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ORIGINAL ARTICLE

Influence of *ghrelin* gene polymorphisms on hypertension and atherosclerotic disease

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Ghrelin is involved in several metabolic and cardiovascular processes. Recent evidence suggests its involvement in blood pressure regulation and hypertension. The aim of the study was to determine associations of single-nucleotide polymorphisms (SNPs) and haplotypes of the ghrelin gene (*GHRL*) with hypertension and atherosclerotic disease. Six *GHRL* SNPs (rs27647, rs26802, rs34911341, rs696217, rs4684677 and a –473G/A (with no assigned rsID)) were investigated in a sample of 1143 hypertensive subjects and 1489 controls of Caucasian origin. Both single-locus and haplotype association analyses were performed. In single-locus analyses, only the non-synonymous rs34911341 was associated with hypertension (odds ratio (OR)=1.95 (95% confidence interval (CI): 1.26–3.02), *P*=0.003). Six common haplotypes with frequency >1% were inferred from the studied *GHRL* SNPs, and their frequency distribution was significantly different between hypertensive subjects and controls (χ^2=12.96 with 5 d.f. (degree of freedom), *P*=0.024). The effect of rs26802 was found to be significantly (*P*=0.017) modulated by other *GHRL* SNPs, as its C allele conferred either an increased risk (OR=1.30 (1.08–1.57), *P*=0.005) or a decreased risk (OR=0.50 (0.23–1.06), *P*=0.07) of hypertension according to the two different haplotypes on which it can be found. No association of *GHRL* SNPs or haplotypes with atherosclerotic disease was observed. In conclusion, we observed statistical evidence for association between *GHRL* SNPs and risk of hypertension.
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Keywords: ghrelin; haplotype analysis; polymorphism

INTRODUCTION

Ghrelin, a 28-amino acid peptide from the stomach, is an endogenous ligand for the growth hormone secretagogue receptor (GHS-R1a) that stimulates growth hormone release.[1] Recent findings have expanded the actions of ghrelin to include metabolic, immunologic, orexigenic, reproductive and behavioral effects, as well as modulation of gastrointestinal motility.[2,3] Moreover, there is increasing evidence for a cardiovascular function of ghrelin. Ghrelin has been postulated to exert a protective effect against atherosclerosis by anti-inflammatory,[4] vasodilatory[5] and antioxidant effects,[4] as well as through improved endothelial function.[6] Furthermore, a radiolabeled ghrelin [^{125}I-His9] has been shown to bind to the heart and peripheral vascular tissues. The signal was augmented in atherosclerotic regions, suggesting that ghrelin receptor expression is upregulated in such areas, thus implicating ghrelin in the development of atherosclerosis.[7] Expression of both ghrelin and its receptor has been identified in the heart and blood vessels, and a decrease in blood pressure was observed after intravenous injection of ghrelin in humans.[1] Moreover, low plasma ghrelin levels have been found to be associated with hypertension in a Finnish cohort of hypertensive subjects.[8] In contrast, high ghrelin concentrations were associated with increased mean intima-media thickness in Finnish males.[9]

Recently, several single-nucleotide polymorphisms (SNPs) of the ghrelin gene (*GHRL*) have been shown to be associated with blood pressure levels.[10] In particular, the combination of the most common genotypes at rs27647, rs26802, rs696217 (Leu72Met) and rs4684677 (Gln90Leu) was associated with the lowest systolic and diastolic blood pressure levels in a sample of Finnish subjects with impaired glucose tolerance.[10] In addition, in another Finnish study, the Gln51 allele of the non-synonymous rs34911341 (Arg51Gln) SNP was associated with an increased risk of hypertension.[11] The proposed underlying mechanism involved its association with either low IGF-I concentrations (as IGF-I has been reported to stimulate endothelial nitric oxide formation) or lower circulating ghrelin levels, as previously reported for this SNP.[11]

However, little is known about the potential association of these *GHRL* SNPs with atherosclerosis and its cardiovascular complications. To our knowledge, only rs696217 has been examined in relation to coronary heart disease (CHD) without positive findings,[12,13] but no data are available about the association of this variant or any other

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GHRL SNPs with overall atherosclerotic disease (including other manifestations beyond CHD).

