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A Polymorphism in the Human Agouti-Related Protein Is Associated with Late-Onset Obesity

GEORGE ARGYROPOULOS, TUOMO RANKINEN, DONI R. NEUFELD, TREVA RICE, MICHAEL A. PROVINCE, ARTHUR S. LEON, JAMES S. SKINNER, JACK H. WILMORE, D. C. RAO, AND CLAUDE BOUCHARD

Pennington Biomedical Research Center, Louisiana State University (G.A., T.Ra., D.R.N., C.B.), Baton Rouge, Louisiana 70808; Division of Biostatistics and Departments of Genetics and Psychiatry, Washington University Medical School (T.Ri., M.A.P., D.C.R.), St. Louis, Missouri 63110; School of Kinesiology and Leisure Studies, University of Minnesota (A.S.L.), Minneapolis, Minnesota 55455; Department of Kinesiology, Indiana University (J.S.S.), Bloomington, Indiana 47405; and Department of Health and Kinesiology, Texas A&M University (J.H.W.), College Station, Texas 77843-4243

The mouse agouti-related protein (AGRP) is a powerful appetite effector that results in hyperphagia and the development of obesity when administered intracerebroventricularly or when overexpressed in transgenic mice. Animal studies have also shown that exogenous administration of AGRP predisposes toward hedonic intake of high fat and high sucrose diets. The human ortholog (hAGRP) maps on chromosome 16q22 and has similar physiological properties, as tested in animal models. A polymorphism was identified in the third exon of hAGRP, c.199G→A, that resulted in a nonconservative amino acid substitution, Ala^{67}Thr. Computational analysis of the protein showed significant differences in the coils of the two polymorphic isoforms of the protein. Human studies showed no genotype effects in individuals with a mean age of 25 yr. However, the *G/G* genotype was significantly associated with fatness and abdominal adiposity in the parental population with a mean age of 53 yr. The c.199G→A polymorphism in hAGRP could, therefore, play a role in the development of human obesity in an age-dependent fashion. (*J Clin Endocrinol Metab* 87: 4198–4202, 2002)

THE HYPOTHALAMUS PLAYS an important role in the regulation of energy homeostasis (1, 2). Human agouti-related protein (hAGRP) is expressed in the arcuate nucleus of the hypothalamus, testes, and adrenal gland and is up-regulated in obese and diabetic mice (3, 4). AGRP has been characterized as a potent anabolic effector of food intake (5). The murine and human orthologs stimulate hyperphagia when administered intracerebroventricularly (6, 7) or when overexpressed in transgenic mice (8). Streptozotocin-induced diabetes resulted in up-regulation of AGRP (9), whereas chronic intracerebroventricular administration of AGRP resulted in a decrease in the expression of uncoupling protein 1 in the rat (10), suggesting a role for AGRP in energy expenditure. Leptin down-regulates AGRP expression (11–13), whereas hAGRP can itself be a negative regulator of leptin action (14). The carboxyl-terminus region has been shown to be more active than other portions of the protein. A synthetic isoform of the hAGRP protein containing the 46 carboxyl-terminus cysteine-rich residues, hAGRP-(87–132), was able to effectively bind the melanocortin receptors MC3R, MC4R, and MC5R and inhibit the binding of αMSH (15, 16). The minimal promoter of hAGRP was recently characterized, and two putative binding sites were identified for the signal transducers and activators of transcription (17) that have binding sites for the long isoform of the leptin receptor (18, 19). AGRP is thought to exert its orexigenic effects by antagonizing the action of αMSH at its receptors, MC3R and MC4R. This takes place by the activation of AGRP/neuropeptide Y neurons (20, 21), which results in increased expression of the two neuropeptides. In the paraventricular nucleus, increased amounts of AGRP/neuropeptide Y block the action of αMSH by binding its receptor MC4R (22–27), which leads to an increase in appetite and food intake.

A single nucleotide polymorphism (SNP) in the minimal promoter of the gene, −38C→T, was recently shown to affect promoter activity (28). Moreover, the genotype with the high promoter activity, *C/C*, was significantly associated with both obesity and type 2 diabetes in Africans (28). This SNP was found in Africans and Africans of the Diaspora, but not in Caucasian Americans. In the present study we report a recently identified polymorphism in the coding region, c.199G→A (17), that was found in Caucasian Americans, but not in Africans and Africans of the Diaspora. Two other groups recently reported the same SNP. Vink *et al.* (29) found that the c.199G→A polymorphism was significantly associated with the eating disorder anorexia nervosa, whereas Dubern *et al.* (30) did not find a significant association with body mass index (BMI) or percent fat mass in obese children. Here we show that the c.199G→A polymorphism could affect the secondary structure of the protein, and that the G/G genotype is significantly associated with human adiposity in an age-dependent fashion.

