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ORIGINAL ARTICLE

LACK OF ASSOCIATION BETWEEN VARIANTS WITHIN THE *AHSG*, *HCRT* AND *NPY2R* GENES AND ANTHROPOMETRICAL PARAMETERS IN CZECH POST-MONICA STUDY

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ABSTRACT

The aim of this study was to examine single nucleotide polymorphisms (SNPs) of candidate genes α*2-Heremans-Schmid glycoprotein* (*AHSG*, rs4917), *Hypocretin* (*HCRT*, rs760282) and *Neuropetide Y2 receptor (NPY2R*, rs 1047214), which are known to have a potential effect on body mass index (BMI) and other indicators of obesity. A population study was performed in 2007/2008 on 2559 adults (1191 males and 1368 females) from the Czech post-MONICA project. The SNPs were examined using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. We did not find any significant association between the examined SNPs and BMI across the whole population. A significantly lower triglyceride level was found in the *AHSG* gene CC homozygotes compared to T allele carriers in the entire population ($p = 0.009$). In conclusion, we are not able to confirm the hypothesis that polymorphisms within the *AHSG*, *HCRT* and *NPY2R* genes are major genetic determinants of BMI and plasma lipids in the Czech-Slavonic population.

Keywords: α*2-Heremans-Schmid glycoprotein* (*AHSG*) gene; Body mass index (BMI); *Hypocretin* (*HCRT*) gene; *Neuropetide Y2 receptor* (*NPYR*) gene; single nucleotide polymorphism (SNP).

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INTRODUCTION

Obesity is a current world problem that has reached pandemic proportions and is considered a complex disorder that is regulated by behavioral, environmental and genetic factors [1-3]. Recent genetic studies focusing on families, twins and adopted children have demonstrated the influence of heritability on body mass index (BMI) that ranged from 50.0 to 90.0% [1]. Among the candidate genes with the potential to influence BMI, there are also genes for α*2-Heremans-Schmid glycoprotein* (*AHSG*), *Hypocretin* (*HCRT*) and *Neuropetide Y2 receptor* (*NPY2R*).

The AHSG (also called Fetuin A) is mainly secreted by hepatocytes and is abundant in blood plasma. The *AHSG* gene is located at chromosome 3q27 whose locus is associated with type 2 diabetes, metabolic syndrome [4] and adipocyte insulin action in humans [5]. Serum AHSG is a natural endogenous inhibitor of the insulin-stimulated insulin receptor tyrosin kinase activity [4-6], insulin receptor autophosporylation, and insulin substrate 1 phosporylation [4]. Rs4917 (Thr248Met) is considered the strongest marker of AHSG plasma levels [7]. In humans, an increased level of AHSG in plasma is associated with insulin resistance and impaired glucose tolerance [8] and plasma cholesterol [6]. However, no relationship between *AHSG* polymorphisms and coronary atherosclerosis has been found [7]. Mice with knockout *AHSG* gene were found to be more sensitive to insulin, resistant to weight gain after a fat enriched diet and also to have a decreased body fat content [9].

The *HCRT* gene for orexin encode prepro-orexine precursor from which orexin A and orexin B neuropep-tides, (also called hypocretins: HCRT1 and HCRT2) that regulate sleep, appetite and energy intake, are derived [10, 11]. The expression of both neuropeptides is highly limited to neurons located in the lateral hypothalamic area [12,11], which is known as the "hunger center" [10,12,13]. Hara *et. al.* [14] generated transgenic mice with ablated orexin-containing neurons by expression of toxic ataxin-3. The transgenic mice exhibited late-onset obesity despite their lower food intake in comparison with non transgenic litter mates.

A negative correlation between orexin A plasma levels and BMI in children [15] and adults [16] has been observed. It is possibly induced by the ability of orexin to regulate the plasma leptin circadian rhythmicity. Patients with narcolepsy accompanied by a low level of HCRT1 also demonstrated lower leptin levels (reduced by 50.0%) without the nocturnal increase that is observed in healthy subjects [17]. Since there exists a premise for a link between orexin levels in the blood and susceptibility to obesity, we focused on an examination of polymorphisms in the regulatory region. Within the 3 kb region upstream of the *HCRT* start codon we found only the rs760282 (–909 T>C) polymorphism with sufficient rate of occurrence. While the rs760282 SNP is unlikely to be able to influence transcription it is used as an informative one [18]. This SNP was chosen because it is near to the regulatory region of the *HCRT* gene and it can be a marker of the regulation of the *HCRT* gene transcription rate.

