

SHORT REPORT

Functional dimorphism of two hAgRP promoter SNPs in linkage disequilibrium

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The agouti related protein (AgRP) exerts its anabolic effects on food intake by antagonising the alpha-melanocyte stimulating hormone (α -MSH) at its receptors, melanocortin receptors 3 and 4 (MC3R and MC4R). A single nucleotide polymorphism (SNP) in the promoter of the human AgRP (hAgRP), $-38C>T$, was associated with low body fatness. The $-38T$ allele that was associated with low body fatness also resulted in lower promoter activity. Here we report a novel SNP, $-3019G>A$, again in the promoter of hAgRP, which is in complete linkage disequilibrium (LD) with the $-38C>T$ SNP (linked alleles: $-3019A/-38T$ and $-3019G/-38C$). Functional analyses in a human adrenal and two mouse hypothalamus cell lines showed that the $-3019A$ allele had significantly higher promoter activity. Hence, the two linked alleles ($-3019A$ and $-38T$) had opposite effects on promoter function and yet they were both associated with low body fatness. The region encompassing the $-38C>T$ SNP had approximately 1000-fold higher activity than the region encompassing the $-3019G>A$ SNP, potentially determining the net functional effect between these two SNPs.

The agouti related protein (AgRP) is expressed in the arcuate nucleus of the hypothalamus, the testes, the lung, and the adrenal gland, and is upregulated in obese and diabetic mice.^{1–3} AgRP exerts its anabolic effects on food intake by antagonising the alpha-melanocyte stimulating hormone (α -MSH) at its receptors, melanocortin receptors 3 and 4 (MC3R and MC4R).^{4,5} When administered intracerebroventricularly (i.c.v.),⁶ or when overexpressed in transgenic mice,⁷ both the murine and human AgRP orthologs stimulate hyperphagia. Streptozotocin-induced diabetes resulted in upregulation of AgRP,⁸ while chronic i.c.v. administration of AgRP resulted in a decrease in expression of the uncoupling protein 1 (UCP1) in the rat,⁹ suggesting a role for AgRP in energy expenditure. AgRP also has an inhibitory paracrine role in the rat adrenal gland by blocking α -MSH-induced corticosterone secretion.¹⁰ Leptin downregulates AgRP expression,¹¹ while human AgRP (hAgRP) can itself be a negative regulator of leptin action.¹² AgRP, therefore, plays a significant role in the regulation of food intake.

Elevated plasma levels of hAgRP have been reported in obese men,¹³ suggesting its involvement in the development of human obesity. Moreover, a 2-h fast resulted in a 73% increase of plasma hAgRP concentration, which is consistent with studies reported in animals.¹⁴ An SNP in the coding region of the gene (Ala67Thr) was associated with anorexia nervosa,¹⁵ implicating hAgRP in the development of eating disorders, while the same SNP was associated with the prevention of late-onset obesity in white subjects.¹⁶ An SNP in the promoter of AgRP, $-38C>T$, was significantly

associated with low BMI in Sierra Leoneans¹⁷ and had a significant protective role against body fatness in blacks of the diaspora (African Americans and African Canadians).¹⁸ The $-38C>T$ SNP is positioned in the minimal promoter region of AgRP that has been shown to have high promoter activity in hypothalamus—and periphery—derived cell lines.^{17,19}

In the present study we report a novel SNP, $-3019G>A$, identified in the distal promoter of AgRP and in a region that has significantly lower activity than the proximal promoter region.²⁰ The $-3019G>A$ SNP was in complete linkage disequilibrium (LD) with the $-38C>T$ SNP, but the linked genotypes of the two SNPs exerted opposite effects on promoter activity (as assessed by *in vitro* cell culture models).

METHODS

Subjects

The HERITAGE Family Study cohort included 259 black subjects (88 men and 171 women) from 114 family units. The study design and inclusion criteria have been previously described.²¹ Although the HERITAGE cohort includes both Caucasian and black subjects, the $-38C>T$ SNP was found in the blacks subjects only. Therefore no Caucasian data were used in the statistical analyses. The study protocols were approved by each of the Institutional Review Boards of the HERITAGE Family Study research consortium. Written informed consent was obtained from each participant.

