Variations in the Ghrelin Receptor Gene Associate with Obesity and Glucose Metabolism in Individuals with Impaired Glucose Tolerance

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Abstract

Background: Ghrelin may influence the development of obesity through its role in the control of energy balance, food intake, and regulation of body weight. The effects of ghrelin are mediated via the growth hormone secretagogue receptor (GHSR).

Methodology/Principal Findings: We genotyped 7 single nucleotide polymorphisms (SNPs) in the GHSR gene and assessed the association between those SNPs and obesity and type 2 diabetes-related phenotypes from 507 middle-aged overweight persons with impaired glucose tolerance participating in the Finnish Diabetes Prevention Study (DPS). Additionally, we performed in silico screening of the 5′-regulatory region of GHSR and evaluated SNPs disrupting putative transcription factor (TF) binding sites in vitro with gelshift assays to determine differences in protein binding between different alleles of SNPs. Rs9819506 in the promoter region of GHSR was associated with body weight (p = 0.036); persons with rs9819506-AA genotype having the lowest body weight. Individuals with rs490683-CC genotype displayed highest weight loss in the whole study population (p = 0.032). The false discovery rate for these results was <10%. Rs490683 and rs509035 were associated with several measures of glucose and insulin metabolism during the follow-up. Rs490683 may be a functional SNP, since gelshift experiments showed differential protein binding between the alleles, with higher binding to the G-allele. Rs490683-C may disrupt a putative binding site for the TF nuclear factor 1 (NF-1), thus rs4906863-GG genotype where the NF-1 site is intact may lead to a higher GHSR gene expression.

Conclusion/Significance: Polymorphisms in the GHSR promoter may modify changes in body weight during long-term lifestyle intervention and affect ghrelin receptor signalling through modulation of GHSR gene expression.

Introduction

Ghrelin is predominantly produced by the stomach and is the only known orexigenic hormone and endogenous ligand for the growth-hormone-secretagogue receptor (GHSR) [1,2]. Ghrelin is composed of 28 amino acids and is uniquely modified by the addition of an octanoyl group to the serine residue at position three. This acylation is necessary for ghrelin to bind to the GHSR and to cross the blood-brain barrier [3].

Ghrelin is implicated in growth hormone release [2], energy balance [4], food intake, and long-term regulation of body weight [5,6]. A major target for ghrelin is the arcuate nucleus of the hypothalamus, where ghrelin acts on appetite-stimulating groups of neurons, which express the potent orexigenic neuropeptides neuropeptide Y (NPY) and agouti-related protein (AGRP) [7]. These NPY/AGRP-containing neurons also express ghrelin receptors [8].

The GHSR gene, located on chromosome 3q26.31, encodes a protein that belongs to the family of G protein-coupled receptors [9]. Two distinct GHSR transcripts are known: GHSR type 1a acts as ghrelin receptor, whereas type 1b is a truncated form of the GHSR 1a and pharmacologically inactive [9]. GHSR 1a is expressed mainly in the hypothalamus and pituitary [10,11], whereas the GHSR type 1b mRNA expression has also been found in many peripheral organs [10], including immune cells [12,13], indicating that ghrelin may have multiple functions in these tissues but with thus far unknown importance.
Models of disrupted ghrelin signalling can show complex energy homeostasis-related phenotypes. GHSR-deficient mice show resistance to diet-induced obesity when supplied with a high-fat diet early in life, which implicates that functional ghrelin signalling is required for the full development of diet-induced obesity [1,14].

In humans, the GHSR gene lies within a quantitative trait locus strongly linked to multiple phenotypes related to obesity and the metabolic syndrome [13]. Baessler et al. have described a “susceptible” and “non-susceptible” haplotype for obesity of five GHSR single nucleotide polymorphisms (SNPs) [15]. Variainen et al. showed an association between insulin values and a GHSR variant. A recent study reports a weak association between one GHSR variant and BMI [17]. In recently conducted genome-wide association (GWA) studies, GHSR was not among the few genes associated with obesity [18,19] or type 2 diabetes [20,21]. Although GWA studies are very elegantly conducted to unravel the genetics of chronic diseases, candidate gene analyses also can often contribute to our knowledge about these disorders. Thus, we investigated SNPs in the GHSR gene and their association with obesity, glucose and insulin metabolism, and the conversion from impaired glucose tolerance (IGT) to type 2 diabetes in participants of the Finnish Diabetes Prevention Study (DPS). We demonstrated an association between one GHSR promoter SNP and weight loss during a 3-year follow-up and consequently showed that this SNP might have functional consequences on the expression of the GHSR gene.

Results

We genotyped 7 SNPs in the GHSR gene, four of which are located in the 5′-region, one in exon 1, one intronic and one SNP in the 3′-region (Fig. 1A). Rs490683, which was associated with weight loss and measures of glucose metabolism, is in strong linkage disequilibrium (LD) with rs509035 (Fig. 1B).