The aim of this study, then, was to explore the influence of previously studied *GHRL* SNPs on hypertension and atherosclerotic disease in a large cohort of Caucasian subjects by means of single-locus analyses, as done in previous studies, while also using more efficient haplotype analyses.

METHODS

Study population

A total of 2632 Caucasian subjects (54.5% male, age 57 ± 12 years, range: 15–100) were included in the study. Their data were obtained from the Lipid Analytic Cologne (LIANCO), a database described elsewhere.[14,15] In brief, LIANCO was designed to assess the relationships between genetic mutations, serum lipoproteins, other biochemical parameters and clinical data on hypertension, diabetes and atherosclerotic disease. Approval of the study protocol was obtained from the Ethics Committee of the University of Cologne. Between spring 1999 and March 2002, a total of about 5000 patients were recruited in the Cologne (Germany) area by hospitals and office-based physicians.

Subjects with hypertension (N=1143, 52.5% male, age 60 ± 12 years) and normotensive controls (N=1489, 56.0% male, age 55 ± 13 years) participated in this case–control study. Hypertension was operationally defined as having a measured systolic blood pressure of >140 mm Hg and/or diastolic blood pressure of >90 mm Hg; patients taking antihypertensive drugs were also classified as hypertensive, irrespective of their measured blood pressure. Subjects were on different antihypertensive medications, including diuretics (11.1%), angiotensin-converting enzyme inhibitors (17.0%), angiotensin II receptor 1 antagonists (5.8%), calcium channel blockers (7.1%), β-blockers (18.8%), α-blockers (1.3%), antihypertensive agents with central mechanisms of action (0.5%), peripheral vasodilators (0.2%) and combination therapies. Altogether, about 39.6% of the participants in the hypertension group were on antihypertensive drug treatment.

Atherosclerotic disease was defined as the presence and/or history of at least one of the following parameters: CHD, stroke, transient ischemic attack, prolonged reversible ischemic neurological deficit or peripheral arterial vascular disease. CHD was defined as the presence of at least one of the following conditions: angiographic evidence of CHD, myocardial infarction, angina pectoris, coronary bypass surgery or positive stress test. Subjects were defined as diabetic through oral glucose tolerance test (2 h plasma glucose concentration $\geqslant$200 mg dl^{-1} ($\geqslant$11.1 mmol l^{-1})) or if receiving antidiabetic drug treatment. The baseline characteristics of the study population are given in Table 1.

Genotyping

Six *GHRL* SNPs were investigated in this report: rs27647, rs26802, rs696217, rs4684677, rs34911341 and a G/A SNP located at position −473 from the transcription site (referred to as −473G/A, as it has not yet been assigned an rsID). Genomic DNA was prepared from peripheral blood using standard techniques. The six SNPs were detected by PCR followed by restriction fragment length polymorphism as previously described.[10]

The genomic DNA was amplified by PCR followed by digestion with specific restriction enzymes (Fermentas, St Leon-Rot, Germany), as previously described.[10] The PCR products were automatically sequenced (ABI Prism Genetic Analyzer model 310, Applied Biosystems, Foster City, CA, USA). The sequence of both strands was determined.

Quality checks to ensure correctness of the genotypes were carried out by independent rating of the results by two investigators. Discrepancies were resolved by either reaching consensus or re-genotyping. Furthermore, to assess genotyping reproducibility, a random 10% of the samples were re-genotyped, yielding 100% concordance. The percentage of missing genotype data ranged from 0 to 0.7% (mean value 0.3%) for the six SNPs.

