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SHORT COMMUNICATION

The agouti-related protein and body fatness in humans

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OBJECTIVE: The objective of this study was to examine the impact of a single nucleotide polymorphism (SNP) (−38C>T) in the promoter of the human agouti-related protein (hAgRP) gene on promoter affinity for transcription factors (TFs) and its possible association with body composition phenotypes.
DESIGN: Electrophoretic mobility shift assays for the functional studies and association analyses for the population studies.
SUBJECTS AND METHODS: Nuclear extracts were isolated from the mouse hypothalamus cell line GT1-7 and subjected to binding assays using oligonucleotide probes corresponding to the −38C>T region and an antibody for the E12/E47 TFs. Individuals ($n=259$) from the HERITAGE Family Study were genotyped for the −38C>T SNP and used in the association studies.
RESULTS: Electrophoretic mobility shift and supershift assays confirmed binding of the E12/E47 TF to the −38C>T site in a genotype-dependent manner. The *T* allele was found exclusively in the black subjects while the genotype with the higher binding affinity, *CC*, was significantly associated with high BMI, fat mass, and percent body fat in the black subjects of the HERITAGE Family Study.
CONCLUSIONS: The E12/E47 TF could play a role in the regulation of hAgRP expression while the population studies suggest that the *TT* genotype of the −38C>T SNP could play a protective role against the development of obesity in the black population of the HERITAGE Family Study.
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Keywords: AgRP; E12/E47; promoter; polymorphism

Introduction

The murine and human AgRP orthologs stimulate hyperphagia when administered intracerebroventricularly (i.c.v.)[1–3] or when overexpressed in transgenic mice.[4] AgRP is expressed in the arcuate nucleus of the hypothalamus, the testes, and the adrenal gland, and is upregulated in obese and diabetic mice.[5,6] AgRP exerts its anabolic effects on food intake by antagonizing the alpha-melanocyte stimulating hormone (α-MSH) at its receptors, melanocortin receptors 3 and 4 (MC3R and MC4R).[7–9] An SNP in the coding region of the gene (Ala67Thr) has been associated with anorexia nervosa,[10] implicating hAgRP in the development of eating disorders. In addition, elevated plasma levels of hAgRP have been reported in obese men,[11] further suggesting an involvement of this gene in the development of obesity. Moreover, a 2-h fast resulted in a 73% increase of plasma hAgRP concentration, which is consistent with studies reported in animals.[12] The genomic structure of hAgRP has been determined including upstream sequences with significant promoter activities.[13] An SNP, −38C>T, was identified in the promoter of the gene that significantly affected promoter activity and binding affinity for TFs, as tested in hypothalamus- and periphery-derived cell lines.[14] This SNP was found in Africans and Africans of the Diaspora but not in Caucasian Americans.[14] The genotype with the higher promoter activity, *CC*, was found at significantly higher frequencies in obese and diabetic Africans (Sierra Leoneans) but not in Africans of the Diaspora (Jamaicans and the Gullah-speaking African Americans).[14]

In the present study, further functional *in vitro* experiments were performed to identify candidate TFs that could bind to the site of the polymorphism. In addition, black subjects from

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the well-characterized HERITAGE (HEalth, RIsk factors, exercise Training And GEnetics) Family Study were used to examine whether the *CC* genotype was associated with obesity phenotypes. Additional questions addressed were whether high mean BMI and genetic substructure in sample populations of black people impeded the detection of associations between genotypes and obesity-related phenotypes.

Methods

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were obtained from the mouse hypothalamus cell line, GT1-7,[15] as previously described.[16] Binding reactions were carried out in buffer (20 mM HEPES-KOH pH 7.9, 25% v/v glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 300 μg/ml BSA), 50 000 cpm of radiolabeled probes, 1.5 μg of poly[d(I-C)], and 5 μg of nuclear extract in a total volume of 35 μl. The 50-fold excess unlabeled competitor probes were used for competition on ice, for 15 min, prior to addition of the radiolabeled probes. Supershifts with the E12/E47 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were performed by precompetition of the nuclear extracts for 15 min, also on ice, prior to the addition of the radiolabeled probes. The following two pairs of primers were used to make the probes: [AgRPCF: 5′-ttt cag ggc cgc **c**tg cct t-3′ and AgRPCR: 5′-aag gca ggc **g**gc cct gaa a-3′] and [AgRPTF: 5′-ttt cag ggc **t**gc ctg cct t-3′ and AgRPTR: 5′-aag gca ggc **a**gc cct gaa a-3′] with the complementary mutant nucleotides shown in bold. Primers were end-labeled with T4 polynucleotide kinase as prescribed by the manufacturer (New England Biolabs, Beverly, MA, USA). Primers were annealed by mixing equimolar amounts of labeled primers (10 pmol/μl) in a tube and placing the tube in boiled water, where it was allowed to cool to room temperature for 3 h. Annealed cold competitor probes were prepared in the same fashion using concentrated stocks of primer (100 pmol/μl). Reactions with the probe were carried out for 15 min at room temperature and resolved on 6% nondenaturing acrylamide–bisacrylamide gels in 0.5 × TBE buffer (run at 20 mA for 1 h at 4°C). Gels were dried, and DNA–protein complexes were visualized by autoradiography. Gel-band densities, representing binding affinities, were evaluated with the NIH IMAGE program.