Association with obesity-related phenotypes

At baseline, no significant differences were observed between the genotypes of all seven SNPs for obesity-related traits (Table 1 for rs490683, data not shown for other SNPs). However, using repeated measures ANOVA we observed that during the 3-year follow-up weight was significantly different between genotypes of rs9819506 and BMI [17]. During the 3-year intervention, all subjects being analyzed were analyzed separately, differences in weight loss according to the rs490683-CC genotype and weight loss during a 3-year follow-up and consequently showed that this SNP might have functional consequences on the expression of the GHSR gene.

Association with phenotypes related to glucose metabolism

At baseline, no significant associations with measures of glucose metabolism and the seven genotyped GHSR polymorphisms were observed (Table 1 for rs490683, data not shown for other SNPs).

Discussion

We have shown that two SNPs (rs490683 and rs9819506) in the promoter region of the human GHSR gene are associated with the change in body weight and BMI. The GHSR SNP rs9819506, which has not been examined in previous studies, showed an association and individuals with rs9819506-LI genotype showed lowest values for body weight and a trend for lowest BMI during a 3-year follow-up. Interestingly, subjects with rs490683-CC genotype displayed highest weight loss in the whole study group as well.
as in the intervention group, when assessed separately. Previously, Baessler et al. [15] showed an association between GHSR haplotypes and obesity. The so-called “susceptible” haplotype tested in that study was found more often in obese individuals and included rs490683-C and rs509035-T alleles. These results might be considered contradictory to ours, where we see the rs490683-CC genotype as “beneficial” genotype. However, it is difficult to compare data from a longitudinal intervention study with individuals at high risk of type 2 diabetes describing the effect of the certain genotype on the weight development and a cross-sectional study carried out in a different population. A more recent study, however, could not point out a clear relationship between GHSR SNPs and obesity [17]. Wang et al. [23] showed that obese children and adolescents had a higher rs495225-T allele frequency than underweight subjects, but this trend could not be confirmed in their further studies. Thus, they concluded that there is no

Figure 1. The human GHSR gene and location of SNPs and their LD measures. A. Schematic representation of the human GHSR gene indicating the locations of analyzed single nucleotide polymorphisms (SNPs) that belong to two haploblocks. (black star), SNPs genotyped in the Finnish Diabetes Prevention Study population. # SNPs from HapMap database in transcription factor (TF) binding sites and tested in gelshift assays.

B. Standardized pair-wise linkage disequilibria (LD) measures of D’ and r2 for seven GHSR single nucleotide polymorphisms (SNPs) of the Finnish Diabetes Prevention Study. Major/minor alleles and the minor allele frequency (in parentheses) are included below each SNP identification number. For each cell, the number on the top line is D’ and the number on the bottom line is r2. Strength of LD is indicated in greyscale with black denoting strong and white weak LD.

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evidence for involvement of GHSR SNPs in body weight regulation. In our study, SNPs rs495225 and rs565105 were not unequivocally associated with measures of obesity or type 2 diabetes.

Vartiainen et al. [16] showed that subjects with rs495225-CC genotype had highest area under the insulin curve values and IGFBP-1 concentrations. In the present study, in the DPS population rs6772767, rs490683 and rs509035 were associated with several measures of glucose metabolism over four consecutive measurements. Beneficial changes in glucose metabolism could be observed specifically in individuals with rs490683-CC and rs509035-TT genotypes, especially in the control group. The control group in the DPS trial can be considered as a prospective cohort of people at high risk of diabetes. Since changes in measures of glucose metabolism during the study period were only observed in the control group, it indicates that the rs490683-CC genotype and the rs509035-TT genotype are beneficial or protective genotypes. A reduction in body weight was, however, evident when the whole study population was analysed, but this was mostly explained by the change in the intervention group. This finding is in some discrepancy with the association shown with measures of glucose metabolism. Thus, it might be that the different association with body weight and glucose metabolism are mediated via different mechanisms. In addition, we cannot exclude the impact of the moderate population size contributing to these varying associations. Unfortunately, we do not have the possibility to increase the size of the DPS population, but hopefully in the future it is possible to carry out joint analyses with the data from several other similar type of lifestyle intervention studies. However, we feel that the nature of DPS, as being a longitudinal study with an intensive lifestyle intervention in a carefully phenotyped high risk population, is unique and therefore our results offer valuable contribution and addition to the knowledge about weight and glucose control.

Rs490683 and rs509035 are in strong LD with each other and this may further explain the similar results concerning glucose metabolism phenotypes. Rs509035 is located in the intron of the GHSR gene, thus it was not included in our analysis for putative TF binding sites. Nevertheless, it cannot be excluded that this SNP has functional consequences, but we did not test this. Thus, based on our results we may speculate that rs490683 could be the causal SNP accounting for the effects observed.

As a limitation we need to point out that due to the distribution of these data, only non-parametric tests could be carried out where adjustments for covariates were not possible. To account for multiple testing and to control false positive rate, or type I error, we calculated the false discovery rate (FDR), expressed as q-value, for given p-values. Associations with a significant p-value, but a high q-value must be cautiously interpreted.