Statistical analysis

Descriptive statistics are given, unless otherwise indicated, as proportions (percent or mean values ± s.d.). As a large proportion of the patients were also being treated with lipid-lowering drugs, we corrected the low-density lipoprotein cholesterol levels according to previously described methods[14] to obtain values simulating untreated conditions. Triglyceride concentrations were log-transformed before analysis. Contingency tables and χ^2-tests were used to analyze differences between proportions. Comparison of means was performed by the unpaired Student's t-test or by the Mann–Whitney U-test. Allele frequencies were estimated by gene counting, and SNP case–control comparisons were tested using the Cochran–Armitage trend test.

Statistical analyses were carried out using the Statistical Package for the Social Sciences Version 16.1.2 (SPSS, Munich, Germany), except for linkage

Table 1 Subjects' characteristics

Parameter	*All subjects*	*Subjects with hypertension*	*Control subjects*	P-*value*
Sex	2632	1143 (52.5% male)	1489 (56.0% male)	0.031
Age (years)	57 ± 12	60 ± 12	55 ± 13	<0.0001
Body mass index (kg m^{-2})	27.0 ± 4.4	28.1 ± 4.6	26.2 ± 4.0	<0.0001
Systolic blood pressure (mm Hg)	136 ± 19	146 ± 19	129 ± 15	<0.0001
Diastolic blood pressure (mm Hg)	82 ± 10	86 ± 11	79 ± 8	<0.0001
Type II diabetes	473 (18%)	277 (27%)	196 (12%)	<0.0001
Atherosclerotic disease	593 (23%)	316 (32%)	277 (17%)	<0.0001
Family history of diabetes	763 (29%)	316 (31%)	447 (28%)	0.036
Family history of premature CVD	498 (19%)	198 (20%)	300 (19%)	0.43
Smoking status				0.0068
Never smoker	1855 (71%)	745 (74%)	1110 (68%)	
Ex-smoker[a]	217 (8%)	69 (7%)	148 (9%)	
Current smoker	559 (21%)	193 (19%)	366 (23%)	
Total cholesterol (mg dl^{-1})	256 ± 70	250 ± 63	260 ± 73	0.0007
LDL cholesterol (mg dl^{-1})	162 ± 60	157 ± 48	164 ± 66	0.0046
HDL cholesterol (mg dl^{-1})	57 ± 18	54 ± 17	58 ± 19	<0.0001
Triglycerides (mg dl^{-1})	217 ± 443	221 ± 233	214 ± 535	0.68
Plasma glucose (mg dl^{-1})	96 ± 108	101 ± 50	92 ± 133	0.0868

Abbreviations: CVD, cardiovascular disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
[a]Having stopped smoking for >1 year.

disequilibrium and haplotype analyses, which were carried out using the THESIAS software.[16] All haplotype analyses were adjusted for age, gender, body mass index, diabetes and atherosclerotic disease when appropriate. Because the natural history of hypertension and atherosclerotic disease differs in males and females,[17] analyses were carried out separately in males and females, and the Mantel–Haenszel statistic was used for testing the homogeneity of the results across gender.

To deal with the number of tested SNPs, statistical significance was defined as $P<0.01$ (=0.05/5, 5 being the number of SNPs apart from rs4684677, which was in complete association with rs696217). All tests were two-sided.

RESULTS

For ease of presentation, the results presented are those obtained in the pooled sample of males and females after checking for the homogeneity of the associations across gender.

Association of *GHRL* SNPs with hypertension

Genotype and allele frequencies of the six *GHRL* SNPs are presented in Table 2. In the whole cohort, all observed genotype distributions were compatible with Hardy–Weinberg equilibrium, and only rs26802 showed a slight deviation from Hardy–Weinberg equilibrium in hypertensive patients (P=0.036). Only rs34911341 showed an association with hypertension, in which carrying the Gln51 allele was associated with an increased risk of hypertension (odds ratio (OR)=1.761 (95% confidence interval (CI): 1.151–2.695), P=0.0084). After adjusting for age, gender, smoking, body mass index and diabetes, this OR was still significant (OR 1.949 (1.257–3.021), P=0.003).