Subjects and Methods

Subjects

The HERITAGE Family Study cohort consists of 483 white subjects (233 men and 250 women) from 99 nuclear families and 259 black subjects (88 men and 171 women) from 105 family units. The study design and inclusion criteria have been described previously (31). To be

Abbreviations: AGRP, Agouti-related protein; BMI, body mass index; hAGRP, human agouti-related protein; SNP, single nucleotide polymorphism.

eligible, the individuals were required to be in good health, *i.e.* free of diabetes, cardiovascular diseases, or other chronic diseases that would prevent their participation in an exercise training program. Subjects were also required to be sedentary, defined as not having engaged in regular physical activity over the previous 6 months. Individuals with resting systolic blood pressure greater than 159 mm Hg and/or diastolic blood pressure more than 99 mm Hg were excluded. The study protocol had been approved by each of the institutional review boards of the Heritage Family Study research consortium. Written informed consent was obtained from each participant.

Body composition

Stature was measured to the nearest 0.1 cm with the subject standing erect on a flat surface, with heels, buttocks, and back pressed against the stadiometer, and the head positioned in the Frankfort horizontal plane. Body mass was recorded to the nearest 100 g using a balance scale with subjects clothed only in a light-weight bathing suit. BMI was calculated by dividing body mass (kilograms) by stature squared (meters). Body density was assessed by underwater weighing (32). Body density was converted to percent body fat (32). The reproducibility of the body density and fat mass measurements was very high, with intraclass correlations for repeated measures ranging between 0.97 and 1.00 without significant differences among the four clinical centers involved in this study (31). Computed tomography scans were used to determine abdominal total fat, abdominal sc fat, and abdominal visceral fat as previously described (33). The computed tomography scans were obtained between the fourth (L4) and fifth (L5) lumbar vertebrae. Areas were calculated by delineating them with a graph pen and then computing using an attenuation range from −30 to –190 Hounsfield units (34).

Genotyping for the c.199G→A polymorphism

The c.199G→A polymorphism was scored by amplification of the second coding exon with the following primers: *agrpga1*, 5′-agt ctc ccc tgg cat aaa cc-3′; and *agrpga2*; gta gtg tcg tcg ctg gtg ag-3′, essentially as previously described for this polymorphism (17). Briefly, PCR cycling conditions were as follows: 1 cycle at 94 C for 4 min, followed by 35 cycles, each consisting of a step at 94 C for 60 sec, a step at 60 C for 60 sec, and a step at 72 C for 60 sec. A final extension step at 72 C for 5 min was also applied. PCR was carried out in 20-μl volumes. Amplicons were digested in a 30-μl volume containing 1 U of the enzyme *Bsm*AI, as prescribed by the manufacturer (New England Biolabs, Inc., Beverly, MA). The *G/G* genotype does not digest with *Bsm*AI. Genotyping was performed in a blinded fashion without prior knowledge of the participants or their phenotypes.

Statistical analyses

A χ^2 test was used to confirm that the observed genotype frequencies were in Hardy-Weinberg equilibrium. The normality of the distributions was checked with the Shapiro-Wilk statistic of the univariate procedure of the SAS statistical software package (SAS Institute, Inc., Cary, NC). Associations between the AGRP c.199G[arrow]A SNP and adiposity phenotypes were analyzed using a mixed procedure in the SAS software package. Nonindependence among family members was adjusted for using a sandwich estimator, which asymptotically yields the same parameter estimates as ordinary least squares or regression methods, but the SE values and consequently hypothesis tests are adjusted for the dependencies. The method is general, assuming the same degree of dependency among all members within a family. Body composition phenotypes were adjusted for age and sex; abdominal visceral fat was adjusted also for total fat mass. Generation-specific associations between genotype and body composition phenotypes were tested by adding a generation by genotype interaction term into the mixed model. Values are given as the mean and SEM.

