Two ethnic-specific polymorphisms in the human Agouti-related protein gene are associated with macronutrient intake[1–3]

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ABSTRACT

Background: The Agouti-related protein (AGRP), an appetite modulator, induces hyperphagia when administered intracerebroventricularly or when overexpressed in transgenic mice. Exogenous administration of AGRP in rodents predisposes to high fat and high sugar intakes.

Objective: The objective was to examine the potential associations of 2 ethnic-specific polymorphisms in the *AGRP* gene (Ala67Thr in whites and −38C>T in blacks) in the Health, Risk Factors, Exercise Training, and Genetics (HERITAGE) Family Study.

Design: We examined the effect of the 2 polymorphisms in the *AGRP* gene on self-reported macronutrient intakes in 478 white and 272 black participants in the HERITAGE Family Study.

Results: Both *AGRP* polymorphisms showed a significant association with energy intake. In whites, a smaller proportion of total energy was derived from fat by the Ala67Thr heterozygotes ($\bar{x}$ ± SEM: 29.4 ± 0.7%) than by the Ala67Ala homozygotes (31.5 ± 0.5%; $P = 0.009$), mainly because of a lower intake of saturated ($P = 0.06$) and monounsaturated ($P = 0.01$) fats by the Ala67Thr heterozygotes. The percentage of energy from carbohydrates was 2.6% greater in the Ala67Thr heterozygotes (55.1 ± 1.1%) than in the Ala67Ala homozygotes (52.5 ± 0.6%; $P = 0.03$). In blacks, protein intake was associated with the −38C>T promoter polymorphism. *T/T* homozygotes had a significantly lower protein intake than did the *C*-allele carriers (*C/C*: 16.8 ± 0.4%; *C/T*: 17.2 ± 0.2%; *T/T*: 15.4 ± 0.7%; $P = 0.04$). No significant differences in total energy and alcohol intakes existed between genotype groups in blacks or whites.

Conclusions: The present study suggests that 2 ethnic-specific *AGRP* variants, previously shown to be associated with leanness in the HERITAGE Family Study, are also associated with macronutrient intake. *Am J Clin Nutr* 2005;82:1097–1101.

KEY WORDS Agouti-related protein, *AGRP*, Ala67Thr, −38C>T, energy intake, macronutrient intake, food-frequency questionnaire, Québec Family Study

INTRODUCTION

The Agouti-related protein (AGRP) is expressed in the arcuate nucleus of the hypothalamus and projects to other key feeding hypothalamic nuclei and sites throughout the brain (1–3). AGRP has been characterized as a potent anabolic effector of food intake (4). The murine and human orthologs stimulate hyperphagia when administered intracerebroventricularly (5, 6) or when overexpressed in transgenic mice (7). AGRP is thought to exert its orexigenic effects by antagonizing the action of the α-melanocyte–stimulating hormone at the type 3 and type 4 melanocortin receptors (8–10). An additional alternate mechanism that has been proposed for AGRP's orexigenic action is through the central nervous system opioid system (6). Animal studies have shown that administration of opioid receptor antagonists block AGRP-induced food intake when given simultaneously but not 24 h after AGRP injection, which indicates that the short-term effects of AGRP are mediated by the activity of the opioid receptors (6). The opioid system not only mediates food intake but also food selection (11, 12). Intracerebroventricular administration of AGRP in rats selectively increased food intake during a high-fat but not a low-fat diet, and antagonism of AGRP preferentially suppressed the high-fat diet over the low-fat diet (6). In addition, when given ad libitum access to a standard nonpurified diet and a 20% sucrose solution, injection of AGRP selectively increased the intake of the nonpurified diet (6). Agouti (Ay/a) mice treated with melanocortin antagonist consumed a greater proportion of their daily intake from fat and less from carbohydrate than did their wild-type littermates when fed a 3-choice macronutrient diet (13). In rat pups, hypothalamic AGRP concentrations peak at weaning, which helps mediate the transition from suckling of a fat-rich diet to independent feeding of a carbohydrate-rich diet (14).

Studies in humans have shown that plasma AGRP concentrations are elevated in obese compared with lean subjects (15, 16).

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In addition, there is increasing evidence that plasma AGRP concentrations increase during an extended period of fasting (16–18), which corroborates with the findings in rodents (19–21). Although the physiologic role of peripheral AGRP remains to be elucidated, these findings suggest that AGRP may represent an important peripheral marker of changes in energy balance.