The *NPY2R* gene encodes the Neuropeptide Y2 receptor which belongs to a heterogeneous family of G-protein-coupled receptors [19]. Four binding subtypes of neuropeptide receptors (Y1-Y4) were described. The Y1 and Y2 receptors bind to peptide YY (PYY) and neuropeptide Y (NPY) [20]. These abdominal hormones are members of the pancreatic polypeptide family together with pancreatic polypeptide (PP). All these polypeptides have been described as strong central regulators of gastric function [1,21]. Neuropeptide Y is an appetite hormone and is widely distributed throughout the central and peripheral nervous systems. The NPY is the most abundant neuropeptide in the brain and is associated with the regulation of blood pressure, appetite, mood and circadian rhythms [19]. Single nucleotide polymorphisms in the *NPY2R* genes have been found to play a role in obesity, having an additive effect on other genes [22]. In a study performed on French Caucasians, rs1047214 was selected for genotyping from 12 investigated SNPs [21]. In accordance with the French and other studies, we decided to investigate this SNP on our database [23]. In our study, we analyzed the effect of the SNPs within the *AHSG* (rs4917), *HCRT* (rs760282) and *NPY2R* (rs1047214) genes on anthropo-metrical and biochemical parameters in the Czech-Slavonic population.

MATERIALS AND METHODS

We analyzed a randomly selected representative sample of adult individuals (1191 males and 1368 females), aged 25-64 years at the time of first examination (Table 1). All individuals had participated in the Czech post-MONICA study, performed according to the World Health Organization MONICA Project protocol, which was aligned to examine risk factors of cardiovascular disease development, including BMI and plasma lipids, after overnight fasting [24,25]. The subjects were examined in nine Czech districts (Kromeriz, Chrudim, Cheb, Jindrichuv Hradec, Pardubice, Litomerice, Plzen, Prague East, and Benesov) in 1997/1998 and were reexamined in 2000/ 2001 and 2007/2008. For our purposes, the most recent data (2007/2008) were used. Written informed consent was given by all individuals. The study was approved by the Institutional Ethics Committee and is in agreement with the Helsinki Declaration of 1975.

Table 1. Basic characteristics of the analyzed individuals (values are expressed as mean ± SD).

	Males	**Females**
Number of subjects	1191	1368
Age (years)	49.00 ± 10.80	48.60 ± 10.60
Total cholesterol (mmol/L)	5.76 ± 1.06	5.80 ± 1.15
Triglycerides (mmol/L)	1.97 ± 1.28	1.47 ± 0.82
Body mass index (kg/m^2)	28.20 ± 4.00	27.60 ± 5.50
Non smokers (%)	67.30	78.50
Diabetes prevalence (%)	8.90	6.80
Hypertension prevalence (%)	40.70	33.10

Table 2. Primers sequence, restriction enzymes and size of the restriction fragments used for detection of polymorphisms in genes for AHSG, prepro-orexine and NPY2R.

Polymorphism	Primer Sequence (5'>3')	PCR Product	Enzyme	Size	Allele	
AHSG rs4917	F: TGG GGC AGA GGT TGC AGT GAC CTC C R: AAT GTC CTG TCA TCA CTA CTG CAC TGC	132 bp	*Nco*I	132 105 + 27	C T	Thr Met
HCRT rs760282 – Nested PCR	F: ATA AGA AGC TTG GGC CTG GA R: ACC TTG AAA ACT CAG CCT AGA CAG T F: GCG GGG GGG *CCT* GGT GCA GTG GCC R: ACT AAA TTT TGG TCT TGT TGC TCA G	1575 bp 295 bp	*Hin*1II	 295 108 + 187	 C T	 Regulatory region
NPY2R rs1047214	F: ATC AGC TTC CTG ATT ATT GGC TTG GC R: AAG ACT ATA GAC AGT GCC ATA GAT GC	174 bp	*Kpn*2I	174 95 + 79	C T	Ile Ile

Polymorphisms were genotyped using the the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (for more details see Table 2) in a total volume of 25 μL. All PCR chemicals and restriction enzymes used were from Fermentas International Inc., Burlington, Ontario, Canada. The PCR reactions were performed on the PCR cyclers MJ Research DYAD Disciple. Restriction fragments were separated *via* horizontal electrophoresis on 10.0% polyacrylamide gel using the MADGE platform.