Genotyping

Initial screening for polymorphisms in the promoter region of AgRP was carried out using a representative cohort of 40 individuals consisting of diabetic, obese, and lean white and black subjects. Several combinations of primers were used for amplification of genomic DNA by polymerase chain reaction (PCR) and bidirectional sequencing of the amplicons on an ABI 3700 DNA Analyzer (Applied Biosystems, Foster City, CA). Once the $-3019G>A$ SNP was identified, genotyping was performed by amplification of genomic DNA and direct sequencing using a probe on the LI-COR DNA Analyzer 4200 (Lincoln, NE). The following primers were used: forward primer: 5'-ATT TAA CGC GTC CAT CTT CTC CTC TCC CTG-3'; reverse primer: 5'-CTT GGG CTA GTC AGC TGA GAT CGT GCC ACT G-3'; LI-COR probe: 5'-CCT GGA CAA GGT GGG AAC AG-3'. PCR was performed as previously described.¹⁷ Since its identification in our laboratory, SNP $-3019G>A$ has appeared in dbSNP with the rs#: 8047574. The nomenclature adopted for referencing gene names, symbols, and polymorphism descriptions was according to den Dunnen and Antonarakis.²²

Abbreviations: AgRP, agouti related protein; α -MSH, alpha-melanocyte stimulating hormone; LD, linkage disequilibrium; MC3R and MC4R, melanocortin receptors 3 and 4; SNP, single nucleotide polymorphism; UCP1, uncoupling protein 1

Transfection constructs and cell culture

Constructs to examine the impact of the $-3019G>A$ SNP on promoter activity were made by amplification of genomic DNA from a heterozygous individual and directional cloning of the PCR products into the pGL3-basic vector (Promega, Madison, WI). The amplified region containing the SNP was 203 nucleotides (nt) long ($-3165/-2962$). The forward and reverse primers contained recognition sites for the *Mlu*I and *Nhe*I restriction endonucleases for digestion of the PCR products to facilitate directional cloning into the vector. The sequence of the primers (inclusive of the restriction sites for the enzymes) was as follows: forward primer: 5'-TTT GAA CGC GTC ACA GGA AAC ACA TGG CTGT-3'; reverse primer: 5'-CTT GGG CTA GCG CTC AAA CCC TCT CCC TTCT-3'. Constructs to examine promoter activity for the $-38C>T$ SNP were prepared as previously described.¹⁷ Cell culture was carried out under standard conditions in a humidified incubator at 37°C and 5% CO₂. The GT1-7 cells were the kind gift of Dr Mellon²³ and were grown in DMEM medium (Cellgro, cat. no 10-017-cv), 10% FBS, penicillin-streptomycin (Gibco, cat. no 15140-122) to 1%. The human adrenal NCI-h295R cells were purchased from the American Type