Thus far, no studies of the association between *AGRP* gene variants and macronutrient intake in humans have been reported. In the present study, we examined the effect of 2 ethnic-specific polymorphisms in the *AGRP* gene (Ala67Thr in whites and −38C>T in black) on self-reported macronutrient intakes in blacks and white participants in the HERITAGE Family Study. We previously showed that the −38C>T single nucleotide polymorphism (SNP) is associated with reduced body fatness in blacks, whereas the Ala67Thr genotype is associated with leanness in whites in an age-dependent fashion (22, 23).

SUBJECTS AND METHODS

Subjects

This study was based on data from 272 black subjects (90 men and 182 women) and 478 white subjects (230 men and 248 women) from the HERITAGE Family Study. The study design and inclusion criteria were described previously (24; Internet: http://www.pbrc.edu/heritage). Briefly, eligible individuals were required to be between the ages of 17 and 65 y, to be healthy but sedentary (no regular strenuous physical activity over the previous 6 mo), to have a BMI (in kg/m^2) < 40, and systolic and diastolic blood pressures $\leq$159 and 99 mm Hg, respectively. Participants with BMIs slightly >40 ($n = 6$), who were considered by the supervising physician to be healthy and able to perform the required exercise regimen, were included in the study. Furthermore, individuals with confirmed or possible coronary heart disease, chronic or recurrent respiratory problems, and uncontrolled endocrine and metabolic disorders (including diabetes, hypoglycemia, and the use of antihypertensive or lipid-lowering drugs) were excluded. The study protocol was approved by each of the Institutional Review Boards of the HERITAGE Family Study research consortium. Written informed consent was obtained from all participants.

Dietary record

Daily energy, macronutrient, and micronutrient intakes were estimated by using Willett's food-frequency questionnaire (25). The questionnaire provided data on a subject's usual eating habits, dietary supplement use, food items in 5 major food groups, food preparation, seasonings, and favorite foods. The questionnaire also included questions regarding consumption of caffeine and alcohol-containing beverages over the past year. The questionnaire was self-administered by the subjects and reviewed with the subjects by an interviewer for completeness. The questionnaire scores and nutrient intakes were calculated at the Channing Laboratory, Harvard University, where the questionnaire was developed and validated. The reproducibility and validity of the food-frequency questionnaire were documented elsewhere (25). In brief, the intraclass correlations between 2 measurements at an interval of 1 y for nutrient intake estimated by the Willett questionnaire were similar to those computed from a 7-d diet record. Correlation coefficients between the mean calorie-adjusted intakes of macronutrients from 1-wk diet records and those from the questionnaire completed after the diet records ranged from 0.43 to 0.59. In the present study, the dietary phenotypes were expressed as a percentage of total daily energy intake derived from each energy nutrient. Besides the main energy nutrients (ie, fat, carbohydrate, protein, and alcohol), we also included the following subphenotypes: saturated, monounsaturated, and polyunsaturated fats; sucrose; and other carbohydrates.

Genotyping

A c.199G→A transition in the third exon of the human *AGRP* gene results in a nonconservative amino acid substitution, Ala67Thr (dbSNP database: rs5030980). This polymorphism was scored by amplification of the second coding exon with the primers AGRPga1, 5'-*agt ctc ccc tgg cat aaa cc*-3' and AGRPga2, 5'-*gta gtg tcg tcg ctg gtg ag*-3' and by restriction digestion of the amplicons by the *Bsm*AI enzyme. The −38C>T polymorphism (dbSNP database: rs5030981), in the minimal promoter of the AGRP, was genotyped on a LI-COR Analyzer 4200 (Lincoln, NE). The following primers were used: AGRPct1, 5'-*ctt gac ccg aat tct tgg aa*-3'; AGRPct2, 5'-*gtg aag gac cct tcc tgg ag*-3'; and the sequencing primer AGRPsnpCT, 5'-*aca aat taa att aag ctt tca gg*-3'. These polymorphisms were not the only 2 that were detected after the *AGRP* gene was sequenced in a representative cohort of whites and African Americans. However, because all other SNPs detected were in linkage disequilibrium with either the promoter or the Ala67Thr polymorphism, we opted to refer only to the latter 2. Furthermore, they are the only 2 polymorphisms that have been studied extensively and, thus, the present data can provide meaningful information for comparisons with other relevant publications. The genotyping of both polymorphisms was described previously (22, 23).