To check our PCR-RFLP results, we repeated 5.0% of the samples in each polymorphism. The results of SNPs in the *AHSG*, *HCRT* and *NPY2R* genes were identical in 100.0% of cases.

Hardy-Weinberg equilibrium was proved by the χ^2 test to confirm the independent segregation of the alleles. Differences between genotypes were calculated by analysis of variance (ANOVA), logarithmic transformation was used for triglyceride levels. Mean values ± SD were calculated, two-tailed *p* values of less than 0.05 were considered to be significant (Table 3).

RESULTS

The frequencies of the examined genotypes were similar to the previously analyzed populations in *AHSG* [4,7,8], *HCRT* [18] and *NPY2R* [1,21,23]. The number and percentage of individual genotypes and call rates are summarized in Table 3. No association between polymorphisms and BMI or waist-hip ratio (WHR) was found. No interaction between gene and gender was found.

The relationship between genotype and phenotype was observed for the *AHSG* gene. A significantly lower triglyceride level was found in the CC genotype ($p = 0.0302$) compared with CT and TT genotypes in the group involving both genders. After using the CC *vs.* T carriers model, the *p* value decreased to 0.0088.

Table 3. Genotypes and frequencies of examined SNPs in the Czech post-MONICA study.

	Genotype; *n* (%)			Call Rate
Genes	TT	TC	CC	%
AHSG, rs4917	199 (9.4)	959 (45.1)	969 (45.6)	83.1
Males Females	91 (9.5) 108 (9.2)	424 (44.4) 535 (45.7)	441 (46.1) 528 (45.1)	
HCRT, rs760282	1062 (53.4)	795 (40.0)	132 (6.6)	77.7
Males Females	454 (51.9) 608 (54.5)	349 (39.9) 446 (40.0)	71 (8.1) 61 (5.5)	
NPY2R, rs1047214	716 (32.9)	1058 (49.4)	366 (17.7)	98.4
Males Females	331 (34.4) 385 (32.7)	472 (49.0) 586 (49.8)	160 (16.6) 206 (17.5)	

Table 4. Impact of genotype and gender on anthropometrical parameters. [The asterisk (*) denotes statistical significance.]