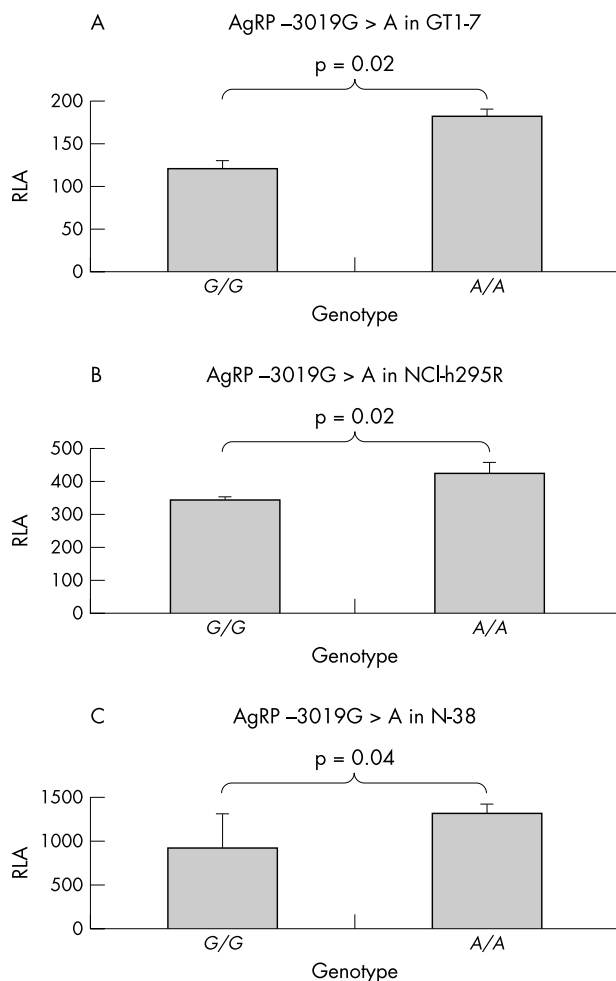


Figure 1 Mean relative luciferase activities for the two genotypes of the $-3019G>A$ SNP, in three cell lines: GT1-7 (A), NCI-h295R (B), and N-38 (C). Luciferase activities were normalised by β -galactosidase measurements for transfection efficiencies and the values shown are the means of a minimum of nine independent experiments for each genotype in each cell line. The standard deviations are represented by the vertical bars on the columns. Comparisons between genotypes in each cell line were performed by Student's *t*-test and the *p*-values are shown above the bars.

Table 1 Genotype frequencies for the $-3019G>A$ AgRP SNP, allele frequencies for SNPs $-3019G>A$ and $-38C>T$, and linkage disequilibrium test for the two SNPs

Genotype frequencies	AA	GA	GG
($-3019G>A$)	16 (6.6%)	106 (43.4%)	122 (50%)
Locus/allele	1	2	
$-3019G>A$	0.2828 (A)	0.7172 (G)	
$-38C>T$	0.7172 (C)	0.2828 (T)	

Linkage disequilibrium test: $\chi^2 = 434.33$, $p < 0.0000001$, $D' = 1.0$.

Tissue Collection (Manassas, VA) and were grown in the following media: DMEM/F12 50/50 MIX medium 500 ml (Cellgro, cat. no 15-090-cv) by adding BD ITS+culture supplement 5 ml (BD Biosciences, cat. no 354352) and Nuserum I culture supplement 13 ml (BD Biosciences, cat. no 35-5100) and 0.75 M HEPES 10.5 ml penicillin-streptomycin (GIBCO, cat. no 15140-122) to 1%. The N-38 is a newly developed cell line that was generated through immortalisation of mouse primary hypothalamic cells at embryonic day 17 with SV40 T-antigen introduced through retroviral infection. The N-38 cell line, a clonal population of hypothalamic neurons, has been propagated to P-44 and maintains SV40 T-antigen expression after 2 years of continuous growth.²⁴ Cell culture conditions for the N-38 cells were identical to those for the GT1-7 cells given above.

All cells were serum starved for 24 h prior to transfection. Transient cotransfections with the AgRP promoter constructs and β -gal plasmids were carried out for 24 h in the absence of serum using the Geneporter2 transfection reagent as prescribed by the manufacturer (Gene Therapy Systems, San Diego, CA). Subsequently, the media were supplemented with 20% FCS for 24 h. Cells were harvested using 1 \times Geneporter2 lysis buffer and the lysates were assayed for luciferase and β -galactosidase activities, as prescribed by the assay manufacturer (Promega, Madison, WI) in a luminometer (ZyLux, Pforzheim, Germany). All luciferase activity measurements were normalised to β -galactosidase values. Measurements were averaged from a series of a minimum of nine independent experiments per cell line.

Statistical analyses

AgRP $-38C>T$ and $-3019G>A$ haplotype frequencies were assessed using the EH program.²⁵ Linkage disequilibrium (LD) was calculated as $D' = x_{ij} - p_i p_j$, where x_{ij} is the $A_1 B_1$ haplotype frequency, and p_1 and p_2 are the frequencies of alleles A_1 and B_1 at loci A and B, respectively. A standardised LD coefficient D' was defined as $D' = D/D_{max}$, where $D_{max} = \min(p_1 p_2, q_1 q_2)$, when $D < 0$, or $D_{max} = \min(q_1 p_2, p_1 q_2)$, when $D > 0$, and q_1 and q_2 are the frequencies of alleles A_2 and B_2 at loci A and B, respectively.²⁶

RESULTS AND DISCUSSION

An SNP was identified at position -3019 upstream of the putative transcription start site of the promoter of hAgRP ($-3019G>A$). The functional impact of the SNP was assessed by examining the promoter activity of the region ($-3165/-2962$) containing the SNP. Two constructs were made in the pGL3-basic luciferase reporter vector representing the two genotypes. The A/A construct had significantly higher promoter activity than the G/G construct in the mouse hypothalamus GT1-7 and the human adrenal NCI-h295R cell lines (fig 1A,B). This result was repeated in another mouse hypothalamus neuronal cell line, N-38 (fig 1C). The three cell lines endogenously express AgRP as assessed by real-time quantitative RT-PCR (data not shown).