Statistical analyses

All analyses were performed with the SAS Statistical Software (version 8.2; SAS Institute Inc, Cary, NC). Data were analyzed separately by race. Associations between the *AGRP* polymorphisms and dietary phenotypes were quantified by using the mixed-model procedure with a post hoc Tukey's test. The dietary phenotypes were adjusted for sex, age, and BMI by including them into the model. Because the study was designed as a family study, the subjects were related within families. Nonindependence among family members was adjusted for by using a "sandwich estimator," which asymptotically yields the same parameter estimates as does ordinary least-squares or regression methods, but the SEs and resulting hypothesis tests are adjusted for the dependencies (26, 27). The method is a generalization of analysis of variance and regression that estimates group differences and regression coefficients while accounting for multiple levels of analysis. In this study, there were 2 levels: families and subjects within family. The method assumed that the same degree of dependency existed among all subjects within a family. Possible sex-by-gene, BMI (<30 compared with ≥30)-by-gene, and generation-by-gene interactions were tested with the mixed-model procedure by including main effects and interaction terms in the same model.

TABLE 1
Characteristics of the HERITAGE Family Study subjects[1]

	Blacks (n = 90 M, 182 F)	Whites (n = 230 M, 248 F)
Age (y)	33.7 ± 11.8	35.9 ± 14.5[2]
BMI (kg/m^2)	27.9 ± 6.0	25.9 ± 5.0[3]
Total energy intake (kcal)	2259 ± 1449	2249 ± 909
Fat intake (% of energy)	30.9 ± 6.6	31.4 ± 5.7
Saturated fat (% of energy)	10.8 ± 2.9	11.8 ± 2.4[3]
Monounsaturated fat (% of energy)	11.9 ± 2.7	11.4 ± 2.6
Polyunsaturated fat (% of energy)	5.4 ± 1.4	5.4 ± 1.5
Carbohydrate intake (% of energy)	53.1 ± 9.4	52.1 ± 7.6
Sucrose (% of energy)	11.3 ± 3.6	9.6 ± 3.1[3]
Other carbohydrates (% of energy)	41.8 ± 8.1	42.5 ± 6.4
Protein intake (% of energy)	16.8 ± 3.9	16.4 ± 3.0[3]
Alcohol intake (% of energy)	0.9 ± 1.9	1.9 ± 3.0[3]

[1] All values are $\bar{x}$ ± SD.
[2,3] Significantly different from blacks (Student's t test): [2] $P < 0.05$, [3] $P < 0.01$.

RESULTS

The phenotypic characteristics of the 478 white subjects and the 272 black subjects are shown in **Table 1** and were previously described in detail (28). The Ala67Thr polymorphism was present only in whites, and the −38C>T polymorphism was identified only in blacks; the allele frequencies of the common alleles were as follows: 0.95 for Ala67 in whites and 0.71 for the −38C allele in blacks. The genotype frequencies were in Hardy-Weinberg equilibrium.

Both AGRP polymorphisms showed significant associations with macronutrient intake. In whites, the Ala67Thr polymorphism was significantly associated with fat and carbohydrate intakes. The Thr67 allele was associated with a 2.1% of energy lower fat intake and a 2.6% of energy higher carbohydrate intake (**Table 2**). The difference in fat intake can be attributed to differences in energy from saturated (−0.8% of energy) and monounsaturated fats (−0.9% of energy), whereas no differences were observed for polyunsaturated fat. There were no differences in total energy, protein, and alcohol intakes.

In blacks, the protein intake in the *T*/*T* homozygotes of the −38C>T polymorphism was 1.3% and 1.8% of energy lower than that in the *C*/*C* homozygotes (post hoc Tukey's test, P = 0.14) and *C*/*T* heterozygotes (post hoc Tukey's test, P = 0.03), respectively (**Table 3**). When the *C*-allele carriers were grouped together and compared against the *T*/*T* homozygotes, the effect of the *T*/*T* genotype on reducing protein intake was significant (P = 0.036); the *C*/*C* plus *C*/*T* group had a higher protein intake ($\bar{x}$ ± SEM: 16.98 ± 0.37% of energy) than did the *T*/*T* homozygotes (15.47 ± 0.70% of energy). Total energy, fat, carbohydrate, and alcohol intakes were not significantly different between the 3 genotype groups. No significant interactions between the genotypes and sex, race, generation, or BMI were observed.

