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A missense mutation in the human melanocortin-4 receptor gene in relation to abdominal obesity and salivary cortisol

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Abstract

Aims/hypothesis. The melanocortin-4 receptor (MC4-R) regulates food intake and possibly energy expenditure, and the inactivation of the MC4-R by gene targeting results in obesity, a phenotype strongly associated with Type II (non-insulin-dependent) diabetes mellitus. In our study, we addressed the hypothesis that a *G→A* substitution at codon 103 (Val-103Ile) of the *MC4R* gene influences abdominal obesity, insulin, glucose, and lipid metabolism as well as circulating hormones, including salivary cortisol.
Methods. We genotyped the missense variant at codon 103 of the *MC4R* gene in 284 unrelated Swedish men born in 1944 by using polymerase chain reaction amplification followed by digestion with the restriction enzyme *Hinc*II.
Results. The frequency of allele *G* was 0.97 and 0.03 for allele *A*. The observed genotype frequencies were 95% and 5% for *G/G* and *G/A*, respectively. The heterozygotes had lower waist-to-hip ratio ($p = 0.023$) and trends for lower body mass index ($p = 0.054$) and abdominal sagittal diameter ($p = 0.095$) compared to *G/G* homozygotes. Moreover, *G/A* subjects had borderline lower serum leptin concentrations ($p = 0.087$) and total cholesterol ($p = 0.082$). The heterozygotes had also, in comparison to *G/G* subjects, significantly ($p < 0.01$) higher mean cortisol concentrations in the morning (21.4 vs 14.6 nmol/l), at ll:45 h (11.6 vs 7.0 nmol/l), 30 min after a standardized lunch (15.3 vs 8.0 nmol/l) and finally, 60 min after lunch (10.8 vs 6.7 nmol/l).
Conclusion/interpretation. These findings suggest that the missense mutation in the *MC4R* gene could contribute to the variability in body mass, abdominal fat distribution and leptin concentrations in the general population. Moreover, the *G/A* mutation exhibits evidence of associations with diurnal cortisol levels. [Diabetologia (2001) 44: 1335–1338]

Keywords Abdominal obesity, genes, melanocortin receptor, polymorphism, salivary cortisol.

Melanocortins are regulatory peptides formed by post-translational processing of pro-opiomelanocortin (POMC) and are known to have a broad range of physiological effects [1]. To date, five melanocortin (MC) receptor subtypes have been identified, cloned and characterized including the MC4-R [2–6]. MC4-R is a 332 amino acid seven-transmembrane G-protein linked receptor and is expressed essentially in the brain [5, 7]. The MC4-R contributes to the regulation of food intake and possibly energy expenditure [8]. The gene for human MC4-R is located on the long arm of chromosome 18 [9].

Recent animal studies suggest that inactivation of the MC4-R by gene targeting results in a maturity-onset obesity syndrome associated with hyperphagia, hyperinsulinaemia, and hyperglycaemia [10]. Moreover, MC4-R deficiency enhances caloric efficiency in mice, in a way similar to that seen in the agouti

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Abbrevations: BMI, body mass index; bp, base-pair; MC, melanocortin; MC4-R, melanocortin-4 receptor; PCR, polymerase chain reaction; POMC, pro-opiomelanocortin; WHR, waist-to-hip ratio.

obesity syndrome, which results from ectopic overexpression of agouti protein, an antagonist at the MC receptors [11].

A study of 306 extremely obese patients detected three obese probands with haploinsufficiency mutations in the *MC4R* gene [12]. In an additional study group of 186 obese individuals, they identified an additional haploinsufficiency carrier, bringing the total number of mutation-carrying obese patients identified to four [13]. Recent human studies have shown that *MC4R* mutations result in a syndrome of hyperphagic obesity [14], whose features closely resemble several of those of the phenotype reported previously in *MC4R* knockout mice [10]. To date, mutations in *MC4R* seem to be the most frequent, although heterogeneous, genetic cause of morbid obesity [15].

A restriction fragment length polymorphism at codon 103 (Val103Ile) of the *MC4R* gene has been described [16]. DNA sequencing analysis has made it possible to identify a $G \rightarrow A$ base substitution that results in a loss of a *Hinc*II restriction site [16]. In this study, we tested the hypothesis that this polymorphism influences abdominal obesity and estimates of insulin, glucose, and lipid metabolism as well as circulating hormones, including salivary cortisol.

Subjects and methods

Subjects. For this study, the subjects ($n = 284$) were randomly selected from a larger geographically defined total population cohort of men born in Gothenburg, Sweden, in 1944. The design has been described elsewhere [17, 18]. All subjects gave written informed consent before participating in the study, which was approved by the local ethics committee.

Phenotypic measurements. Body mass index (BMI, kg/m^2), waist-to-hip ratio (WHR), and abdominal sagittal diameter were measured as described previously [17, 18]. Salivary cortisol was measured repeatedly over a random working day. Endocrine measurements, beside cortisol, included testosterone, insulin-like growth factor I, and leptin as described previously [17, 18]. Insulin, glucose, triglycerides, and total, high- and low-density lipoprotein cholesterol were measured in the overnight fasting state as described previously [17, 18]. The serum lipids were determined by an enzymatic procedure in a Roche Molecular Biochemicals Cobas Fara II (Roche Molecular Biochemicals, Mannheim, Baden-Würtemberg, Germany).