To test the potential functional relevance of SNPs in the 5′-region of GHSR we first examined the regions where SNPs are available from a human source. The DNA sequences (oligos) used for the gelshift experiments were designed to match the human sequence. We observed that nuclear proteins were binding to the sequence containing the rs490683-TG allele with much higher abundance compared to the sequence containing the rs490683-TT allele. We used the rs490683-TG allele as an example for further in silico promoter analysis, which showed that this SNP disrupts putative TF-binding sites. Nevertheless, it cannot be excluded that this SNP possibly disrupting putative TF-binding sites with high q-value must be cautiously interpreted.

The whole study population was analysed, but this was mostly explained by the change in the intervention group.

To test the potential functional relevance of SNPs in the 5′-region of GHSR we first examined the regions where SNPs are possibly disrupting putative TF-binding sites with in silico promoter analysis. We tested oligonucleotides with each allele of each SNP in gelshift assays using nuclear protein extracts from rat hypothalamus. We chose to use hypothalami of male rats as a comparable protein source, due to the fact that this tissue is unavailable from a human source. The DNA sequences (oligos) used for the gelshift experiments were designed to match the human sequence. We observed that nuclear proteins were binding to the sequence containing the rs490683-TG allele with much higher

### Table 1. Baseline characteristics of the DPS study subjects according to the rs490683 polymorphism of the GHSR gene.

| Genotype          | P-value* |
|-------------------|----------|
| rs490683-CC (n)   | rs490683-CG (n) | rs490683-GG (n) |
| Sex (M/F)         | 13/33 (46)   | 68/147 (215) | 85/161 (246) | 0.635b |
| Age (years)       | 54±7 (46)    | 56±7 (215)  | 55±7 (246)  | 0.246c |
| Weight (kg)       | 88.5±13.8 (46) | 84.4±12.9 (215) | 87.4±15.2 (246) | 0.080a |
| BMI (kg/m²)       | 32.4±4.9 (46) | 30.8±4.2 (215) | 31.4±4.7 (246) | 0.132a |
| Waist circumference (cm) | 101.5±10.2 (46) | 100.7±10.8 (213) | 101.6±11.3 (246) | 0.062 |
| Waist-to-hip ratio | 0.91±0.07 (46) | 0.92±0.07 (213) | 0.92±0.08 (246) | 0.415 |
| Fasting plasma glucose (mmol/l) | 6.3±0.7 (46) | 6.1±0.7 (215) | 6.1±0.8 (246) | 0.653 |
| 2-h plasma glucose (mmol/l) | 9.0±1.6 (46) | 9.0±1.6 (215) | 8.8±1.4 (246) | 0.198 |
| Fasting serum insulin (pmol/l) | 16.5±8.7 (40) | 14.5±6.5 (193) | 14.7±7.9 (228) | 0.574 |
| 2-h serum insulin (pmol/l) | 105.4±61.2 (39) | 92.9±57.3 (192) | 95.9±71.7 (227) | 0.728 |
| HOMA-IR (mmol²xmU⁻¹x1⁻²) | 4.61±2.43 (40) | 4.01±2.01 (193) | 4.09±2.47 (228) | 0.550 |
| HOMA-IS (U/mmol) | 126.8±78.2 (40) | 119.6±73.2 (193) | 115.7±58.7 (228) | 0.567 |
| Total serum cholesterol (mmol/l) | 5.81±0.91 (46) | 5.64±0.96 (215) | 5.55±0.89 (245) | 0.147 |
| HDL cholesterol (mmol/l) | 1.25±0.32 (46) | 1.21±0.29 (215) | 1.20±0.29 (245) | 0.523 |
| LDL cholesterol (mmol/l) | 3.68±0.79 (45) | 3.64±0.86 (215) | 3.59±0.82 (244) | 0.681 |
| Triglycerides (mmol/l) | 1.95±0.92 (46) | 1.74±0.76 (215) | 1.68±0.75 (245) | 0.139 |
| Systolic blood pressure (mmHg) | 138±17 (45) | 137.4±17.8 (213) | 139±18 (244) | 0.458 |
| Diastolic blood pressure (mmHg) | 87±8 (45) | 85±10 (213) | 86±10 (244) | 0.592 |

Values are means±SD; ANOVA for comparison among three genotype groups, adjusted for age, sex and BMI as main effects.

*p-value adjusted for age and sex.

Chi-square Test.

Kruskal-Wallis Test.

FDR was >60% for all p-values.

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the NF-1 half site is intact, GHSR expression is enhanced, thus speculated that in individuals with NF-1 has been shown to activate transcription [24] and it can be sites (TTGGC or GCCAA) with somewhat reduced affinity [24].