Algorithmic analyses to identify predicted recognition binding sites for TFs in the promoter of hAgRP were performed with the following software: 'TESS: Transcription Element Search Software on the WWW', Jonathan Schug and G Christian Overton, Technical Report CBIL-TR-1997-1001-v0.0, of the Computational Biology and Informatics Laboratory, School of Medicine, University of Pennsylvania, 1997, URL: http://www.cbil.upenn.edu/tess.

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.[17] Although the HERITAGE cohort includes both Caucasian and black subjects, the −38C>T SNP was identified in the black people 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.

Body composition

Stature was measured to the nearest 0.1 cm with the subject standing erect on a flat surface, 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 lightweight bathing suit. Body mass index (BMI) was calculated by dividing body mass (kg) by stature squared (m^2). Body density was assessed by underwater weighing.[18] Body density was converted to percent body fat (%FAT),[18] from which fat mass and fat-free mass were calculated. Reproducibility of the body density and fat mass was very high with intraclass correlations for repeated measures ranging between 0.97 and 1.00 without significant differences between the four clinical centers involved in this study.[18] The data presented are from baseline (ie sedentary state before training) measurements.

Genotyping

The −38C>T SNP was genotyped on a LI-COR DNA Analyzer 4200 (Lincoln, NE, USA). The following primers were used: AgRPCTF: 5′-ctt gac ccg aat tct tgg aa-3′; AgRPCTR: 5′-gtg aag gac cct tcc tgg ag-3′; and the sequencing primer AgRPsnpCT: 5′-aca aat taa att aag ctt tca gg-3′. The nomenclature adopted for referencing gene names, symbols, and polymorphism descriptions is that of the Nomenclature Working Group (http://archive.uwcm.ac.uk/uwcm/mg/docs//mut_nom.html).

Statistical analyses

Associations between the −38C>T SNP and body composition phenotypes were analyzed using a MIXED procedure in the SAS software package. Nonindependence among family members was adjusted using a 'sandwich estimator', which asymptotically yields the same parameter estimates as ordinary least-squares or regression methods, but the standard errors and consequently hypothesis tests are adjusted for the dependencies. Age and gender were included as covariates in the MIXED model for all body composition phenotypes.

Results and discussion

In the present study, the involvement of a promoter polymorphism in hAgRP (−38C>T) in adiposity was examined. The −38C>T SNP was previously reported to be associated with obesity and type II diabetes in Sierra Leoneans from West Africa but not so in the cases of

Jamaicans or Gullah-speaking African Americans.[14] Additional functional assays were carried out in an effort to identify the TFs whose binding might be affected by the mutation. Algorithmic analysis had previously identified[14] that the recognition binding site for the helix–loop–helix type of TFs (E12/E47) was abrogated when substituting the *C* by the *T* allele. Here, we present supershift EMSA data that confirm this prediction and show specificity of the −38C>T site for the E12/E47 type of transcription factor (Figure 1).

E12 and E47 belong to the family of the helix–loop–helix TFs and they are alternate splicing products of the E2A gene.[19] E12/E47 heterodimerize with the MyoD TF, which also belongs to the helix–loop–helix family of TFs, to bind E-box sequences within promoters of genes.[20] E-box binding TFs, like E12/E47, are usually involved in transcriptional activation, but their role in the repression of gene expression has also been reported.[21,22] Insulin, which downregulates NPY/AgRP expressing neurons in the arcuate nucleus,[23] is transcriptionally activated by the E12/E47 TFs that bind to the E-box in the promoter of the insulin gene, in a beta-cell-line-selective manner.[24,25] The E12/E47 TFs could, therefore, play a direct role in the regulation of hAgRP as well as an indirect role by regulating insulin expression. Precompetition of nuclear extracts from the mouse hypothalamus GT1-7 cell line with anti-E12/E47-specific antibody showed a reduction in binding (Figure 1). Specifically, in the uncompeted nuclear extracts, the *CC* genotype exhibited 10- and 2-fold higher affinities for bands A and B, respectively, than the *TT* genotype (Figure 1). In the nuclear extracts that were precompeted with anti-E12/E47, affinity for band A in the case of the *CC* genotype was reduced 5-fold in comparison to the uncompeted band A, but band B was unaffected. In the case of the *TT* genotype, band A was completely abrogated by the anti-E12/E47 antibody, whereas band B was again unaffected (Figure 1). These data show that the E12/E47 TF is competitively binding to the −38C>T site, in a genotype-dependent manner, diminishing the binding of the radio-labeled probe to the DNA/protein complex represented by band A. Yet, although there was a reduction in affinity for both *CC* and *TT* genotypes, binding to bands A and B was not completely abrogated or supershifted by the antibody for E12/E47, suggesting that there might be additional TFs binding to this site. Pull-down experiments would be required to confirm this hypothesis. These data, however, demonstrate the functional potential of the −38C>T site and suggest that the E12/E47 type of TFs (along with other transcriptional activators and/or repressors) could play a role in the transcriptional activation of hAgRP.