DISCUSSION

In the present study, we reported associations between self-reported macronutrient intake and 2 ethnic-specific polymorphisms in the *AGRP* gene (Ala67Thr in whites and −38C>T in blacks). The association was more pronounced in whites, in whom the rare allele of the Ala67Thr *AGRP* variant was associated with less energy derived from fat, specifically saturated fat, and more from carbohydrates, as recommended (29). In addition, we found a significant association between the −38C>T *AGRP* variant and protein intake in blacks. To our knowledge, this is the first study that reports such associations in humans, namely ethnic-specific polymorphisms in the same gene associated with macronutrient intake.

TABLE 2
Association of dietary phenotypes with the Ala67Thr single nucleotide polymorphism in *AGRP* in whites[1]

	Ala67Ala (n = 429)	Ala67Thr (n = 49)	P[2]
Total energy intake (kcal)	2364 ± 91	2390 ± 152	0.88
Fat intake (% of energy)	31.5 ± 0.46	29.4 ± 0.71	0.009
Saturated fat (% of energy)	11.4 ± 0.21	10.5 ± 0.39	0.06
Monounsaturated fat (% of energy)	12.0 ± 0.22	11.2 ± 0.30	0.015
Polyunsaturated fat (% of energy)	5.4 ± 0.12	5.1 ± 0.20	0.28
Carbohydrate intake (% of energy)	52.5 ± 0.55	55.1 ± 1.09	0.032
Sucrose (% of energy)	9.8 ± 0.26	10.6 ± 0.56	0.18
Other carbohydrates (% of energy)	42.7 ± 0.55	44.5 ± 0.8	0.033
Protein intake (% of energy)	16.2 ± 0.28	15.7 ± 0.41	0.28
Alcohol intake (% of energy)	1.6 ± 0.2	1.7 ± 0.49	0.83

[1] All values are $\bar{x}$ ± SEM.
[2] ANOVA with mixed model was used to compare the Ala67Ala and Ala67Thr genotypes.

TABLE 3
Association of dietary genotypes with the −38C >T single nucleotide polymorphism in *AGRP* in blacks[1]

	Genotype			
	C/C (n = 140)	*C/T* (n = 112)	*T/T* (n = 20)	P[2]
Total energy intake (kcal)	2164 ± 127	2064 ± 152	2253 ± 256	0.77
Fat intake (% of energy)	32.3 ± 0.69	32.0 ± 0.71	30.6 ± 1.39	0.51
Saturated fat (% of energy)	11.6 ± 0.30	11.5 ± 0.34	10.9 ± 0.57	0.44
Monounsaturated fat (% of energy)	12.3 ± 0.29	12.2 ± 0.29	11.5 ± 0.67	0.52
Polyunsaturated fat (% of energy)	5.5 ± 0.23	5.4 ± 0.20	5.6 ± 0.38	0.86
Carbohydrate intake (% of energy)	51.5 ± 0.94	51.3 ± 1.04	54.9 ± 2.05	0.24
Sucrose (% of energy)	11.1 ± 0.47	11.0 ± 0.45	11.7 ± 0.75	0.63
Other carbohydrates (% of energy)	40.4 ± 0.87	40.2 ± 0.92	43.5 ± 2.1	0.32
Protein intake (% of energy)	16.8 ± 0.38	17.2 ± 0.47	15.4 ± 0.68	0.042[3]
Alcohol intake (% of energy)	1.2 ± 0.34	1.1 ± 0.21	1.1 ± 0.24	0.87

[1] All values are $\bar{x}$ ± SEM.
[2] ANOVA with mixed model was used to compare *C/C, C/T,* and *T/T* genotypes.
[3] Post hoc Tukey adjustments: *C/C* compared with *C/T,* P = 0.60; *C/C* compared with *T/T,* P = 0.14; *C/T* compared with *T/T,* P = 0.03. P = 0.036 for the comparison between *C*-allele carriers and *T/T* homozygotes.

Both *AGRP* variants examined in the present study are suggested to have functional effects (30) and were associated with obesity-related phenotypes in the HERITAGE Family Study (22, 23). The Ala67Thr *AGRP* variant is located in the third exon and outside of the protein fragment that has been shown to retain activity (31). Nonetheless, algorithmic analysis of the secondary structure of the 2 resulting isoforms of the protein showed differences in coils (22). These conformational changes might alter the functional properties of AGRP, but functional testing is required to confirm this hypothesis. In the current study, the Ala67Thr heterozygotes followed a diet in which less energy was derived from fat (less saturated and monounsaturated fats) and more energy was derived from carbohydrates compared with the diet of the Ala67Ala homozygotes. We previously showed, in the same population, that the Ala67Thr heterozygotes of the parental generation had a lower BMI, total fat mass, and abdominal fat (22). Furthermore, the Ala67Thr genotype was found to be more frequent in anorexia nervosa patients than in a control group (32).

