Association study of *ghrelin receptor* gene polymorphisms in rheumatoid arthritis

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Abstract

Objective

Ghrelin is a newly characterised growth hormone (GH) releasing peptide widely distributed that may play an important role in the regulation of metabolic balance in inflammatory diseases such as rheumatoid arthritis (RA) by decreasing the pro-inflammatory Th1 responses. In this study we investigated the possible contribution of several polymorphisms in the functional Ghrelin receptor to RA susceptibility.

Methods

A screening of 3 single nucleotide polymorphisms (SNPs) was performed in a total of 950 RA patients and 990 healthy controls of Spanish Caucasian origin. Genotyping of all 3 SNPs was performed by real-time polymerase chain reaction technology, using the TaqMan 5´-allele discrimination assay.

Results

We observed no statistically significant deviation between RA patients and controls for the GHSR *SNPs analysed. In addition, we performed a haplotype analysis that did not reveal an association with RA susceptibility. The stratification analysis for the presence of shared epitope (SE), rheumatoid factor (RF) or antibodies anti cyclic citrullinated peptide (anti-CCP) did not detect significant association of the* GHSR *polymorphisms with RA.*

Conclusion

These findings suggest that the GHSR *gene polymorphisms do not appear to play a major role in RA genetic predisposition in our population.*

Key words

Rheumatoid arthritis, ghrelin, ghrelin receptor (*GHSR*) gene, single nucleotide polymorphism.

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Introduction

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease, characterised by inflammation of synovial joints (1), affecting approximately 1% of the population worldwide. Both genetic and environmental factors seem to be involved in its complex etiology, although a strong genetic component has been previously described (2). HLA genes, mainly *HLA-DRB1* alleles play an important role in the genetic association to this disorder (3, 4). The contribution of other genes outside the HLA region to RA susceptibility has been aim of study in the last years. (5-7).

Ghrelin is a newly characterised 28-amino-acid peptide, isolated from the stomach (8). This hormone has been studied primarily in relation to the control of appetite and fat metabolism but to date, other ghrelin functions have been reported: release of various hormones like GH, ACTH, cortisol, and prolactin (9); modulation of cell proliferation and survival; glucose metabolism, pancreatic function, and gastric acid secretion (10, 11), and immune modulation increasing anti-inflammatory cytokine and chemokine levels in experimental arthritis (12); Ghrelin and *GHSR* have been detected on the surface of immune cells: T cells, B cells, neutrophils, and monocytes (13, 14). This may explain some of the anti-inflammatory effects of ghrelin. *GHSR* has been found in blood vessels and endothelial cells, suggesting that ghrelin modulates cardiovascular function (15).

In addition, Cortistatin, a recently discovered cyclic-neuropeptide related to somatostatin, emerged as a potential endogenous anti-inflammatory factor based on its production by and binding to immune cells that exerts its effects on synovial cells through both somatostatin and ghrelin receptors (12). Ghrelin is located in the chromosome 3q26-29 region that has been identified as associated to RA (16). This locus is also associated with specific traits of metabolic syndrome and body mass index (BMI). Chronic inflammation is associated with a significant loss of body mass.

Ghrelin exerts most of its known effects on the body through the growth hormone secretagogue receptor (*GHSR*) type 1a (17), a highly functional conserved G-protein-coupled receptor, which was described earlier than ghrelin (18). Association studies for *GHSR* were previously carried in human obesity (19) and in cardiovascular diseases such as myocardial infarction, coronary artery disease (20) and left ventricular hypertrophy (21). We investigated the possible association of *GHSR* polymorphism with RA susceptibility since ghrelin and its receptor may play an important role in inflammatory diseases.

Material and methods

Patients

The study population consisted of 950 patients independently recruited from Hospital Virgen de Las Nieves and Hospital Clínico San Cecilio (Granada), Hospital Xeral-Calde (Lugo), and Hospital Clínico San Carlos and Hospital La Paz (Madrid) and 990 blood bank donors from the corresponding hospitals, who were included as healthy controls. All the patients met the American College of Rheumatology revised criteria for RA (22). Rheumatoid factor (RF) was measured by nephelometry (Behring, Nephelometer Analyzer II) with a detection limit of 15 u/ml and anti-citrullinated protein/peptide antibodies (ACPAs) were determined by a second generation anti-CCP-2 antibody ELISA (Immunoscan RA Mark 2; Eurodiagnostica, Arhem, The Netherlands) with a cut-off level of 25 arbitrary units/ml, according to manufacturer's instructions. The range of measurement was 0-1600 U/ml and all values higher than this upper limit were truncated and considered as 1600 U/ml for the analysis. HLA class II alleles were determined using a reverse dot-blot kit with sequence-specific oligonucleotide (SSO) probes (Dynal RELI™ SSO *HLA-DRB1* typing kit, Dynal Biotech, Bromborough, UK). When necessary, high resolution typing of *HLA-DRB1*03* samples was performed using Dynal AllSet™ SSP *DRB1*03*. The following alleles were considered as SE positive: *DRB1*0101, *0102, *0401, *0404, *0405, *0408, *0410, *1001, *1402*. Of the characterized patients, 68% were positive for rheumatoid factor, 62% were seroposi-