As indicated in Table 3, little pair-wise linkage disequilibrium was observed between *GHRL* polymorphisms, except between the rs696217 and rs4684677 variants that were in complete association. As a consequence, results involving rs4684677 are not shown in this report, as they are exactly the same as those observed for rs696217. Six haplotypes with frequencies >1% were inferred from the three common *GHRL* SNPs (rs27647, rs26802 and rs696217) and accounted for more than 99% of the whole chromosome (Table 4). The frequency distribution of these six haplotypes significantly differed between cases and controls (χ^2=12.96 with 5 d.f. (degree of freedom), P=0.024). Although the AALeu haplotype was less frequent in cases than in controls (0.261 *vs.* 0.293), the opposite was observed for the ACLeu haplotype (0.268 *vs.* 0.244). These two haplotypes only differ at the rs26802 position, suggesting that the rs26802 C allele confers an increased risk of hypertension (OR=1.303 (1.083–1.567), P=0.005) when carried on the A-Leu background. Conversely, on the A-Met background, the rs26802 C allele tended to be associated with a decreased risk of hypertension (OR 0.496 (0.231–1.061), P=0.071), as the ACMet haplotype was nearly half as frequent in cases as in controls (0.012 *vs.* 0.021), and the frequency of the AAMet haplotype was quite similar in cases and in controls (0.054 *vs.* 0.050). The test for homogeneity of these two ORs was significant (χ^2=5.66 with 1 d.f.,

Table 3 Pairwise LD between *GHRL* polymorphisms in the pooled sample (*n*=2633)

	rs27647	*rs26802*	*–473G/A*	*rs34911341*	*rs696217*	*rs4684677*
rs27647	—	–0.638	0.185	–0.371	–0.835	–0.835
rs26802	0.124	—	0.048	–0.453	–0.087	–0.087
–473G/A	0.001	0.000	—	–1	–1	1
rs34911341	0.002	0.002	0.000	—	–1	–1
rs696217	0.036	0.000	0.000	0.001	—	1
rs4684677	0.036	0.000	0.000	0.001	1	—

Abbreviation: LD, linkage disequilibrium.
In the upper-right triangle of the table, LD is expressed in terms of D′, which is the ratio of the unstandardized coefficient to its maximal/minimal value, whereas in the lower-left triangle of this matrix, LD is expressed in terms of the r^2 statistic.

Table 2 Genotype and allele frequencies of ghrelin polymorphisms in hypertensive patients and non-hypertensive controls

SNP	*Group*	*Genotype frequencies, n (%)*			*MAF*	*P-value*[a]
rs27647		GG	GA	AA	A	
	Hypertensives	178 (17.2)	482 (46.5)	376 (36.3)	0.596	0.364
	Controls	225 (15.4)	695 (47.5)	542 (37.1)	0.608	
rs26802		AA	AC	CC	C	
	Hypertensives	454 (43.6)	490 (47.0)	98 (9.4)	0.329	0.090
	Controls	696 (47.4)	641 (43.7)	130 (8.9)	0.307	
–473G/A		GG	GA	AA	A	
	Hypertensives	1014 (97.1)	30 (2.87)	0	0.014	0.743
	Controls	1429 (97.3)	39 (2.66)	0	0.013	
rs34911341		Arg51Arg	Arg51Gln	Gln51Gln	Gln51	
	Hypertensives	993 (95.3)	49 (4.70)	0	0.024	0.0084
	Controls	1428 (97.3)	40 (2.73)	0	0.014	
rs696217		Leu72Leu	Leu72Met	Met72Met	Met72	
	Hypertensives	980 (86.2)	154 (13.5)	3 (0.26)	0.070	0.439
	Controls	1265 (85.4)	207 (14.0)	9 (0.61)	0.076	
rs4684677		Gln90Gln	Gln90Leu	Leu90Leu	Leu90	
	Hypertensives	980 (86.2)	154 (13.5)	3 (0.26)	0.070	0.439
	Controls	1265 (85.4)	207 (14.0)	9 (0.61)	0.076	

Abbreviation: MAF, minor allele frequency.
[a]Cochran–Armitage test *P*-value.