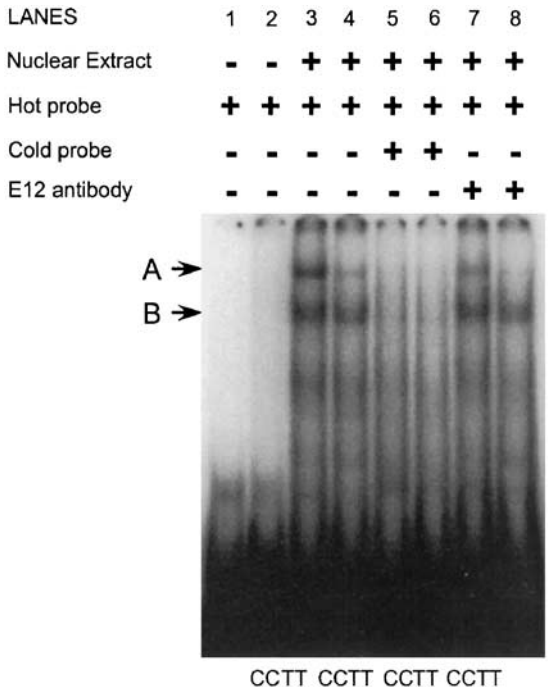

Figure 1 Electrophoretic mobility shift and supershift assay (EMSA). Nuclear extracts from the mouse hypothalamus cell lines GT1-7 were either precompeted with the cold probe (lanes 5 and 6), precompeted with the E12/E47 antibody (lanes 7 and 8), or not competed at all (lanes 2 and 3) before addition of the hot oligonucleotide probe. Two binding complexes were observed, A and B (indicated by the arrows), with the specificity for complex A being reduced for the *TT* genotype (lane 6). Precompetition with the E12/E47 antibody inhibited significantly the binding of the probe with the nuclear extracts and it abrogated complex A for the *TT* genotype (lane 8). Lanes 1 and 2 represent the hot probe in a reaction with the binding buffer but in the absence of nuclear extracts, antibody, and cold probe.

The HERITAGE Family Study was subsequently employed to examine for possible associations between the *TT* genotype (ie the genotype with the lower affinity for TFs) and obesity-related phenotypes. Basic characteristics of the study population are provided in Table 1. The −38C>T polymorphism was found in the black subjects but not in the Caucasian Americans of the HERITAGE Family Study. This is in agreement with the previous report that had evaluated the presence of this polymorphism in Caucasian and Hispanic Americans.[14] *TT* homozygous individuals (ie those with the low promoter activity[14] and reduced affinity for the E12/E47 TF genotype) had significantly lower mean BMI, fat mass, and percent body fat than either *CC* homozygotes or *CT* heterozygotes ($P<0.05$, Table 2). A single *C* allele, therefore, was sufficient to abolish the effect of the *TT* genotype for low BMI and adiposity. These data are in agreement with those previously reported for the Sierra Leoneans,[14] and show further a significant association of the *TT* genotype with low fat mass and percent body fat. By design, there were no individuals with diabetes or cardiovascular disease recruited in the HERITAGE Family Study, and thus no association analyses could be performed with these phenotypes. We also examined the possibility that the polymorphism may play a role in training-induced BMI reduction,[26] but there were no significant associations in men or women.

