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A novel missense mutation T101N in the melanocortin-4 receptor gene associated with obesity

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ABSTRACT. Mutations in the melanocortin-4 receptor (MC4R) are associated with severe obesity, independent of their effect on cortisol or thyroid-stimulating hormone levels. We examined a morbidly obese male (BMI = 62 kg/m^2) with a binge-eating disorder and eight family members for mutations in the MC4R gene and potential differences in leptin levels. Fifty healthy individuals served as controls. Sequence analysis revealed a novel heterozygous missense mutation (c.302 C>A, p.T101N) located in the second transmembrane domain of the receptor, which was not detected in controls. The Fisher exact test revealed an association between the T101N mutation and history of obesity ($P < 0.05$) in the family. The Kruskal-Wallis test showed an association between the mutation and the leptin/BMI ratio ($P < 0.05$), while there was no association between the T101N mutation and diabetes or arterial hypertension in the family. Although the available family was small, we could show a significant association between the heterozygous T101N mutation and obesity.

Key words: MC4R gene; BMI; Leptin; Obesity

INTRODUCTION

Obesity is a multifactorial disease with complex interactions between genetic predisposition and environmental factors. Mutations in the melanocortin-4 receptor (MC4R) are the commonest monogenic cause of human obesity with 1.6-5.8% prevalence and autosomal dominant heredity (Vaisse et al., 2000; Branson et al., 2003; Farooqi et al, 2003; Larsen et al., 2005; Rettenbacher et a., 2007; Wangensteen et al. 2009). Carriers are afflicted with hyperphagia, hyperinsulinemia and increased fat mass with normal cortisol, gonadotropin, thyroid and sex steroid levels (Farooqi et al. 2003). At least 95 mutations in the MC4R are associated with obesity, comprising nonsense, missense and frameshift mutations. The quantitative effect of MC4R mutations on body weight is so far unknown.

Binge-eating disorder occurs in 2.5% of non-obese subjects, but in 30% of individuals with obesity (Hsu et al., 2002). In a study with 300 severely obese patients undergoing laparoscopic banding, 19 carriers of MC4R mutations (6.3%) were identified (Potoczna et al., 2004). All MC4R variant carriers suffered from binge-eating disorder compared with 18.1% of noncarriers. MC4R mutation carriers lost less weight, showed less improvement in metabolic syndrome, had a dilated esophagus, and had 5-fold more gastric complications than did noncarriers.

MC4R is a 332-amino acid protein encoded by a single exon. It is a seven-transmembrane G protein-coupled receptor expressed in the hypothalamic nuclei, which are important in the regulation of food intake and body weight (Huszar et al. 1997). The receptor signals through activation of adenylate cyclase in response to its agonist, the satiety neuropeptide α-melanocyte-stimulating hormone (α-MSH) (Fan et al., 1997). MC4R signaling is modulated by the antagonist agouti-related protein, which is also expressed in the hypothalamic arcuate nucleus (Mountjoy et al., 1994). MC4R is also a target of leptin, which acts through the activation and inhibition of pro-opiomelanocortin (POMC) and agouti-related protein. The present report describes a novel missense mutation in a patient with severe adiposity and the clinical impact on the context of his pedigree.

MATERIAL AND METHODS

Index patient

A 27-year-old male patient developed, after normal development, binge-eating syndrome and severe obesity at 10 years of age. At 18 years of age, type 2 diabetes became manifest. Today, the patient [body mass index (BMI) = 62 kg/m^2] is afflicted with diabetic nephropathy, neuropathy and diabetic foot syndrome.

Classification of normal glucose tolerance, impaired fasting glycemia or type 2 diabetes was performed according to World Health Organization (WHO) and American Diabetes Association (ADA) criteria (Alberti et al., 1998).