Table 4 Haplotype frequencies distribution of *GHRL* polymorphisms in hypertensive and control population

Polymorphisms			*Haplotype frequencies*			
rs27647	*rs26802*	*rs696217*	*Controls* (n=*1489*)	*Hypertensives* (n=*1143*)	*Haplotypic OR (95% CI)*[a]	P-*value*
G	A	Leu	0.348	0.354	Reference	
G	C	Leu	0.039	0.048	1.189 [0.842 to 1.681]	0.325
A	A	Leu	0.293	0.261	0.883 [0.749 to 1.039]	0.135
A	A	Met	0.050	0.054	1.108 [0.825 to 1.489]	0.494
A	C	Leu	0.244	0.268	1.157 [0.987 to 1.356]	0.072
A	C	Met	0.021	0.012	0.530 [0.273 to 1.029]	0.061

Abbreviations: BMI, body mass index; CI, confidence interval; d.f., degree of freedom; OR, odds ratio.
The rs4684677 is not shown in this table as it was in complete association with the rs696217. The –473G/A and rs34911341 polymorphisms are not shown in this table because their rare alleles were inferred to be present on several very rare (<1%) haplotypes.
[a]Haplotypic OR with their 95% CI adjusted for age, sex and BMI. These are haplotypic ORs by comparison with the most frequent GALeu haplotype, under the assumption of haplotypic additive effects. The global test of haplotypic association was significant (χ^2=12.96 with 5 d.f., *P*=0.024).

Table 5 Association of *GHRL* polymorphisms with SBP and DBP

	N[a]	*SBP*		*DBP*	
		Controls	*Hypertensives*	*Controls*	*Hypertensives*
rs27647					
AA	542/376	124.6 (8.38)	148.1 (16.2)	77.6 (5.23)	86.6 (8.97)
AG	696/482	125.0 (7.29)	146.8 (15.3)	78.0 (4.74)	86.7 (8.68)
GG	225/178	124.3 (8.58)	147.2 (15.2)	77.3 (6.11)	86.9 (10.0)
P[a]		*P*=0.439	*P*=0.519	*P*=0.172	*P*=0.935
rs26802					
AA	696/454	124.8 (8.24)	146.8 (15.8)	77.6 (5.28)	86.4 (9.03)
AC	642/490	124.4 (7.80)	147.1 (15.5)	77.8 (5.04)	86.7 (8.98)
CC	130/98	126.0 (6.85)	149.8 (15.4)	78.0 (5.45)	87.5 (9.36)
P[b]		*P*=0.045	*P*=0.267	*P*=0.701	*P*=0.425
–473G/A					
GG	1430/1014	124.7 (7.93)	147.1 (15.6)	77.7 (5.16)	86.6 (9.01)
GA	39/30	124.4 (8.32)	150.4 (17.0)	77.2 (6.17)	88.5 (10.0)
P[c]		*P*=0.755	*P*=0.281	*P*=0.462	*P*=0.344
rs34911341					
ArgArg	1428/993	124.8 (7.92)	147.2 (15.6)	77.7 (5.18)	86.6 (9.07)
ArgGln	40/49	123.9 (8.77)	147.5 (17.1)	78.0 (5.67)	87.4 (8.38)
P[c]		*P*=0.470	*P*=0.783	*P*=0.795	*P*=0.473
rs696217					
LeuLeu	1265/980	124.7 (7.94)	146.9 (15.9)	77.7 (5.27)	86.3 (9.20)
LeuMet	207/154	124.9 (7.58)	146.3 (14.1)	78.2 (4.72)	86.7 (8.39)
MetMet	9/3	126.0 (12.2)	140.0 (17.3)	76.8 (3.98)	83.3 (5.77)
P		*P*=0.957	*P*=0.701	*P*=0.362	*P*=0.911

Abbreviations: DBP, diastolic blood pressure; d.f., degree of freedom; SBP, systolic blood pressure.
Mean (s.d.) are shown. Test were performed on adjusted values for age, gender, body mass index, diabetes and atherosclerotic disease.
[a]Number of controls/number hypertensives according to genotypes.
[b]Genotypic test of association (2 d.f.).
[c]Test of association with 1 d.f., comparing carriers of the rare allele with noncarriers.