DNA, although NF-1 can also bind specifically to individual half symmetric consensus sequence TTGGC(N5)GCCAA on duplex CAAT-box TF [26,27]. NF-1 protein binds as a dimer to the dyad equivalent to both the TGGCA-binding protein [25] and the adenovirus DNA replication [24]. Binding specificity of NF-1 is site-specific DNA-binding proteins functions both as cellular TFs changing the sequence from GCCA to CCCA. The NF-1 family of NF-1 binding site exists and is disrupted by the SNP rs490683, affinity than to that of rs490683-C allele. At this position a putative NF-1 binding site exists and is disrupted by the SNP rs490683, changing the sequence from GCCA to GCCA. The NF-1 family of site-specific DNA-binding proteins functions both as cellular TFs in the regulation of gene expression and as replication factors for adenovirus DNA replication [24]. Binding specificity of NF-1 is equivalent to both the TGGCA-binding protein [25] and the CAAT-box TF [26,27]. NF-1 protein binds as a dimer to the dyad symmetric consensus sequence TTGGC(N3)GCCAA on duplex DNA, although NF-1 can also bind specifically to individual half sites (TTGGC or GCCAA) with somewhat reduced affinity [24]. NF-1 has been shown to activate transcription [24] and it can be speculated that in individuals with rs490683-GG genotype, where the NF-1 half site is intact, GHSR expression is enhanced, thus potentially leading to an increase in receptor signalling and ultimately to an increase in appetite [28]. In fact, our study shows that subjects with rs490683-GG genotype lost less weight during a lifestyle intervention than individuals with rs490683-CC genotype.

It has been shown recently that GHSR signals with ~50% activity even in the absence of an agonist [29]. That implies that control of the expression level of the receptor is directly correlated to signalling activity [7]. It has been shown that during prolonged fasting, GHSR expression in the hypothalamus is increased, which could contribute to the amplification of ghrelin action [20] and could be expected to result in a ghrelin-independent increase in receptor signalling and thereby an increase in appetite [7]. The GHSR polymorphism rs490683 was associated with weight change in our study population and the rs490683-GG genotype, where the NF-1 site is intact, might lead to increased ghrelin receptor expression.

The alleles of rs519394 did not show differential protein binding, indicating that the SNP is not in an important region for GHSR gene expression. Although rs9819506 was associated with weight and BMI, gelshift assays showed no difference in protein binding between the alleles, which may be due to species differences. Although we could observe a difference in protein binding between the alleles of rs6772676, it cannot explain the observed association with glucose levels.

TFs and more importantly their DNA binding domains are highly conserved between humans and rodents. For example, the nucleic acid sequence of the rat NF-1 is 99% identical to the human sequence and on amino acid level there is a 91% overlap between the sequences. The variations in the rat NF-1 protein (compared to the human sequence) are not located within its DNA binding domain, thus they are unlikely to affect its binding to DNA. Due to the tissue unavailability from humans the rat protein extract provides a good model to test for differential protein binding to the alleles because of the high degree of conservation between the two species. We used hypothalami of male rats only, but obviously the autosomes of male and female rats are identical. Only genes located on the Y-chromosome will differ between the two sexes, whereas e.g. NF-1 is located on chromosome 17. Some TFs might show a difference in their expression levels. Thus, the gelshift bands might be stronger or weaker comparing males and females but the clear difference of protein binding to the different SNP alleles will still occur.

To our knowledge, no GWA study for obesity has been carried out in Finns alone; however, samples from Finnish studies have been used in replication studies (e.g. [18,19]). The primary GWA studies investigating the genetics of type 2 diabetes are available. The studies regarding type 2 diabetes in Finnish subjects [20,21,30] have used the Illumina HumanHap300 Genotyping BeadChips containing over 317,000 SNPs. Two SNPs in the GHSR gene overlap with the SNPs investigated in our study, that is, rs9819506 and rs565105. One more SNP, rs10513702, is in high LD (r2 = 0.966) with rs6772676, which was also genotyped in our study. Other GWA studies investigating obesity have used variably the Affymetrix GeneChip Human Mapping 10K, 100K or 500K Array Set [31,32,33,34]. In the 500K Array Set are 6 SNPs of the GHSR gene included and one of those overlaps with the SNPs genotyped in our study (rs509035). The other GHSR SNPs are not in LD with SNPs which we genotyped. In GWA studies stringent quality control criteria are applied for the given setting. Thus it is possible that the few SNPs used in this study from the GHSR gene did not match those criteria (e.g. call rate, Hardy-Weinberg equilibrium for the given study population).

In summary, results of our study suggest that polymorphisms in the GHSR gene are longitudinally associated with obesity and glucose metabolism in a prospective study setting. Genetic
Variation in the promoter may have an effect on appetite regulation by modulating gene expression of GHSR and subsequently ghrelin receptor signalling. This study is the first to show potential functional consequences of a GHSR SNP. Further studies are warranted to confirm our results and to find SNPs that are causal and have clinical relevance.