Nomenclature

The nomenclature adopted for referencing gene names, symbols, and polymorphism descriptions was according to den Dunnen and Antonarakis (34a) and the Nomenclature Working Group (http://archive.uwcm.ac.uk/uwcm/mg/docs//mut_nom.html).

Results

An SNP was identified in the third exon of the gene, c.199G→A (counting as nucleotide 1 of the cDNA the adenine in the translation initiator, ATG) that resulted in a nonconservative amino acid substitution of the monocarboxylic alanine at position 67 by the hydroxyl-containing threonine (Ala^{67}Thr). Algorithmic analysis (35, 36) was undertaken to predict the impact of the polymorphism on the secondary structure of the molecule, which could impact the functional activity of the protein. Windows 1 and 2 for the predicted coils were only slightly affected, but window 3 was significantly affected, with the probability score dropping by approximately 50% (Fig. 1). These data were confirmed using an alternative algorithm (37) (data not shown).

The well characterized HERITAGE family study (*HE*alth, *RI*sk factors, exercise *T*raining, *A*nd *GE*netics) (31) was used to examine the association of the c.199G→A SNP with BMI, adiposity, and abdominal fat. The basic characteristics of the study population are presented in Table 1. Four hundred and eighty-two individuals were genotyped, including 183 parents (unrelated individuals) and 299 offspring (related individuals). There were no *A/A* homozygotes identified in the Caucasian sample, whereas a sample of 225 African-Americans showed complete absence of this SNP. There was a significant interaction between genotype and generation in the Caucasian sample for adiposity phenotypes ($P = 0.0097$). The offspring were therefore analyzed as a separate group. The mean age of the offspring was 25 yr, and that of the parents was 53 yr (Table 1). There were no significant associations between the c.199G→A polymorphism and measures of human fatness (BMI, fat mass, percent body fat, and abdominal visceral fat) in the offspring (all offspring or a single offspring per family; Table 2). However, all 4 measures of human fatness were significantly higher in the homozygous, *G/G*, parents even when abdominal visceral fat was adjusted for fat mass (Table 2).

Discussion

In the present study we report a polymorphism in the coding region of AGRP, c.199G>A, that was significantly associated with the development of obesity in humans, but in an age-dependent fashion. The plasma levels of hAGRP have previously been reported to be higher in obese men (38), but in the case of the c.199G→A polymorphism it would more likely be the activity and binding affinity for the melanocortin receptors that could be affected. It should be pointed out that the amino acid substitution Ala^{67}Thr is outside of the protein fragment that has been shown to retain activity (residues 83–132) (16). Nonetheless, algorithmic analysis of the secondary structure of the two isoforms of the polymorphic protein revealed differences in the coils of the two proteins (35, 36). These findings were confirmed by another algorithm also predicting coiled coils (37). We further performed multiple alignments (data not shown) using AGRP protein sequences from different species (human, mouse, bovine, and porcine). The alanine at position 67 was 100% conserved, but