Genotyping. Genotyping of the DNA sequence variation at codon 103 was carried out by PCR and restriction enzyme digestion. Genomic leukocyte DNA (150 ng in a final volume of 10μl) was amplified by PCR using the following primers: 5'-ATGGAGGGTGCTACGAGCAACT-3' (forward) and 5'-TCTGTACTGTTTAATAGGGTGTTG-3' (reverse) [16]. The primers amplified a product of 223 bp. The reverse primer has sequence mismatch (underlined) in order to create an artificial restriction site for *Hinc*II in the PCR product derived for the *G* allele. PCR conditions were one cycle at 95 °C for 3 min, 35 cycles at 95 °C for 15 sec, 50 °C for 15 sec and 72 °C for 45 sec with a final extension at 72 °C for 10 min. The PCR product was digested at 37 °C overnight with 10U of *Hinc*II (New England Biolabs, Beverly, Mass., USA), creating two fragments of 200 bp and 23 bp for the *G* allele. If the *A* allele was present, the *Hinc*II restriction site disappeared leaving a 223 bp fragment. The fragments were separated on a 3 % agarose gel.

Statistical analysis. All statistical analyses were done using SPSS for Windows, release 10.0 (SPSS, Chicago, Ill., USA). The results are presented as mean and standard deviation (SD). Data comparisons were carried out with the General Linear Model, with genotypes as independent factors and BMI and WHR as covariates. *P* values are two-sided throughout, and a *p* value of less than 0.05 was considered significant. All *p* values were adjusted for multiple tests by using Spjotvoll-Stoline post hoc correction [19].

Results

The frequency of allele *G* was 0.97 and 0.03 for allele *A*. The observed genotype frequencies were 95 % and 5 % for *G/G* and *G/A*, respectively. Table 1 presents the differences in anthropometric, endocrine, and metabolic measurements between the two *MC4R* genotypes. The heterozygotes ($n = 13$) had lower WHR ($p = 0.023$) and borderline lower BMI ($p = 0.054$) and abdominal sagittal diameter ($p = 0.095$) compared to *G/G* homozygotes ($n = 255$). In addition, *G/A* subjects tended to have lower serum leptin concentrations ($p = 0.087$) and lower total cholesterol ($p = 0.082$).

The differences in diurnal salivary cortisol measurements between the two *MC4R* genotypes are displayed in graph form in Figure 1. The heterozygotes had, in comparison to *G/G* homozygotes, ($p < 0.01$) higher mean cortisol concentrations in the morning (21.4 vs 14.6 nmol/l), at ll:45 h (11.6 vs 7.0 nmol/l), 30 min after a standardized lunch (15.3 vs 8.0 nmol/l) and finally, 60 min after lunch (10.8 vs 6.7 nmol/l).

Discussion

The examined men were selected from an ongoing cohort study, and 80 % volunteered to participate in the first part of the study. The second part, which was laboratory-based, attracted fewer participants but the non-responders were similar to the responders to such an extent that selection bias is thought to be negligible [17, 18].

Variation in human obesity-related phenotypes is known to be partly heritable and results from the additive and interactive effects of a substantial number of genes [20]. Some mutations in the *MC4R* gene predispose heterozygous carriers to obesity, most likely as a result of haploinsufficiency [14, 15]. However, the variable penetrance and expressivity of obesity in heterozygous subjects indicates that some of these mutations have little or no effect or that the *MC4R* gene acts in concert with a range of other genes to regulate energy balance [14, 15].

Table 1. Differences in anthropometric, endocrine and metabolic measurements between the two genotypes of the *G/A* polymorphism at codon 103 of the *MC4R* gene

	Genotypes		
	G/G (*n* = 255)	*G/A* (*n* = 13)	*p*[a]
Body mass index (kg/m^2)	26.3 (3.9)	24.1 (3.2)	0.054
Waist-to-hip ratio	0.94 (0.07)	0.89 (0.06)	0.023
Abdominal sagittal diameter (cm)	22.7 (3.7)	21.0 (2.3)	0.095
Testosterone (nmol/l)	19.6 (5.5)	21.1 (4.7)	> 0.20
Insulin-like growth factor I (μg/l)	205.5 (65.7)	206.6 (50.8)	> 0.20
Leptin (μg/l)	6.3 (4.3)	4.2 (2.3)	0.087
Fasting insulin (mU/l)	12.8 (11.1)	8.8 (2.7)	0.193
Fasting glucose (mmol/l)	4.6 (1.0)	4.4 (0.6)	> 0.20
Triglycerides (mmol/l)	1.8 (1.1)	1.4 (0.6)	0.163
Total cholesterol (mmol/l)	6.2 (1.1)	5.7 (1.0)	0.082
High-density lipoprotein cholesterol (mmol/l)	1.2 (0.3)	1.3 (0.4)	> 0.20
Low-density lipoprotein cholesterol (mmol/l)	4.1 (1.0)	3.7 (1.1)	0.132