Figure 3. Glucose metabolism measures according to rs490683 genotypes in DPS. A. 2-h plasma glucose according to rs490683 genotypes in all subjects. Mean±SEM are estimated marginal means calculated from repeated measures ANOVA with group, age and BMI at baseline as significant covariates. rs490683-CC (n = 42, filled circles); rs490683-CG (n = 171, open circles); rs490683-GG (n = 210, filled triangles). Pair-wise comparisons all subjects: CC vs. CG p = 0.020, CC vs. GG p = 0.295, CG vs. GG p = 0.190. B–E. Relative changes fasting plasma glucose (B), 2-h plasma glucose (C), fasting serum insulin (D) and 2-h serum insulin (E) according to the different genotypes of rs490683 in all subjects and separately in intervention and control group. rs490683-CC (black bars); rs490683-CG (striped bars); rs490683-GG (white bars). The q-value denotes the false discovery rate (FDR) and is reported only for p < 0.1. n.s., non-significant. doi:10.1371/journal.pone.0002941.g003
Materials and Methods

Subjects and study design

The Finnish DPS is an intervention study carried out in five centres in Finland (clinical trial reg. no: NCT00518167). The main aim of the study was to assess the efficacy of an intensive individually designed diet and exercise program to prevent or delay the onset of type 2 diabetes in subjects with IGT. The study design, subjects, inclusion and exclusion criteria and intervention program have been described earlier in detail [35,36]. In brief, 522 overweight (BMI ≥ 25 kg/m²) men and women aged 40 to 65 years with IGT participated in the study. DNA samples were available from 507 individuals (intervention group 259 and control group 248 subjects). IGT was defined as a 2-h plasma glucose concentration of 7.8 to 11.0 mmol/l (oral glucose tolerance test [OGTT] 75 g) with a fasting plasma glucose concentration less than 7.8 mmol/l [37]. The persons enrolled in the study were randomly assigned to one of the two treatment modalities, the intervention group or the conventional care control group. The study protocol was approved by the Ethics Committee of the National Public Health Institute in Helsinki, Finland, and conducted in accordance with the guidelines proposed in the Declaration of Helsinki. All participants gave written informed consent. We certify that all applicable institutional and governmental regulations concerning the ethical use of human volunteers were followed during this research.

Selection of GHSR SNPs and genotype analysis

For selection of GHSR SNPs for genotype analysis the HapMap [38], NCBI [39] and Ensembl [40] databases were used. Originally, six tag SNPs from two haploblocks covering 15 kb of the gene including three SNPs in haploblock 1 in the 5’ end of the gene (rs474225, rs490683 [alternative name in HapMap rs863441], rs9819506), and three SNPs in haploblock 2, one in the coding region in exon 1 leading to a synonymous amino acid substitution (rs495225), one in the intron (rs509035) and one in the 3’ end of the GHSR gene (rs565105) were selected (Fig. 1A). In addition, all SNPs in haploblock 1 in the 5’ regulatory region (spanning over 17 kb, chr 3: 173648897–17365897; NCBI accession number for GHSR NM_198407.1) available in the HapMap database and located in putative TF-binding sites were tested for their functionality in gelshift experiments. SNPs which showed different protein binding in vitro were genotyped additionally (rs6772676).

Genotyping was performed with TaqMan Allelic Discrimination Assays (Applied Biosystems, Foster City, CA, USA). The PCR amplification was performed in a GeneAmp PCR system 2700 (Applied Biosystems, Foster City, CA, USA) in following conditions: 95°C for 10 min and 40 cycles of denaturation 92°C for 15 sec and annealing/extension 60°C for 1 min. Allele-specific fluorescence was detected on an ABI Prism 7000 sequence detector. A subset of randomly selected samples representing 6.3% of the study cohort was repeated.

Figure 4. Differential protein binding of rat hypothalamus nuclear protein extract. Gelshift experiments were performed with 32P-labeled oligonucleotides containing one or the other allele of respective single nucleotide polymorphism (SNP). Numbers below the gels indicate the means of three independent experiments with standard deviation (SD) in parenthesis. Protein binding of the "wild-type" allele was normalized to 100%. Representative gels are shown. NS denotes non-specific DNA-protein interactions, which are not due to specific binding between the labelled DNA and the proteins contained in the hypothalamus extract. * p<0.05; ** p<0.001.

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Promoter analysis

In silico promoter analysis of the sequences containing all SNPs in haploblock 1 for putative TF-binding sites was performed using Genomatix MatInspector Release professional 7.4.8.2 [22]. We determined all SNPs that lie in possible TF-binding sites and consequently all TF-binding sites that are disrupted by either one of each SNP allele. We used a threshold of 0.75 for core similarity and the optimized setting for matrix similarity.