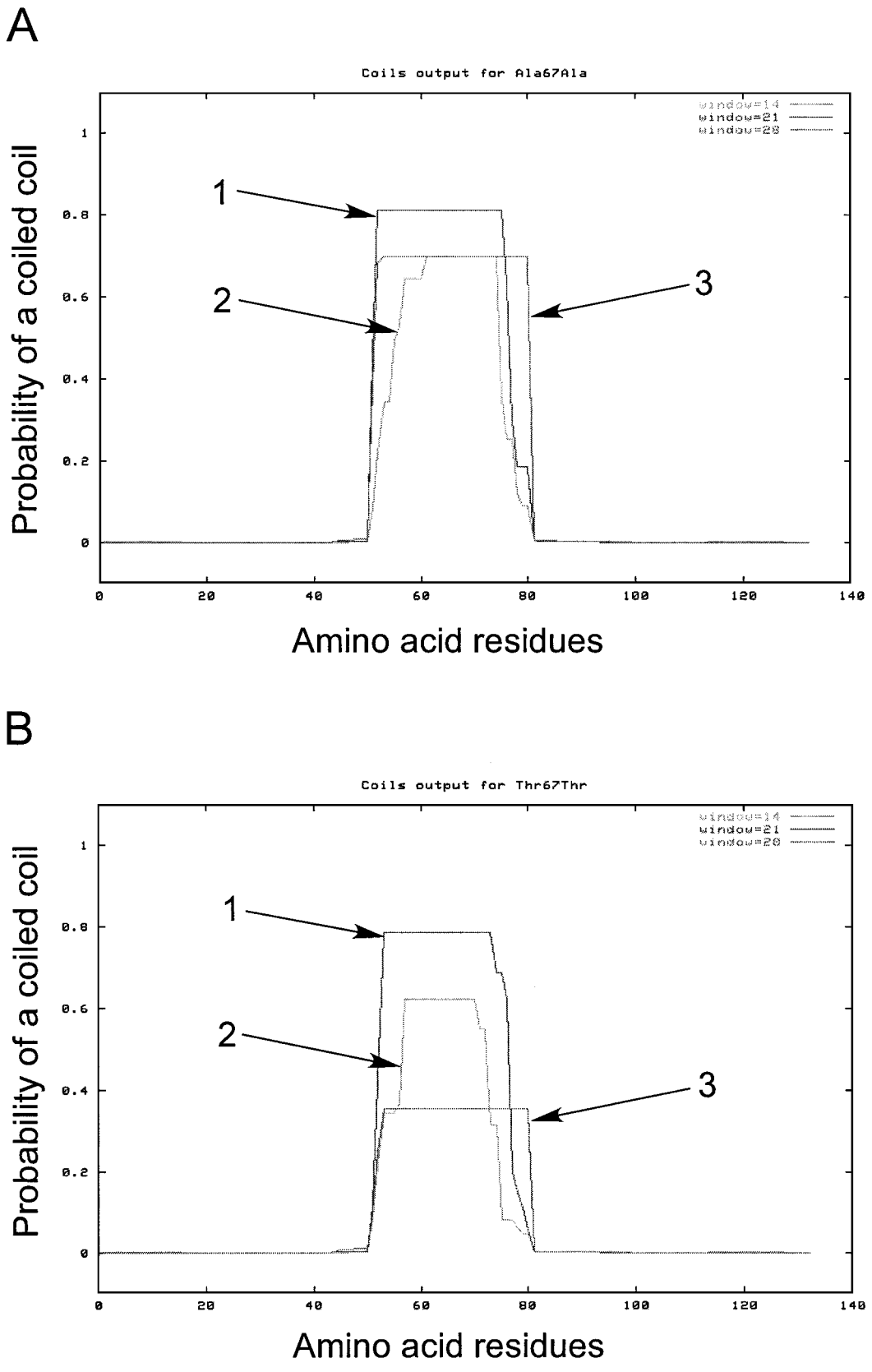

FIG. 1. Graphical presentation of the impact of the Ala^{67}Thr polymorphism on the secondary structure of the protein. Different windows of the predicted coiled coils are numbered and pointed to by the *arrows*. A, Coiled coils for the Ala^{67}Ala isoform; B, coiled coils for the Thr^{67}Thr isoform. The algorithm used for the prediction of the coils was previously described (35, 36).

TABLE 1. HERITAGE Family Study population characteristics

Generation	Gender	No.	Variable	Mean	SD
Parent	Male	93	Age (yr)	53.7	5.3
			Height (cm)	175.7	6.1
			Mass weight (kg)	87.7	15.2
			BMI	28.4	4.5
			Fat weight (kg)	24.5	9.1
			Percent body fat (%)	27.5	6.5
			Fat-free weight (kg)	62.3	7.5
	Female	90	Age (yr)	51.9	4.9
			Height (cm)	162.3	6.2
			Mass weight (kg)	72.3	13.5
			BMI	27.5	4.8
			Fat weight (kg)	26.8	10.5
			Percent body fat (%)	36.4	7.9
			Fat-free weight (kg)	44.5	4.8
Offspring	Male	140	Age (yr)	25.5	6.0
			Height (cm)	179.0	6.0
			Mass weight (kg)	82.5	16.7
			BMI	25.7	4.9
			Fat weight (kg)	17.6	11.1
			Percent body fat (%)	20.1	9.2
			Fat-free weight (kg)	64.3	7.9
	Female	159	Age (yr)	25.6	6.4
			Height (cm)	164.6	6.5
			Mass weight (kg)	64.5	13.2
			BMI	23.7	4.4
			Fat weight (kg)	18.2	10.0
			Percent body fat (%)	26.8	9.0
			Fat-free weight (kg)	46.1	5.2