Mutation screening

The index patient and available family members were tested for mutations in the

MC4R gene after informed consent was given. Fifty non-obese individuals served as controls. Genomic DNA was extracted from whole blood using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) following manufacturer instructions. Approximately 50 ng DNA was amplified with the primers 1F 5'-CCCTGACCCAGGAGGTTAAA-3', 1R 5'-CTGTGCATCCGTATCTGTAC-3', 2F 5'-GGCTGATATGCTGGTGAGCG-3', 2R 5'-GAACATGTGGACATAGAGAG-3', 3F 5'-CTCAGATAGTAGTGCTGTC-3', and 3R 5'-TGCAGAAGTACAATATTCAGG-3'.

Reactions were performed in a total volume of 50 µL containing 10X polymerase chain reaction (PCR) buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 3 mM $MgCl_2$, 1.25 mM dNTPs, 10 pM of each primer and 2 U Taq DNA polymerase (Fermentas Life Sciences, St. Leon-Rot, Germany). The gel-verified PCR products were purified using QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer. Sequencing reactions utilized the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystem, Foster City, CA, USA). Dye-terminator removal was done using Dye Ex™ 2.0 Spin Kit (Qiagen) following manufacturer instructions. Analysis was performed on the ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems). A published wild-type sequence was used as reference sequence (accession No. AY236539).

Laboratory analysis

Serum and EDTA plasma samples from the index patient were tested for cortisol and ACTH levels by Modular Analytics (Roche Diagnostics). TSH concentrations were measured by Advia® 2400 Chemistry System (Bayer HealthCare Diagnostics, Germany). Serum samples from the index patient and available family members were tested for leptin levels by ELISA (Mediagnost, Reutlingen, Germany).

Statistical analysis

Statistical analysis was carried out using the SAS system software, version 9.1 (SAS Institute Inc., Cary, NC, USA). The Fisher exact test was used to detect possible associations between MC4R mutations and BMI, type 2 diabetes and hypertension. The Kruskal-Wallis test was performed to find a possible correlation between MC4R mutations and leptin/BMI ratio. The statistical significance level was determined as $P < 0.05$.

RESULTS

Cortisol, ACTH and TSH levels from the index patient were within the normal range. Sequencing analysis of the MC4R gene revealed a heterozygous missense mutation (c.302 C>A, p.T101N) located in the second transmembrane domain of the receptor (Figure 1).

The mutation has not been previously described and was not detected in 50 controls. A pedigree analysis was performed (Figure 2). The parents and the sister of the index patient suffer from type 2 diabetes, and the mother and the sister are obese. The index patient, his sister and his mother are afflicted with arterial hypertension. His mother carries the T101N mutation. The grandmother on the mother's side died because of breast cancer at 67 years,

and the grandfather on the mother's side died of unknown causes at 58 years. The two aunts and the first cousin carrying the T101N mutation are overweight, but do not have diabetes and hypertension. None of the examined family members are affected with binge-eating disorder. The clinical details are given in Table 1.