Protein extraction

Hypothalami of three male Sprague-Dawley rats (supplier Harlan, Netherlands) were pooled and the frozen tissue was homogenized in 0.5 ml of low salt buffer (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 50 mM KCl, 0.5 mM dithiothreitol, proteinase inhibitors) using a rotor-stator homogenizer (ART Labortechnik, Müllheim, Germany). Cells were then resuspended in 2–3 packed volumes of the same low salt buffer with 0.5% Nonidet P-40 and homogenized by pipetting on ice. The nuclear pellet was subsequently resuspended in one packed cell volume of high salt buffer (10 mM Hepes, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, proteinase inhibitors). Extraction was performed for 30 min on ice.

Gelshift assay

Gelshift assays were performed with 10 μg protein of the nuclear extracts. The proteins were incubated for 15 min in a total volume of 20 μl of binding buffer (130 mM KCl, 1 mM dithiothreitol, 25 ng/ml herring sperm DNA, 5% glycerol, 10 mM Hepes, pH 7.9). Constant amounts (1 ng) of 32P-labeled double-stranded oligonucleotides (50,000 cpm) containing either one of the respective SNP alleles (supplementary Table S1) were then added, and incubation was continued for 20 min at room temperature. Protein-DNA complexes were resolved by electrophoresis in 8% non-denaturing polyacrylamide gels (mono- to bisacrylamide ratio 19:1) in 0.5×TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3) for 90 min at 200 V and quantified on a FLA-3000 reader (Fuji, Tokyo, Japan) using ScienceLab99 software (Fuji).

Statistical analysis

The significances of differences in genotype and allele frequencies were analyzed using a chi-square test and a two-sided Fisher’s exact test, respectively. Normal distributions were tested with the Kolmogorov-Smirnov test with Lilliefors correction. To normalize the skewed distributions, logarithmic transformations or reciprocal transformations were applied when needed. The differences in continuous variables between genotypes of single SNPs at baseline were evaluated with the general linear model (GLM) for the univariate analysis of variance (ANOVA). Biochemical measurements were adjusted for age, sex and BMI as main effects and weight-related variables for age and sex only. When variables could not be transformed to be normally distributed, Kruskal-Wallis test was used. GLM repeated measures ANOVA was used to analyze differences between genotypes of each SNP during the follow-up period concerning obesity-related and insulin- and glucose-metabolism related phenotypes. A final model was built manually with a backward selection procedure with following covariates included: age at baseline, sex, study group and BMI at baseline, as appropriate. Genotype*group interaction was tested and groups were analyzed separately if the

Table 2. Single nucleotide polymorphisms (SNPs) in the GHSR promoter and putative transcription factor (TF) binding sites.

| SNP     | Allele | Putative TF binding sites                                                                 |
|---------|--------|------------------------------------------------------------------------------------------|
| rs6772676 | C      | Activating transcription factor, ATF-1                                                      |
|         |        | Pax-3 paired domain protein, expressed in embryogenesis, mutations correlate to Waardenburg Syndrome |
|         | T      | PBX - HOXA9 binding site                                                                 |
| rs9881097 | T      | Avian C-type LTR TATA box                                                                  |
| C       | Pu.1 (Pu120) Ets-like TF identified in lymphoid B-cells PAX 2/5/8 binding site          |
| rs474225  | T      | Fkh-domain factor FKHL1 (FOXO)                                                             |
| C       | Interferon regulatory factor 3 (IRF-3)                                                     |
|         |        | -                                                                                        |
| rs490683  | C      | Orphan nuclear receptor rev-erb alpha (NR1D1), monomer binding site                        |
| G       | Non-palindromic nuclear factor 1 binding sites                                             |
| rs11918879 | C      | Bm-S, POU-VI protein class (also known as emb and CNS-1)                                  |
| T      | Bm-S, POU-VI protein class (also known as emb and CNS-1)                                  |
| rs477441  | A      | Hepatic nuclear factor 1                                                                   |
| C       | Ecotropic viral integration site 1 encoded factor, amino-terminal zinc finger domain     |
| rs9819506  | G      | TEA domain-containing factors, transcriptional enhancer factors 1,3,4,5                   |
|         | Octamer binding site (OCT1/OCT2)                                                         |
|         | Octamer-binding factor 1, POU-specific domain                                             |
| A      | -                                             |
| rs519384  | A      | Liver enriched Cut - Homeodomain TF HNF6 (ONECUT)                                         |
| T      | Liver enriched Cut - Homeodomain TF HNF6 (ONECUT)                                         |

1SNPs genotyped from DPS population.
2different protein binding in gelshift assay.

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interaction term was statistically significant. P-values are for trend between all three genotype groups and pairwise comparisons based on estimated marginal means were adjusted for multiple comparisons with Bonferroni correction. Relative changes in weight and measures of glucose and insulin metabolism from baseline to year 3 were analyzed with Kruskal-Wallis test, because data were not normally distributed even after applying several transformations. A p-value of 0.05 or lower was considered statistically significant. Correction for multiple hypothesis testing was performed with FDR using the Q-value 1.0 software [41]. πQ was estimated with bootstrap method using a λ range from 0 to 0.9 by 0.05. The FDR for each p-value is reported as q-value. The q-value threshold is the proportion of significant features that turn out to be false leads. Data are given as means±SD, unless otherwise stated. All data were analyzed using SPSS for Windows 14.0 (SPSS Inc., Chicago, IL, USA). LD statistics were calculated and haplotype blocks were visualized by using the Haploview software [42].