The −38C>T *AGRP* variant is located in the promoter region of the gene (30, 33). The *C/C* genotype was shown to be associated with a significantly higher promoter activity and affinity for transcription factors, whereas the *T/T* genotype had a reduced promoter function that could affect the expression levels of the gene (30). In the black subjects in the current study, *T/T* homozygotes had a lower protein intake. In a previous study, we found that black *T/T* homozygotes seemed to be protected from developing obesity (23). Compared with the *C*-allele carriers, *T/T* homozygotes had a significantly lower BMI, less fat mass, and a lower percentage of body fat (23). Similarly, the C/*C* genotype of the −38C>T polymorphism was associated with obesity and type 2 diabetes in Sierra Leoneans from West Africa but was not in Jamaicans or Gullah-speaking African Americans (30).

The mechanisms by which *AGRP* exerts its effects on macronutrient intake have not been studied thoroughly. A study in rats suggested that AGRP's effect on food selection might be mediated by opioid receptors (6). Intracerebroventricular administration of AGRP was found to preferentially increase the intake of a high-fat diet over a low-fat diet. However, antagonism of the opioid receptor by naloxone changed AGRP's effect on food selection because the preference for the high-fat diet was suppressed and the preference for the low-fat diet was increased. The opioid system is known for mediating fat-selective intake (11, 12).

Interestingly, we found that the 2 AGRP polymorphisms affect different macronutrients; ie, Ala67Thr affected fat and carbohydrate intakes in whites, whereas −38C>T influenced protein intakes in blacks. The reasons for this discrepancy are not clear, but differences in eating habits between ethnic groups might be one explanation (34). Other potential factors are energy requirements or genetic factors regulating taste that are likely to influence food preference, and such factors could be different between blacks and whites (35, 36). Some (37–39), but not all (40), studies have shown that the validity and reproducibility of food-frequency questionnaires are lower in minority populations, which might be another explanation for the race differences we found.

On the basis of these results, we hypothesized that subtle differences in food preference in carriers of the rare *AGRP* alleles (*T/T* and Ala67Thr in blacks and whites, respectively) could over time result in lower adiposity. In rodents, it has been proposed that AGRP may play a role in food selection (6, 13, 14). In support of this hypothesis, high concentrations of AGRP—such as those achieved by intracerebroventricular AGRP administration in rats (6) or observed in Agouti (Ay/a) mice with chronic melanocortin antagonism (13)—are associated with a preference for foods high in fat or high in carbohydrates when the animals are given the option. In addition, hypothalamic AGRP concentrations peak at weaning to help mediate the transition from a fat-rich diet to independent feeding of a carbohydrate-rich diet (14).

The limitations of self-reported dietary intake are well recognized (41). Although the food-frequency questionnaire used in the present study has reasonable reproducibility and validity (25), recalled data have inevitably a larger variation. This results in a larger SE of measurement, which makes it harder to find significant differences between the genotype groups. Therefore, the current associations may have been underestimated compared with more precisely assessed macronutrient intakes. In addition, we assume that the recall bias was similar for each genotype and, therefore, did not affect the general outcome of our findings.

It is important to remember that the *P* values were not adjusted for multiple testing. Because this was the first study to examine AGRP polymorphisms in relation to macronutrient intake, we elected to report differences that were significant at the unadjusted 0.05 α-level to make sure that all potentially useful associations were identified for subsequent studies. Bonferroni-adjusted α levels would have been 0.005 compared with 0.05.

In summary, the present study showed for the first time that 2 ethnic-specific *AGRP* variants, previously associated with body leanness in the HERITAGE Family Study, are also associated with differences in macronutrient intake. This is the first study to report such associations in humans, and replication of the findings in other populations is needed to confirm our findings.

RJFL reviewed the relevant literature, performed the statistical analyses, interpreted the data, and drafted the manuscript. TR, TR, ASL, JSS, DCR, CB, and GA were involved in the study design, data collection, and drafting and revision of the manuscript. None of the authors had a direct conflict of interest due to relations with industry or financial interests. However, CB is a member of the Mars Nutrition Research Council.

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