tive for cyclic citrullinated peptide and 66% encoded the shared epitope (SE), All study subjects were of Spanish Caucasian origin and were included in the study after giving their written informed consent. We obtained approval for the study from all the local ethics committees.

GHSR genotyping

We selected the rs509035, rs512692 and s2922126 *GHSR* SNPs as genetic markers for screening and association studies (dbSNB,http://www.ncbi.mlh.nih.gov/SNP/). The two first are tag SNPs of a 5 SNPs haplotype block spanning a 11.63-kb region and encompassing most of the intron, exon 1, and 5´UTR region of the *GHSR* gene that has been associated with susceptibility human obesity (19). In addition, we analysed a third SNPs (rs2922126) located in a putative binding site for the transcriptional factor PEA3-C/EBP involved in transcription of matrix metalloproteinase's. DNA from patients and controls was obtained from peripheral blood, using standard methods. The minor allele frequencies were >5%. Samples were genotyped for these variants using a TaqMan 5' allelic discrimination Assay-By-Design method (Applied Biosystems, Foster City, CA, USA). All samples were genotyped for the three *GHSR* SNPs in the same centre to avoid genotyping inconsistencies. The genotype call rate was up to 93% for all the tested *GHSR* genetic variants. The probes were labelled with the fluorescent dyes VIC and FAM and a PCR reaction was carried. Post-PCR, the genotype of each sample was attributed automatically by measuring the allelic specific fluorescence on the ABI PRISM 7500 Sequence Detection Systems using the SDS 1.3.1 software for allelic discrimination (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Statistical analysis to compare allelic and genotypic distributions was performed using the Unphased and Statcalc software packages (Epi Info 2002; Centers for Disease Control and Prevention, Atlanta, GA). In the case of no departure from Hardy-Weinberg equilibrium, significance was calculated with 2X2 contingency tables and Fisher's exact test; *p*-values, odds ratios (ORs), and 95% confidence intervals (95% CIs) were calculated.

The power of the study to detect the effect of these polymorphisms in disease susceptibility was estimated using Quanto version 0.5 software (Department of Preventive Medicine, University of Southern California, Los Angeles).

Results

Association analysis of GHSR gene polymorphisms in RA Spanish patients

We performed single point association analysis of three SNPs along the gene region of *GHSR*. Two variants (rs509035 G/A and rs512692 A/T) had been previously reported to be associated in different human diseases (19-21). The third one (rs2922126 T/A) was localised in the promoter region of the gene and was selected because of its possible functional importance. After applying the statistical analysis no signs of statistically significant association were observed in any of the three RA genotypes separately, between controls and patients in 2x2 or 2x3 contingency tables, considering either allelic or genotypic frequencies (Table I).

Haplotype analysis of GHSR gene polymorphisms

Afterwards we performed a linkage disequilibrium analysis and we could observe than the pairwise maximum-likelihood analysis showed a high degree of linkage disequilibrium between rs509035 and rs512692 (r^2=0.66). However, the rs2922126 genetic variant showed a lower level of LD with the two SNPs defining the GSHR haplotype block previously associated with obesity (r2=0.13 and 0.11 respectively). We carried a combined analysis of the rs509035, rs512692 and rs2922126 SNPs and observed three major combinations in both RA patients and controls. However, no statistical significance was observed in the major GHSR haplotypes (GAT, GAA and ATT) distribution between RA patients and controls (Table II).

Stratification analysis by the presence of shared epitope, RF and anti-CCP antibodies

We performed an analysis after stratification for the presence of SE-alleles,

Table I. Distribution of *GHSR* SNPs in RA patients and healthy controls.