Supporting Information

Table S1 Oligonucleotides used in gelshift assays. List of oligonucleotides which were used to test the difference in protein binding among different GHSR SNPs.

Found at: doi:10.1371/journal.pone.0002941.s001 (0.04 MB DOC)

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The DPS group has been previously described in the earlier publications [35,36]. The Finnish Diabetes Prevention Group consists additionally of following people: Timo Valle, Helena Hamalainen, Pirjo Ilanne-Parikka, Sirrika Keinanen-Kiukkaanniemi.

Author Contributions

Conceived and designed the experiments: UM LP CC MU. Performed the experiments: UM TD. Analyzed the data: UM TD AMT. Contributed reagents/materials/analysis tools: LP MK JL JE CC JT MU. Wrote the paper: UM TD. Read and commented on the paper: LP MK JL JE CC JT MU.

References

1. Murphy KG, Bloom SR (2006) Gut hormones and the regulation of energy homeostasis. Nature 444: 854–859.
2. Koijma N, Hosoda H, Date Y, Nakazato M, Matsuo H, et al. (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402: 656–660.
3. Koijma N, Kangawa K (2005) Ghrelin: structure and function. Physiol Rev 85: 495–522.
4. Inui A (2003) Ghrelin: an orexigenic and somatotrophic signal from the stomach. Nat Rev Neurosci 2: 551–560.
5. Tschop M, Smiley DL, Heimann ML (2000) Ghrelin induces adiposity in rodents. Nature 407: 908–913.
6. Nakazato M, Murakami N, Date Y, Koijma N, Matsuo H, et al. (2001) A role for ghrelin in the central regulation of feeding. Nature 409: 194–198.
7. Holst B, Schwartz TW (2004) Constitutive ghrelin receptor activity as a signaling point in appetite regulation. Trends Pharmacol Sci 25: 113–117.
8. Willersen MG, Kristensen P, Romer J (1999) Co-localization of growth hormone secretagogue receptor and NPR mRNA in the arcuate nucleus of the rat. Neuroendocrinology 70: 306–316.
9. Howard AD, Feighner SD, Cully DF, Arena JP, Liberator PA, et al. (1996) A case-control study nested within the European Prospective Investigation into Cancer and Nutrition (EPIC) of the association of polymorphisms of genes coding for ghrelin and its receptor in relation to anthropometry, circulating levels of IGF-I and IGFBP-3, and breast cancer risk: a case-control study nested within the European Prospective Investigation into Cancer and Nutrition (EPIC). Carcinogenesis 16: 1317–1320.
10. van der Lely AJ, Tscho¨p M, Heiman ML, Ghigo E (2004) Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. Endocr Rev 25: 426–457.
11. van der Lely AJ, Tschop M, Heimann ML, Ghigo E (2004) Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. Endoc Rev 25: 426–457.
12. Hattori N, Saito T, Yagyu T, Jiang BH, Kitagawa K, et al. (2001) GH, GH secretagogue receptor, and ghrelin expression in human T cells, B cells, and neutrophils. J Clin Endocrinol Metab 86: 4284–4291.
13. Dixit VD, Schaffer EM, Yale RS, Collins GD, Salichedel SK, et al. (2004) Ghrelin inhibits leptin- and activation-induced proinflammatory cytokine expression by human monocytes and T cells. J Clin Invest 114: 57–66.
14. Ziegler JM, Kawa K, Coppari R, Balbuzan N, Marce JG, et al. (2005) Mice lacking ghrelin receptors resist the development of diet-induced obesity. J Clin Invest 115: 3564–3572.
15. Baesler A, Hasinoff MJ, Fischer M, Reinhard W, Sonnenberg GE, et al. (2005) Genetic linkage and association of the growth hormone secretagogue receptor (ghrelin receptor) gene in human obesity. Diabetes 54: 259–267.
16. Vartiaenen J, Poykko SM, Raisanen T, Kesaniemla Y, Ukkola O (2004) Sequencing analysis of the ghrelin receptor (growth hormone secretagogue receptor type 1a) gene. Eur J Endocrinol 150: 457–463.
17. Drooui L, McKay JD, Ganzian F, Willkening S, Rinaldi S, et al. (2008) Polymorphisms of genes coding for ghrelin and its receptor in relation to anthropometry, circulating levels of IGF-I and IGFBP-3, and breast cancer risk: a case-control study nested within the European Prospective Investigation into Cancer and Nutrition (EPIC). Carcinogenesis 30: 1093–1097.
18. Loos RJ, Lindgren CM, Li S, Wheeler E, Zhao JH, et al. (2008) Common variants near MC4R are associated with fat mass, weight and risk of obesity. Nat Genet 40: 768–775.
19. Frayling TM, Timpson NJ, Weedon MN, Zeggini E, Freathy RM, et al. (2007) A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. Science 316: 889–894.