		***AHSG*, rs4917 (mean ± SD)**			***HCRT*, rs760282 (mean ± SD)**			***NPY2R*, rs1047214 (mean ± SD)**		
	Sex	**CC**	**CT**	**TT**	**CC**	**CT**	**TT**	**CC**	**CT**	**TT**
Body mass index (kg/m^2)	M F	28.85±4.12 28.07±5.45	28.90±4.04 28.49±5.53	28.75±4.09 28.19±5.78	29.00±4.34 28.69±6.54	28.85±4.10 18.15±5.28	28.82±3.98 28.40±5.53	29.19±4.43 28.53±5.73	28.93±4.12 28.25±5.33	28.62±3.82 28.17±5.67
p Value		***0.56***			***0.73***			***0.34***		
Waist-hip ratio	M F	0.94±0.07 0.83±0.07	0.94±0.06 0.84±0.07	0.95±0.06 0.82±0.08	0.95±0.06 0.83±0.06	0.94±0.07 0.83±0.07	0.94±0.06 0.83±0.07	0.94±0.06 0.84±0.07	0.94±0.06 0.84±0.07	0.94±0.07 0.84±0.07
p Value		***0.96***			***0.27***			***0.33***		
Cholesterol (mmol/L)	M F	5.24±0.99# 5.40±1.02	5.36±1.07 5.44±1.03	5.21±0.88 5.26±1.03	5.59±0.90 5.45±0.97	5.30±1.04 5.44±1.03	5.27±1.01 5.38±1.08	5.33±0.99 5.41±1.00	5.27±1.02 5.42±1.04	5.30±1.01 5.33±1.10
p Value		***0.06***			***0.10***			***0.90***		
High density lipo-proteins (mmol/L)	M F	1.29±0.30 1.59±0.39	1.31±0.34 1.56±0.358	1.24±0.30 1.55±0.36	1.32±0.34 1.59±0.39	1.30±0.33 1.57±0.36	1.29±0.29 1.58±0.38	1.33±0.33 1.54±0.37	1.29±0.32 1.58±0.39	1.28±0.31 1.58±0.36
p Value		***0.18***			***0.75***			***0.90***		
Low density lipo-proteins (mmol/L)	M F	3.20±0.88 3.20±0.97	3.23±0.92 3.26±0.97	3.15±0.78 3.05±0.89	3.51±0.82 3.24±0.89	3.19±0.89 3.24±0.97	3.22±0.91 3.19±0.96	3.24±0.93 3.23±0.94	3.19±0.89 3.22±0.99	3.23±0.87 3.19±0.94
p Value		***0.13***			***0.14***			***0.87***		
Triglycerides (mmol/L)	M F	1.68±1.08 1.33±0.73	1.86±1.37 1.38±0.84	1.92±1.42 1.39±1.57	1.78±1.34 1.30±0.53	1.81±1.17 1.40±0.94	1.76±1.32 1.34±0.89	1.74±1.14 1.39±0.68	1.76±1.18 1.37±0.87	1.84±1.39 1.33±1.00
p Value		***0.03****			***0.38***			***0.98***		
Glycemia (mmol/L)	M F	5.91±1.59 5.51±1.39	5.76±1.18 5.47±1.17	5.77±1.43 5.41±1.00	5.67±0.97 5.59±1.23	5.88±1.47 5.53±1.56	5.81±1.42 5.46±1.07	5.83±1.48 5.52±1.32	5.85±1.37 5.50±1.19	5.82±1.43 5.47±1.40
p Value		***0.23***			***0.55***			***0.89***		

The same SNP showed a slight trend in influencing the total cholesterol level ($p = 0.06$) in codominant model and $p = 0.057$ in model C *vs.* TT carriers.

DISCUSSION

The hypothesis of variants within the *AHSG*, *NPY2R* and *HCRT* genes being significant determinants of plasma lipids or obesity markers in the Czech population was not confirmed by this large population study. An association of AHSG concentration with triglyceride levels has been described [26], which is consistent with our results. In our large study, we also demonstrated a possible increase of total cholesterol in *AHSG* rs4917 T allele carriers. However, in our study, we did not confirm any other previous observations regarding its influence on low density lipo-proteins (LDL)-cholesterol, central obesity or blood glucose [27]. No significant impact of the rs4917 polymorphism on BMI, waist circumference or plasma levels of glucose was found, which supports the results of a previous study on Scandinavian women. In contrast to the Swedish study, where significant association between the rs4917 polymorphism and plasma cholesterol was found [3], in our study only triglycerides were significantly changed.

No significant relationship between rs4917 *AHSG* variant and anthropometrical parameters or lipids was found in an interventional study of 105 unrelated overweight adult Czech females. However, CC homozygotes showed an increase of lean muscle mass, decrease of total body fat and increased basal metabolic rate per kg of body weight in contrast to T carriers [2].

Although no relationship between the *NPY2R* rs1047214 SNP was found in our study, some studies have described some effects. In Swedish men,

the common T allele was associated with lower BMI [1]. In contrast, another study of selected UK adults connects the T allele with higher BMI. This could be explained by the inclusion of morbidly obese and, consequently, a higher BMI median than in the Swedish study [23]. This study also shows, apart from BMI, higher WHR in T allele carriers. Neither triglyceride nor glucose levels were influenced [1]. In the French study of *NPY2R* rs1047214, the T allele was associated with lower WHR, but not with BMI in boys and not with adults [21].

Minimum studies have focused on rs760282 *HCRT* variant. Similar to others [18], we did not find any association with obesity parameters. In conclusion, we are not able to confirm that polymorphisms within the *AHSG*, *HCRT* and *NPY2R* genes are major genetic determinants of BMI and plasma lipids in the Czech-Slavonic population.

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