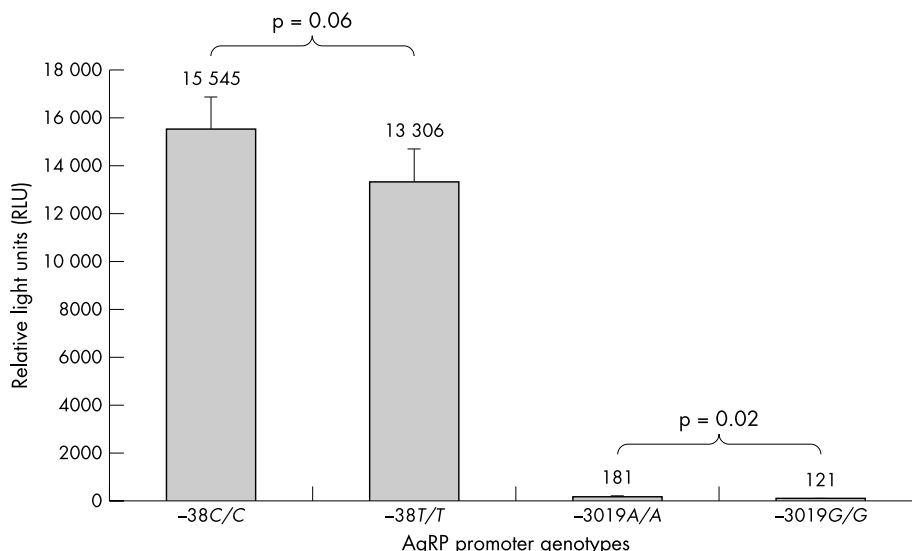


Figure 2 Mean luciferase activities for the genotypes representing the two SNPs $-38C>T$ and $-3019G>A$. The values shown are the actual relative light units (above bars) that were measured by a luminometer. A minimum of eight independent experiments per genotype were performed. The standard deviations are represented by the vertical bars on the columns. Luciferase activities were normalised by β -galactosidase measurements. Comparisons between genotypes were performed by Student's *t*-test.

The promoter activities of the two $-3019G>A$ constructs were considerably lower than the promoter activities we have measured for other regions of the hAgRP promoter.¹⁹ Moreover, studies in our laboratory have shown that SNP $-3019G>A$ lies in a region that might have suppressor effects on gene expression.²⁰

We subsequently genotyped the HERITAGE Family study population to evaluate the possible association of the $-3019G>A$ SNP with obesity-related phenotypes. The SNP was found in black subjects only which was also the case for the $-38C>T$ SNP. Subsequently we compared the allele frequencies of the two SNPs and found them to be identical between the "A-T" and the "G-C" pairs (table 1). This pattern indicates strong linkage disequilibrium (LD), which was confirmed by a formal LD test (table 1).

Since the $-3019G>A$ and $-38C>T$ SNPs are in complete LD and since the *TT* genotype of the $-38C>T$ SNP was associated with low body fatness,¹⁸ then the *AA* genotype of the $-3019G>A$ SNP, by definition, should be associated with low body fatness. However, the functional data (fig 2) suggest the opposite. The results are therefore in disagreement in terms of LD and the functional effects of the two SNPs. However, the region that contains the $-38C>T$ SNP had considerably (~1000-fold) higher promoter activity than the region containing the $-3019G>A$ SNP (fig 2). Therefore, the net effect of the $-3019A/-38T$ haplotype could be determined mostly by the $-38T$ allele at basal conditions. One way to test this hypothesis would be by using longer promoter constructs that contain both SNPs, in all possible haplogroup combinations, and evaluating the impact of each haplogroup on promoter activity. Such studies, however, could be hampered by the presence of additional SNPs in the vicinity of the -3019 or -38 SNPs.

The discovery of disease susceptibility genes is increasingly based on LD mapping with SNPs.²⁷ Past experience with association studies shows that there may be spurious associations with functional SNPs or SNPs in LD with neighbouring loci. Here we present a case of two SNPs that are in complete LD and yet the linked genotypes have opposite functional effects. This puts emphasis on the need to functionally characterise all SNPs in LD in the promoter, as

LD may not necessarily predict the functional properties of the linked SNPs.

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Conflicts of interest: none declared.

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