A striking characteristic of the study that previously identified the −38C>T polymorphism[14] is that the SNP was significantly associated with the frequency of low obesity in the lean Sierra Leoneans (mean BMI = 22.2 kg/μ^2) but not so in the Gullahs (mean BMI = 33 kg/μ^2). The black people of the

Table 1 Heritage Family Study, basic characteristics of the sample population

	Mean	s.d.
Parent, male, $n=20$		
Age (y)	50.8	7.6
Height (cm)	175	5.4
Body weight (kg)	80.4	12.2
BMI	26.3	4.3
Fat mass (kg)	18.9	4.9
Percent body fat (%)	24	3.8
Fat-free weight (kg)	58.9	5.6
Parent, female, $n=49$		
Age (y)	46.6	6.5
Height (cm)	163	6.5
Body weight (kg)	78.4	14.7
BMI	29.5	5.5
Fat mass (kg)	31.1	10.2
Percent body fat (%)	39.1	6.9
Fat-free weight (kg)	46.7	5.2
Offspring, male, $n=61$		
Age (y)	28.2	7.3
Height (cm)	176.9	7.1
Body weight (kg)	86.5	19.5
BMI	27.7	5.7
Fat mass (kg)	21.4	12.6
Percent body fat (%)	22.9	8.5
Fat-free weight (kg)	65.9	9.8
Offspring, female, $n=123$		
Age (y)	27.9	7.7
Height (cm)	162.2	6.6
Body weight (kg)	73.4	17.7
BMI	27.9	6.6
Fat mass (kg)	27.3	13.1
Percent body fat (%)	35.3	9.1
Fat-free weight (kg)	46.2	6.0

n: Sample size; s.d.: standard deviation.

HERITAGE Family Study have a lower mean BMI (BMI = 27 kg/μ^2) (Table 2) compared to the Gullah. The fact that the effect of the *TT* genotype on reduced obesity was detected in the HERITAGE black people but not in the Gullah black people suggests that high BMI (or other obesity-related factors) may be confounding the detection of an association. This could also be caused by insufficient numbers of lean participants representing the three genotypes in the Gullah population, thus providing limited statistical power for an association to be detected.[27–29] However, the effect of the *TT* genotype was not observed in the black Jamaicans, who also have relatively low BMI (mean BMI = 26.5 kg/μ^2).[14] We compared the frequencies of lean (BMI < 25 kg/μ^2), overweight (26 < BMI < 29 kg/μ^2), and obese (BMI > 30 kg/μ^2) individuals (irrespective of genotype) between the HERITAGE and the Gullah and between the HERITAGE and the Jamaicans. We found significant differences between the Gullah and the HERITAGE black people (*P*-value = 9.03×10^{-18}), but there were no significant differences between the HERITAGE and the Black Jamaican. This raises the possibility that the association of the *TT* genotype with low adiposity may require a low mean BMI to yield measurable effects, but may also depend on other (unknown) genetic traits present in some but not all populations.

Table 2 Association of BMI and adiposity parameters with the −38C>T SNP in the promoter of hAgRP

Genotype frequencies[a]	n	Mean ± s.e.m.	P-value
BMI (kg/m^2)			
CC (50%)	127	28.1 ± 0.9	
CT (43%)	109	28.9 ± 1.0	0.015*
TT (7%)	17	25.8 ± 1.1	
Fat mass (kg)			
CC (50%)	109	25.7 ± 2.7	
CT (43%)	88	27.8 ± 2.7	0.028*
TT (7%)	15	21.2 ± 2.7	
Percent body fat (%)			
CC (50%)	109	29.9 ± 1.8	
CT (43%)	88	30.9 ± 1.8	0.013*
TT (7%)	15	26.1 ± 2.0	

Differences between the three genotypes were evaluated by ANOVA and the *P*-values are shown. *Post hoc* analyses showed that the *TT* genotype was consistently lower than either the *CC* or *CT* genotypes. Genotype frequencies are shown in parentheses. Al the phenotypes were adjusted for gender and age. *n*: Sample size; s.e.m.: standard error of the mean.
[a]Genotype frequencies were in Hardy–Weinberg equilibrium (χ^2-test).
*Significant at $P<0.05$.

A confounding factor in association studies is the heterogeneity in allele frequencies as a result of the genetic substructure of the sample populations.[30,31] The genetic substructure of the black subjects in the HERITAGE Study is not well characterized. However, since the majority of participants were recruited in urban areas in the USA and Canada, it is assumed that the mean European admixture in this sample population would be consistent with reported admixture levels for other urban African Americans[32] and likely in the range of 10–25%. In contrast, the black Jamaicans and Gullah subjects have low levels of European admixture,[32] with the Gullahs having the lowest levels compared with any other US black population, at 3.5%.[33] We hypothesize that the genetic substructure in some black populations might impede the analytical approaches to examine genotype/phenotype interactions, thus making it difficult to compare populations with different admixture levels.

In the present study, we present new data that emphasize the functional role of the −38C>T SNP in promoter binding affinity, and identify the E12/E47 helix–loop–helix type of TFs as possible mediators of hAgRP transcriptional activation. In agreement with the functional observations *in vitro*, we found that the *TT* genotype of the −38C>T SNP was significantly associated with low BMI and adiposity *in vivo*, suggesting that it could play a protective role in the development of body fatness in black urban subjects.

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