Genotype	Cases (%)	Controls (%)	*p*	OR (95%CI)	Allele	Cases	Controls	*p*	OR (95%CI)
rs509035	n=945	n=979							
GG	562 (59)	575 (59)	0.74	1 (0.9-1.2)	G	1423 (75)	1482 (76)	0.78	1.0 (0.8-1.1)
AG	299 (32)	332 (34)	0.29	0.9 (0.7-1.1)	A	467 (25)	476 (24)		
AA	84 (9)	72 (7)	0.22	1.1 (0.9-1.3)					
rs512692	n=922	n=929							
AA	580 (63)	590 (63)	0.79	1 (0.8-1.2)	A	1450 (79)	1474 (79)	0.6	1.0 (0.9-1.2)
AT	290 (31)	294 (32)	0.93	1 (0.8-1.2)	T	394 (21)	384 (21)		
TT	52 (6)	45 (5)	0.44	1.2 (0.8-1.8)					
rs2922126	n=894	n=912							
TT	417 (47)	405 (44)	0.34	1.1 (0.9-1.3)	T	1204 (67)	1202 (66)	0.36	1.1 (0.9-1.2)
AT	370 (41)	392 (43)	0.49	0.9 (0.8-1.1)	A	584 (33)	622 (34)		
AA	107 (12)	115 (13)	0.68	0.9 (0.7-1.3)					

RA: rheumatoid arthritis; OR: odds ratio; 95% CI: 95% confidence interval.

Table II. Distribution of *GHSR* haplotypes in RA patients and healthy controls.

Genotype	Cases (%)	Controls (%)	*P*	OR (95%CI)
Haplotype (rs509035/ rs512692/rs2922126)	2n=1223	2n=1577		
GAT	506 (41)	634 (41)	0.5	1 (0.9-1.2)
GAA	384 (32)	520 (33)	0.4	0.9 (0.8-1.1)
ATT	245 (19)	314 (19)	0.9	1 (0.8-1.2)
Other	88 (8)	109 (7)	0.8	1 (0.8-1.4)

RF and anti-CCP, and tested a possible susceptibility for rheumatoid arthritis (RA) combining *GHSR* polymorphisms and the presence of these possible disease markers (Table III).

As expected, the presence of shared epitope (SE) alleles was strongly associated with anti-CCP-positive RA (p=7.05 x 10^{-10}, odds ratio (OR) 4.57, 95% confidence interval (CI) 2.76-7.57) and RF-positive RA (p=1.68 x 10^{-6} , OR 2.99, 95% CI 1.89- 4.74). No association was detected for any SNP with RA when stratification by shared epitope was undertaken in SE-positive RA patients comparing with the controls (p=0.98, p=0.80 and p=0.23 for the allelic frequency respectively). We found an association for the *GHSR* rs509035 GG genotype in SE-negative individuals (p=0.0052; OR 1.58, 95% CI 1.13-2.22). However, we could not observe this association for the rs512692 AA variant (p=0.15, OR 1.27, 95% CI 0.90-1.78) or rs2922126 TT variant (p=0.11, OR 1.34, 95% CI 0.92-1.95). Similarly, no association was detected with RA after stratification of the RA group into RF-positive and RF-negative patients or anti-CCP-positive and anti-CCP-negative patients in genotype or allele frequency. We did not observe a major association of the *GHSR* polymorphisms with RA in combination with the presence of SE, RF or anti-CCP.

Discussion

Rheumatoid arthritis is a complex rheumatic disease characterised by inflammation, abnormal immune responses, and synovial hyperplasia. There is strong evidence that genes contribute to the risk of developing many common diseases. However, the genetic background of patients with RA is still mostly unknown. The presence of a polygenic trait becomes difficult genetic studies of multifactorial diseases.

Recent studies have described new functions for Ghrelin, an orexigenic 28-amino-acid peptide and its receptor GHSR related to immune modulation and pathologic inflammatory states. Ghrelin may inhibit pro-inflammatory cytokines release (IL-1beta, IL-6, and TNF-alpha) by monocytes, T cells and endothelial cells (13) and augment the anti-inflammatory cytokine production and chemokine levels in experimental arthritis (12). Moreover, it has been described that TNF-α increases ghrelin concentrations in patients with severe RA. This may indicate that ghrelin is related to cardiovascular events described in RA (23).

To our knowledge, this is the first study to elucidate the relationship between sequence variants in the *GHSR* gene and RA in humans. We initially were interested in the association between RA and three SNPs along the gene, corresponding to variants in the 5'UTR region (rs512692), intron (rs509035) and in the promoter region (rs2922126). Genetic variants localised within putative regulatory regions might have functional relevance by altering promoter activity or RNA splicing and processing. Although little is known about the functional

Table III. Distribution of the *GHSR* stratified by the presence of shared epitope, RF and anti-CCP antibodies.