20. Scott LJ, Mohlke KL, Bonnyau CC, Willer CJ, Ly LV, et al. (2007) A genome-wide association study of 2 diabetes in Finns detects multiple susceptibility variants. Science 316: 1341–1345.
21. Salonen JT, Uumari P, Aalto JM, Pursiainen K, Kaikkonen J, et al. (2007) Type 2 diabetes whole-genome association study in four populations: the DiaGen consortium. Am J Hum Genet 81: 333–343.
22. Cartharius K, Frech K, Grote K, Klocke B, Halmeyer M, et al. (2005) MaD-inspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics 21: 2953–2954.
23. Wang HJ, Geller F, Demppe A, Schiaible N, Friedel S, et al. (2004) Ghrelin receptor gene: identification of several sequence variants in extremely obese children and adolescents, healthy normal-weight and overweight students, and children with short normal stature. J Clin Endocrinol Metab 89: 157–162.
24. Gronostajski RM (2008) Roles of the NHE1 gene family in transcription and development. Gene 429: 31–45.
25. Bormgmeier U, Nowock J, Sippel AE (1984) The TGGCA-binding protein: a eukaryotic nuclear protein recognizing a symmetrical sequence on double-stranded linear DNA. Nucleic Acids Res 12: 4289–4311.
26. Jones KA, Kadsouga JT, Rosenfeld PJ, Kelly TJ, Tijan R (1987) A cellular DNA-binding protein that activates eukaryotic transcription and DNA replication. Cell 48: 79–89.
27. Santoro C, Mermod N, Andrews PC, Tijan R (1988) A family of human CCAT-box-binding proteins active in transcription and DNA replication: cloning and expression of multiple cDNAs. Nature 334: 218–224.
28. Kim MS, Yoon CY, Park KH, Shin CS, Park KS, et al. (2003) Changes in ghrelin and ghrelin receptor expression according to feeding status. Neuroreport 14: 1317–1320.
29. Holst B, Cygankiewicz A, Jensen TH, Andersen M, Schwartz TW (2003) High constitutive signaling of the ghrelin receptor-identification of a potent inverse agonist. Mol Endocrinol 17: 2201–2210.
30. Zeggini E, Scott LJ, Saxena R, Voight BF, Marchini JL, et al. (2008) Meta-analysis of genome-wide association data and large-scale replication identifies additional susceptibility loci for type 2 diabetes. Nat Genet 40: 638–645.
31. Hinney A, Nguyen TT, Scherag A, Friedel S, Bronner G, et al. (2007) Genome-wide association (GWA) study for early onset extreme obesity supports the role of fat mass and obesity associated gene (FTO) variants. PLoS ONE 2: e1361.
32. Scteteri A, Samana S, Chen WM, Uda M, Allgo W, et al. (2007) Genome-wide association scan shows genetic variants in the FTO gene are associated with obesity-related traits. PLoS Genet 3: e115.
33. Liu YJ, Dina C, Yan H, et al. (2008) Genome-wide association scans identified CFTNNB1 as a novel gene for obesity. Hum Mol Genet 17: 1803–1813.
34. Fox CS, Heard-Costa N, Cupples LA, Dupuis J, Vasan RS, et al. (2007) Genome-wide association to body mass index and waist circumference: the Framingham Heart Study 100K project. BMC Med Genet 8 Suppl 1: S18.
35. Eriksson J, Lindstrom J, Valle T, Aunola S, Ha¨ma¨la¨inen H, et al. (1999) Prevention of Type II diabetes in subjects with impaired glucose tolerance: the Diabetes Prevention Study (DPS) in Finland. Study design and 1-year interim
report on the feasibility of the lifestyle intervention programme. Diabetologia 42: 793–801.
36. Tuomilehto J, Lindström J, Eriksson JG, Valle TT, Hamalainen H, et al. (2001) Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. N Engl J Med 344: 1343–1350.
37. WHO (1985) Diabetes mellitus. Report of a WHO Study Group. World Health Organ Tech Rep Ser 727: i–xii, 1–113.
38. Altshuler D, Brooks LD, Chakravarti A, Collins FS, Daly MJ, et al. (2005) A haplotype map of the human genome. Nature 437: 1299–1320.
39. Wheeler DL, Barrett T, Benson DA, Bryant SH, Canese K, et al. (2007) Database resources of the National Center for Biotechnology Information. Nucleic Acids Res 35: D5–12.
40. Hubbard TJ, Aken BL, Beal K, Ballester B, Caccamo M, et al. (2007) Ensembl 2007. Nucleic Acids Res 35: D610–617.
41. Storey JD, Tibshirani R (2003) Statistical significance for genomewide studies. Proc Natl Acad Sci U S A 100: 9440–9445.
42. Barrett JC, Fry B, Maller J, Daly MJ (2005) Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21: 263–265.