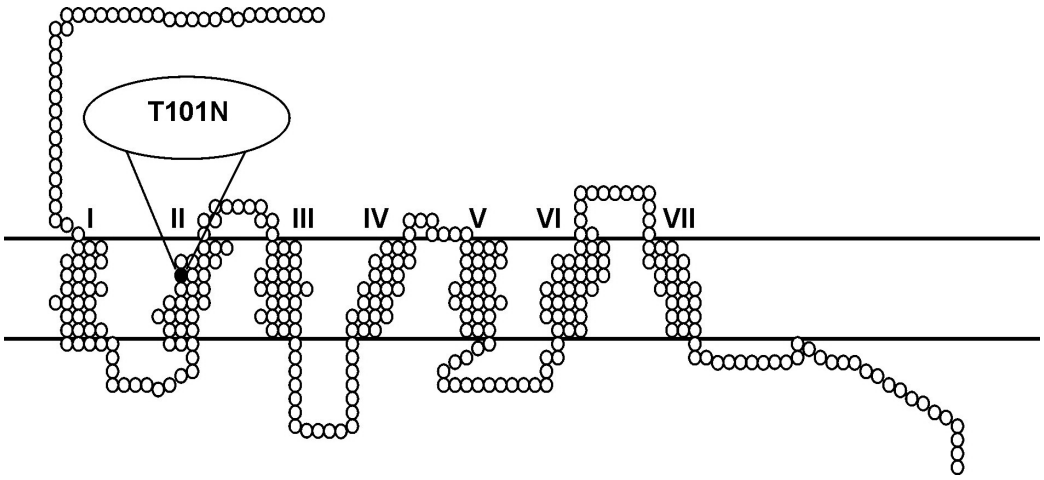

Figure 1. Schematic representation of the human MC4R and location of the novel T101N missense mutation in the second transmembrane domain. The seven transmembrane domains are indicated by Roman numerals.

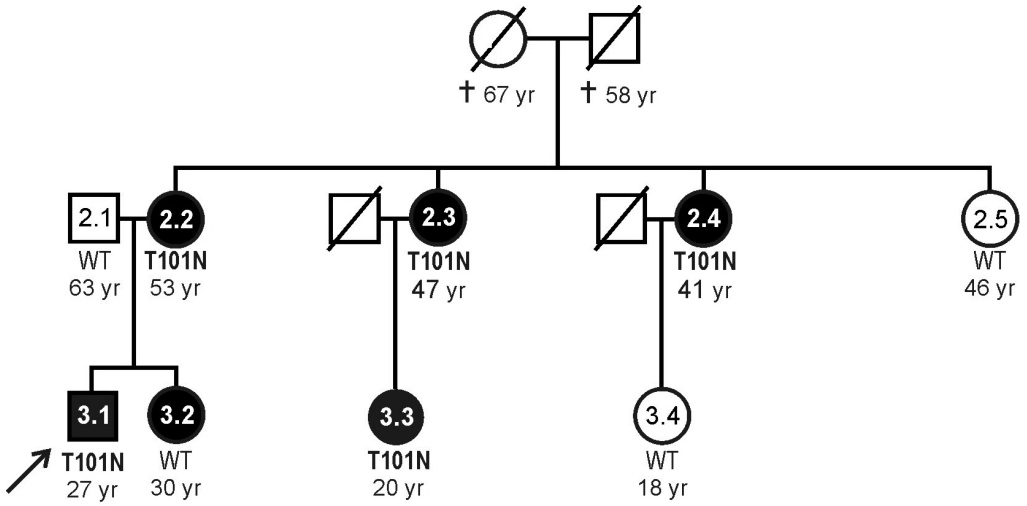

Figure 2. Pedigree of the family examined. Incidence of the heterozygous T101N missense mutation and the current age (years) are shown. The arrow indicates the index patient (3.1). Black symbols = history of obesity. WT = wild-type carriers.

Table 1. Clinical details of the index patient (3.1) and available family members shown in Figure 2.

ID	History of obesity	MC4R mutation	BMI (kg/m^2)	Leptin (ng/mL)	Leptin/BMI x100	Type 2 diabetes	AD	Diabetic complications
3.1	Yes	T101N	62	90.4	145.8	Yes	18	Nephropathy, neuropathy, foot syndrome
3.3	Yes	T101N	30	50.7	169.0	No		
2.2	Yes	T101N	31	17.4	56.1	Yes	28	None
2.3	Yes	T101N	26	19.2	73.8	No		
2.4	Yes	T101N	25	36.6	146.4	No		
3.2	Yes	WT	35	36.0	102.8	Yes	24	None
3.4	No	WT	22	8.39	38.1	No		
2.1	No	WT	26	12.6	48.5	Yes	45	Retinopathy
2.5	No	WT	29	15.3	52.8	No		

BMI = body mass index; AD = age at diagnosis of diabetes, WT = wild-type carriers.