TABLE 2. Association of adiposity parameters with the c.199G>A (Ala67Thr) SNP in hAGRP

Trait	Generation	Genotype[a]	No.	Mean ± SEM	P^b
BMI[c] (kg/m^2)	Parent	*G/A*[d]	19	25.5 ± 0.9	0.012[b]
		G/G	164	28.1 ± 0.4	
	Offspring	*G/A*	30	25.6 ± 1.1	0.34
		G/G	269	24.4 ± 0.4	
Fat mass[c] (kg)	Parent	*G/A*	18	19.8 ± 1.6	0.003[b]
		G/G	150	26.2 ± 0.9	
	Offspring	*G/A*	28	18.6 ± 1.4	0.63
		G/G	262	17.9 ± 0.8	
Body fat[c] (%)	Parent	*G/A*	18	28.0 ± 1.5	0.019[b]
		G/G	150	32.2 ± 0.6	
	Offspring	*G/A*	28	24.7 ± 1.3	0.57
		G/G	262	23.9 ± 0.7	
AVF[c] (cm^2)	Parent	*G/A*	19	95.2 ± 8.5	0.009[b]
		G/G	163	126.4 ± 4.8	
	Offspring	*G/A*	29	51.6 ± 5.0	0.68
		G/G	266	54.0 ± 1.9	
AVF[e] (cm^2)	Parent	*G/A*	18	104.8 ± 6.5	0.043[b]
		G/G	149	122.0 ± 3.5	
	Offspring	*G/A*	27	49.9 ± 3.4	0.28
		G/G	259	54.2 ± 1.3	

No homozygous *A/A* individuals were identified. AVF, Abdominal visceral fat; No., sample size.

[a] Genotype frequencies were in Hardy-Weinberg equilibrium.

[b] Significant at $P < 0.05$.

[c] Adjusted for gender and age.

[d] Allele frequencies were: *A*, 5.5%; *G*, 94.5%.

[e] Adjusted for gender, age, and fat mass.

this is not surprising given that most of the AGRP amino acid residues are 100% conserved between mammalian species. We hypothesize that the functional properties of AGRP could be altered by this polymorphism due to conformational changes made to the protein structure, as has been shown for missense mutations in other genes (39, 40). This hypothesis, however, requires functional testing (*e.g.* x-ray crystallography and measurement of melanocortin binding by the mutant) to confirm the impact of the SNP on the activity of the protein.

AGRP induced feeding when administered in the arcuate nucleus and dorsomedial and ventromedial nuclei (24) and induced c-Fos-like expression in the accumbens shell and central amygdala (41), which are key extrahypothalamic feeding and reward centers. Furthermore, studies in rats have shown that AGRP administration resulted in increased consumption of the high sucrose diet over the low sucrose option (42) and preference for the high fat diet over the low fat option (43), suggesting that AGRP could play a role in macronutrient selection. We hypothesize, therefore, that the *A* allele of this SNP could predispose heterozygous individuals toward a subtle, but chronic, selection of certain foods that might result in lower BMI, lower fat mass, lower percent body fat, and lower visceral adiposity. This hypothesis, however, requires further investigation, in particular of the availability of data that take into consideration the nutritional composition of foods that were consumed by the study population. We also performed a separate analysis of the offspring for ages between 30 and 40 yr to examine whether the *G/A* genotype in the older offspring had the same effect as it did on the parents. There were no significant associations in this subset, but the sample sizes were relatively small (5 *G/A* and 64 *G/G*). These data tentatively suggest that the effects of the *G/A* genotype on visceral adiposity are only evident in the older population.

In the present study we showed that the c.199G→A SNP was consistently associated with four different measures of human fatness: BMI, fat mass, percent body fat, and abdominal visceral fat, all adjusted for gender and age. Importantly, these findings were true in the case of the parents, but not in the case of the offspring, which suggests that the *G/A* genotype could exert its effects in an age-dependent fashion. We conclude that the c.199G→A polymorphism in hAGRP is significantly associated with late-onset obesity in humans and could provide a diagnostic marker for fatness in Caucasian populations.

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Address all correspondence and requests for reprints to: George Argyropoulos, Ph.D., Pennington Biomedical Research Center, 6400 Perkins Road, Baton Rouge, Louisiana 70808. E-mail: argyrog@pbrc.edu.

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