	RA Patients				Controls			
Genotype	SE + (%)	SE- (%)	RF + (%)	RF- (%)	AntiCCP + (%)	AntiCCP - (%)	SE + (%)	SE- (%)
rs509035	n=534	n=277	n=485	n=223	n=358	n=223	n=206	n=361
GG	304 (57)	183 (66)*	282 (58)	126 (57)	216 (61)	142 (64)	116 (57)	199 (55)
AG	182 (34)	71 (26)	162 (33)	70 (31)	105 (29)	64 (29)	73 (35)	142 (39)
AA	48 (9)	23 (8)	41 (9)	27 (12)	37 (10)	17 (7)	17 (8)	20 (6)
G	790 (74)	437 (79)	726 (75)	322 (72)	537 (75)	348 (78)	305 (74)	540 (75)
A	278 (26)	117 (21)	244 (25)	124 (28)	179 (25)	98 (22)	107 (26)	182 (25)
rs512692	n=451	n=238	n=404	n=173	n=310	n=172	n=204	n=351
AA	282 (63)	158 (66)	252 (62)	102 (59)	208 (67)	110 (64)	132 (65)	215 (61)
AT	144 (32)	67 (28)	132 (33)	58 (33)	86 (28)	55 (32)	65 (32)	122 (35)
TT	25 (5)	13 (6)	20 (5)	13 (8)	16 (5)	7 (4)	7 (3)	14 (4)
A	708 (78)	383 (80)	636 (79)	262 (76)	502 (81)	275 (80)	299 (79)	552 (79)
T	194 (22)	93 (20)	172 (21)	84 (24)	118 (19)	69 (20)	79 (21)	150 (21)
rs2922126	n=447	n=248	n=459	n=205	n=296	n=207	n=184	n=323
TT	207 (46)	105 (42)	202 (44)	95 (46)	132 (45)	96 (46)	74 (40)	135 (42)
AT	188 (42)	108 (44)	207 (45)	78 (38)	127 (43)	79 (38)	87 (47)	145 (45)
AA	52 (12)	35 (14)	50 (11)	32 (16)	37 (12)	32 (16)	23 (13)	43 (13)
T	602 (67)	318 (64)	611 (67)	268 (65)	381 (65)	271 (65)	235 (64)	415 (64)
A	292 (33)	178 (36)	307 (33)	142 (35)	201 (35)	143 (35)	133 (36)	231 (36)

SE: Shared epitope; RF: Rheumatoid factor; Anti CCP: Anti cyclic citrullinated peptide antibodies.
*p=0.0052; OR 1.58, 95% CI 1.13-2.22 GG *vs*. AG+AA in SE- individual

relevance of rs509035 and rs512692 SNPs they are localised within two important *GSHR* regulatory regions. The rs509035 is located in *GSHR* intron 2, near to a splice site that leads to the two possible near to the intron 2 splice site that leads to the two different receptor variants, type 1a and 1b (24). Similarly, the rs512692 genetic variant is located within GSHR promoter region. In addition, rs509035 and rs512692 were previously analysed in different association studies related to diseases such as human obesity, myocardial infarction and coronary artery disease or breast cancer (19, 20, 25). Similarly, the rs2922126 SNP is localised in the promoter region in a putative binding site for the transcriptiona factor PEA3-C/EBP. PEA3 is of importance for transcription of matrix metalloproteinases, which were reported to be increased in early rheumatoid arthritis (26). On the other hand, C/EBP is a nuclear transcription factor detected in the synovial tissue of patients with rheumatoid arthritis and plays a potential role in chronic inflammation (27). Thus, it could be hypothesised that the presence of this polymorphism might alter the regulation and expression of the *GHSR* gene.

Nevertheless, no significantly association with RA was found for these three SNPs. We estimated a statistical power of 99%, considering a minor allele frequency of 0.28 (corresponding to a median value of the majority of markers considered) and an OR of 1.5 at the 5% significance level assuming a dominant inheritance model.

We found no evidence of association in the variants of this gene with RA in our cohort although it does not formally exclude *GHSR* as a candidate gene for RA. This could suggest that the true etiological variant of *GHSR* in RA pathogenesis is still unidentified. We could consider the possibility that other polymorphisms within the *GHSR* gene may be involved in the susceptibility to RA.Thus further investigation is needed in this regard.

In conclusion, we analysed for the first time the influence of *GHSR* gene polymorphisms in RA and our findings did not get conclusive evidence for the involvement of *GHSR* in the development of this disease.

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