect any significant relationship between *GHSR* SNPs and weight or BMI.

GHSR mutations have been shown to decrease constitutive activity of *GHSR*, which signals at 50% of its maximal capacity *in vitro*, even in the absence of a ligand (8). Based on the functional analyses of deleterious *GHSR* mutations in short and/or GH-deficient patients showing decreased constitutive *GHSR* activity (9, 10, 11), genetic alterations of *GHSR* would be expected to have an ascertainable growth, even if minor influence on childhood growth. Very

recently, Legendre *et al.* (20) found an up to 5% occurrence of causative *GHSR* mutations in a cohort of 290 patients with short stature and/or GH deficiency. As ghrelin promotes GH secretion by increasing hypothalamic GHRH release and by antagonizing the somatostatin block (21), changes in *GHSR* activity would be expected to change GH secretion, altering GH/IGF-mediated longitudinal growth.

We chose the first decade of postnatal life for analysis. During this time period, rapid infant growth (0–3 years) decelerates to the childhood growth pattern before puberty. As no major influence from sex steroids occurs during this stage, growth largely reflects the role of the GH/IGF system. In fact, the vast majority of subjects were prepubertal at year 10, with menarche having occurred in only 2.6% of girls. Hence, the consistent significance of rs482204 in our cohort may well be explained by stimulatory effects on GH mediated via *GHSR* signaling.

However, GH sensitivity in infants is low and starts to rise continuously from early postnatal life, explaining

Figure 1 Height SDS (mean $\pm$ S.E.M.) in the whole population for each genotype of the rs482204 (left-hand panels) and rs562416 (right-hand panels) SNPs for each observation year. Graphs show mean heights for the whole population of the observation year in addition to mean heights for females and males specifically. The TT genotype (rs482204) was associated with greater length/height at birth, 3, 5, 8, and 10 years without significant gender differences. The TT genotype (rs562416) correlated positively with height at 3, 5, 8, and 10 years. Interestingly, the GG genotype (rs562416), though rare (1.2%), was associated with significantly greater height in males at 1, 5, 8, and 10 years.

the observed minor effect from GH deficiency on growth during early childhood (22). The lack of significance of rs562416 at birth and at 1 year of age as opposed to its influence in later childhood may reflect these developmental changes of GH sensitivity. Correspondingly, no decrease in birth weight was observed in *Ghsr* null mice (2) or in patients with *GHSR* missense mutations (9), as far as reported. Therefore, the finding of a positive association between the rs482204 TT genotype with greater birth length was rather unexpected. One might speculate on a non-GH-dependent mechanism: as ghrelin and *GHSR* mRNAs have been demonstrated in

pancreatic islets and ghrelin stimulates insulin secretion (23), *GHSR* variants may enhance ghrelin-mediated insulin release promoting fetal growth. On the other hand, reduced birth length has also been noted in GH-deficient babies, suggesting a possible minor influence of GH on prenatal growth (24). To our knowledge, rs482204 and rs562416 have not been studied before in relation to stature.

To optimize the screening process to identify allelic differences that were associated with height, we first analyzed the tallest and shortest 27% (Table 2) before looking at the population as a whole, including possible gender differences (Fig. 1). Comparing these two approaches, results of both, rs482204 and rs562416, were largely consistent with positive correlations between heights and the TT genotype vs TC/CC in rs482204 and between heights and the TT genotype vs TG/GG in rs562416. Interestingly, the very small group of GG individuals ($\approx 1.2\%$) showed a large variation in heights with significantly taller stature in GG males compared with GG females. Thus, in our population, height in subjects with rs562416 GG genotype appears to be influenced by gender. The small number of GG individuals and the skewing of the gender ratio are in line with possible confounders such as kindredship. For example, the GG individuals in the study population may have come from two extended families with the taller family contributing mostly boys. Therefore, this result should be viewed cautiously.

As opposed to longitudinal height that represents an ideal model for investigation of common inherited traits, weight regulation is influenced by a complex variety of environmental factors in addition to genetic control. Although ghrelin is an orexigenic hormone primarily synthesized by X/A cells of the gastric mucosa, various attempts to relate genetic alterations of either ghrelin (5, 6) or the *GHSR* (5, 12, 13, 14, 15) to body weight have yielded inconsistent results. Baessler *et al.* (12) found that a disequilibrium block of five *GHSR* SNPs including rs572169 was associated with obesity. However, this association was not confirmed by other studies (5, 13, 14, 15). Looking at a healthy, nonobese cohort, we also found no association between body weight and *GHSR* genetic variants.

We chose an LD-based selection of five SNPs across the whole *GHSR* gene including the promoter and the 3'-UTR region to ensure that potentially important genetic regions were not overlooked. Given that SNPs located within noncoding regions such as promoters, enhancers, and silencers may impact gene regulation (25), it is possible that rs482204 and rs562416, both located within the 3'-UTR outside putative functional domains, may act as regulatory SNPs or affect mRNA stability. Alternatively, they may be in LD with a functional variant. The observation that each SNP affects height at different, though overlapping, time periods would suggest a regulatory role. Undoubtedly,

the molecular mechanism(s) underlying our observations will require further research.

As to limitations of our study, we did not have the opportunity to correlate our findings to hormonal parameters (IGF1) within this cohort, although it is plausible that IGF1 levels may in part be accounted for by variation in GHSR activity. Nevertheless, GH-independent factors such as nutritional status also modulate IGF1 levels, which may partly explain why *GHSR* mutations have not been consistently associated with lower IGF1 levels (9).

Furthermore, to fully characterize *GHSR* polymorphisms, correlation with parental heights and *GHSR* genotypes would have been interesting. However, parental DNA samples could not be obtained.

In conclusion, we found consistent associations between *GHSR* variants and longitudinal height in a population-based cohort of 1362 children followed prospectively from birth to 10 years. Follow-up of this unique cohort to adult height may reveal the functional consequence of these *GHSR* variants on growth and body weight, while further studies addressing the potential underlying molecular mechanisms of these *GHSR* variants on growth will be of great interest.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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