The Fisher exact test revealed an association between the T101N mutation and history of obesity. The correlation with diabetes and arterial hypertension was not statistically significant. The Kruskal-Wallis test showed a significantly increased leptin/BMI ratio in the T101N mutation group compared to the wild-type carriers (118.2 ± 49.9 *vs* 60.5 ± 28.8; mean ± SD) ($P < 0.05$).

DISCUSSION

The novel T101N mutation identified is located in the second transmembrane domain of the receptor, near the conserved amino acid E100, which is crucial for α-MSH binding and signaling (Chen et al., 2007). Codon 101 is a highly conserved amino acid among species. MC4R is a structural and functional conserved protein within 450 million years of vertebrate evolution. More than 90% of all inactivating mutations found in obese patients are located at amino acid positions that are highly conserved (Stäubert et al., 2007). Several mutations are described in the second transmembrane domain in association with obesity, but only in a few cases have pedigree analyses been performed. Kobayashi et al. (2002) described a homozygous G98R mutation in a Japanese woman. She gained weight progressively from 10 months of age, and at 40 years of age her BMI reached 62 kg/m^2. Her parents, who were heterozygous for the mutation, had BMIs of 26 and 27 kg/m^2. Dubern et al. (2007) described a homozygous 2-bp deletion (del 346-347AG) leading to a stop codon and a truncated receptor behind the second transmembrane domain in a 3-year-old boy with severe early-onset obesity. He was hyperphagic and showed a rapid increase in weight in the first months of life. Heterozygous carriers of the mutation (2 parents and 2 adult relatives) displayed a variable degree of severity, two were overweight and two were obese. Patients harboring loss-of-function MC4R mutations do not always exhibit obesity. The mutation I102T characterized as loss-of-phenotype *in vitro* was detected in obese and non-obese individuals (Tao and Segaloff, 2005).

The index patient in this family examined with the T101N mutation is afflicted with hyperphagia and severe obesity. The wide range of the T101N phenotype in this family is explained by the fact that the mutation occurred in heterozygous expression, and thus the affected family members displayed an intermediate phenotype with individual differences. In family studies based on obese index patients, mutation carriers were identified who were moderately overweight or even lean. Transmission of heterozygous MC4R mutations in families of carriers led to variable expressivity that was not correlated to the functional severity of the mutations (Vaisse et al., 2000). The reason for the variability in penetrance and expressivity of MC4R mutations is yet to be elucidated.

As in the family examined here, wild-type relatives of severe obese mutation carriers are also often obese (Dubern et al., 2001). A clinical study comprising 181 phenotypically unselected relatives of extremely obese index patients from 25 pedigrees revealed per-family-differences from 2.02 to 2.7 standard deviation score (SDS) in current BMI SDS between mutation carriers and noncarriers (Dempfle et al., 2004). In 2257 obese individuals and their family members and in 2677 non-obese controls, 108 individuals harboring loss-of-function mutations were identified (Stutzmann et al., 2008). The mutations were found in 1.72% of obese and in 0.15% of non-obese subjects. An age-related penetrance of 40% was observed in MC4R-deficient adults aged >52 years, 60% in 18- to 52-year-old adults and 79% in children.

In this family, the heterozygous T101N mutation was associated with a history of

obesity or current obesity, suggesting a strong influence of the T101N mutation on the phenotype. The T101N mutation was furthermore significantly associated with increased leptin levels. Leptin is secreted by adipocytes as an afferent satiety signal, produced in proportion to the mass of the adipose tissue. Human studies have demonstrated a close association between body fat and plasma leptin levels (Weigle et al., 1995; Halaas et al., 1995; Levin et al., 1996). As leptin levels were not found to be statistically different between MC4R-mutated and non-mutated obese groups, the increase in leptin levels seems to be an unspecific response without any causal relationship to the T101N mutation in the present family.

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