P=0.017), suggesting that the effect of the rs26802 polymorphism on the risk of hypertension could be modulated by other *GHRL* polymorphisms. After adjusting for these haplotypic effects, the effect of the rs34911341 polymorphism was still significant (OR=1.934 (1.210–3.092), *P*=0.006).

Association of *GHRL* SNPs with blood pressure, atherosclerosis and cardiovascular risk factors

No single *GHRL* SNPs (Table 5) or haplotypes (data not shown) were associated with systolic or diastolic blood pressure. Similar negative results were observed when patients taking antihypertensive drugs

were excluded from the analysis (data not shown). No association was observed with atherosclerotic disease, body mass index, glucose plasma concentrations or lipoprotein concentrations (Supplementary information).

DISCUSSION

In this report, six *GHRL* SNPs were studied in relation with the risk of hypertension in a sample of Caucasian subjects.

One non-synonymous SNP, rs34911341 (Arg51Gln), was found to be associated with hypertension, as carriers of the Gln51 allele had an approximately twofold greater risk of hypertension than noncarriers. This result is in complete agreement with that observed by Poykko *et al.*[11] in Finnish subjects. This variant has been shown to be associated with decreased IGF-1 concentrations, and IGF-1 has been shown to stimulate endothelial nitric oxide formation.[18] Such observations might explain the association of rs34911341 with hypertension.[11]

In single-locus analysis, no other studied SNP was associated with hypertension or blood pressure. In particular, in agreement with other studies,[12,19–21] we observed no association with the rs696217 (Leu72Met) variant. However, this variant was marginally associated with hypertension in a sample of obese women[22] and has been shown to interact with dietary fat intake to modulate waist circumference and triglyceride concentrations[23] and to modify the effect of physical activity on changes in weight and waist circumference.[24] Although two studies failed to show any effect of this variant on plasma ghrelin concentrations,[25,26] it cannot be ruled out that its effects, if any, can only be observed under specific environmental conditions. This could also explain why Mager *et al.*[10] have observed an association between rs27647, rs26802, rs696217, rs4684677 and blood pressure levels in a population of overweight Finnish subjects with impaired glucose tolerance.

Interestingly, although rs26802 was not associated *per se* with hypertension in our study, we observed through a haplotype analysis that this polymorphism could modulate the risk of hypertension according to the haplotypes by which it is carried. The most likely explanation is that the observed haplotypic association might be due to other *GHRL* SNPs in linkage disequilibrium. Such a phenomenon may be an explanation for the discrepancy between results showing that this polymorphism is not associated with ghrelin levels[25] but is associated with high-density lipoprotein cholesterol concentrations.[24] According to the HapMap database, the studied SNPs cover about 75% of the haplotypic variability of the coding regions and proximal promoter of the *GHRL* gene. Further investigations and further genotyping would be required to completely characterize the genetic variability of the promoter region. Another limitation of our study is related to the blood pressure measurement methodology. Office-based one-point measurements may limit the accuracy of the blood pressure phenotype and therefore the selection of hypertensives *vs.* normotensives.

Conversely, no association was observed between any of the studied SNPs (or haplotypes) and atherosclerotic disease. These results are in agreement with studies that have reported a similar lack of association with cardiovascular disease in patients with type II diabetes mellitus[12] or with CHD.[13,27]

In conclusion, this study provides statistical support for association of the *GHRL* gene with hypertension. Once clearly identified, the hypertension-associated *GHRL* SNPs might become useful markers in assessing the genetic risk of hypertension.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Hypertension Research website (http://www.nature.com/hr)