Values are given as mean (SD)
[a]Adjusted for BMI and WHR

We examined a *G→A* substitution at codon 103 (Val103Ile) of the *MC4R* gene in 284 unrelated probands and found that *G/A* subjects had lower body mass and estimates of abdominal obesity. The allelic frequency of the substitution is in accordance with allelic frequencies observed in two other studies in Caucasian populations [14, 16]. However, the missense variant Val103Ile was not found to be associated with obesity in white British males [16]. The reasons for this inconsistent result are not clear. One reason for the disparity between our results and those of the previous study could be differences in the degree of obesity in the populations examined, as the range of BMI seemed to diverge [16]. The missense variant Val103Ile has previously been found in extremely obese but also in lean individuals [15]. Interestingly, heterozygous *MC4R* +/- mice also display a broad variability in adult weight [10].

The adipocyte hormone leptin circulates generally in proportion to body adiposity and triggers neuropeptide responses in the brain, which modulate appetite and energy expenditure [21]. Human obesity is characterized by increased circulating concentrations of leptin and 'leptin resistance' [22], which is exemplified in the extreme by the leptin receptor in *db* mice [21]. However, leptin resistance in humans is rarely due to a mutant leptin receptor, and abnormalities in the downstream targets of leptin such as the MC-4 receptor might mediate functional resistance to its action. In the present study, we found that *G/A* subjects had borderline significantly lower serum leptin concentrations compared to homozygotes for the *G* allele. In the same cohort, we have recently found that a microsatellite polymorphism in exon 3 of the *POMC* gene was associated with higher serum leptin concentrations (unpublished data). Given the association between leptin, POMC, and melanocortins, we investigated the potential interaction effects between the *Hinc*II *MC4R* polymorphism and the trinucleotide repeat *POMC* polymorphism. The results showed no interaction with the *G/A MC4R* variant for any of the phenotypes under study including leptin (data not shown).

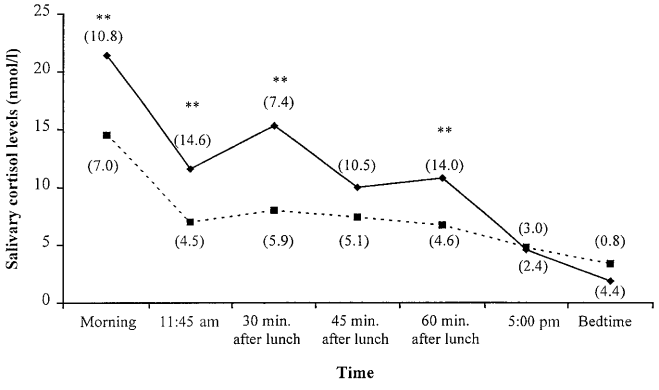

Fig. 1. Salivary cortisol levels (nmol/l) over the day. Lunch at 12:00 h. Results are given as arithmetic means and standard deviation (within parentheses). $^{**}p < 0.01$

We report here findings on the Val103Ile variant in the *MC4R* gene in relation to cortisol in humans. We assessed cortisol by measuring salivary cortisol concentrations under basal conditions and when challenged by a standardized lunch [17, 23]. The assessment of cortisol in saliva is specific for the detection of free cortisol, and cortisol in saliva reflects accurately the free fraction of cortisol in plasma [24]. The test is sufficiently sensitive to measure cortisol concentrations in normal subjects and to distinguish normal secretory patterns from hypocortisolism and hypercortisolism [25, 26]. From a practical point of view, the assessment of cortisol using saliva samples is less of a burden to the subjects and is also simple for the hospital staff to process and analyse. The result shows that *G/A* subjects had higher diurnal cortisol concentrations than homozygote subjects for the *G* allele. As noted above, the heterozygotes were

lean, despite the higher cortisol concentrations. One resolution for this seeming paradox is that the kinetics of the cortisol regulation have a stronger impact on the pathogenesis of obesity and related metabolic abnormalities than the total cortisol secretion in itself [24]. A high morning cortisol peak, a normal circadian rhythm along with a brisk cortisol response to lunch characterized the diurnal cortisol curve in *G/A* subjects. These features are all signs of an excellent regulatory function of the hypothalamic-pituitary-adrenal axis [24, 27].

In summary, our results indicate that a missense mutation in the *MC4R* gene is associated with lower body fat mass, abdominal fat and serum leptin concentrations accompanied by increased salivary cortisol concentrations. In a recent study comparing the activity of the *G→A* mutated *MC4-R* to that of the wild-type receptor a similar dose-response pattern emerged [28]. This suggests that the Val103Ile missense mutation does not impair the function of the MC4-receptor and it is not clear what functional role the mutated receptors have, if any. However, it could indicate functionally important polymorphisms in other neighbouring regions of the gene, including the promoter region, which could be in linkage disequilibrium with the *Hinc*II polymorphism.

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