
Brief Genetics Report

Mutational Screening of the CART Gene in Obese Children

Identifying a Mutation (Leu34Phe) Associated With Reduced Resting Energy Expenditure and Cosegregating With Obesity Phenotype in a Large Family

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Cocaine- and amphetamine-regulated transcript (CART) inhibits feeding and induces the expression of *c-Fos* in hypothalamic areas implicated in appetite regulation. Furthermore, the CART peptide is found in neurons regulating sympathetic outflow, which in turn play an integral role in regulating body temperature and energy expenditure. The CART gene was screened by single-strand conformation polymorphism and automatic sequencing in 130 (72 girls) unrelated obese Italian children and adolescents. Their *Z*-scores (mean \pm SD) of relative to BMI percentiles was 3.9 ± 1.8 , and the average age at obesity onset was 4.7 ± 2.6 years. Two previously described silent polymorphisms were found in the 3' untranslated region: an adenine deletion at position 1457 in 9 patients (allele frequency 0.035) and an A/G substitution at position 1475 in 11 patients (allele frequency 0.042). We found no difference between the obese patients heterozygous for one of these polymorphisms and those patients homozygous for the wild allele with respect to their age of obesity onset, BMI *Z*-scores, and leptin levels. A missense mutation of G729C resulting in the substitution of Leu with Phe at codon 34, within the NH₂-terminal CART region, has been detected in the heterozygous state in a 10-year-old obese boy who has been obese since the age of 2 years. The patient belongs to a large family of obese subjects. The mutation cosegregated with the severe obesity phenotype over three generations and was not found in the control population. Resting metabolic rates were lower than expected in the proband (-14%) and his mother (-16%), who carried the mutation. Leucine at codon 34, conserved in this position in the human and in the rat sequences, immediately precedes a couple of lysine residues that may well represent a dibasic pro-

cessing site. The Leu34Phe mutation might alter the susceptibility to proteolysis of this potential processing site, likely altering the CART effect on thermogenesis and energy expenditure. *Diabetes* 50:2157–2160, 2001

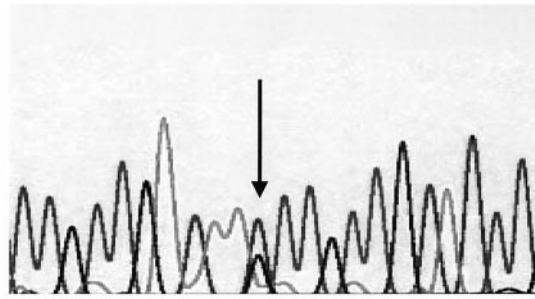
Food intake is regulated via neural circuits located in the hypothalamus. Over the last few years we have gained a good understanding of the specific mediators and neuronal networks that regulate food intake and body weight (1,2). Cocaine- and amphetamine-regulated transcript (CART) was originally described as an mRNA induced in the brain after acute administration of cocaine to rats; it has recently been shown that intracerebroventricular administration of this neuropeptide inhibits feeding and induces the expression of *c-Fos* in several nuclei involved in the regulation of food intake (3). Furthermore, an antiserum against CART increases feeding in normal rats, suggesting that CART may be an endogenous inhibitor of food intake in normal animals (4). This action is modulated by leptin (4,5). In obese animal models with disrupted leptin signaling, CART mRNA is almost absent from the arcuate nucleus (4). Peripheral administration of leptin to obese mice stimulates CART mRNA expression (4). Moreover, in the lateral arcuate nucleus, there is a population of leptin-activated cells containing CART and directly innervating sympathetic preganglionic neurons in the thoracic spinal cord (5). These neurons regulate interscapular brown adipose tissue, which plays an integral role in regulating body temperature, energy expenditure, and diet-induced thermogenesis (5). Because leptin mediates many of its physiological effects by increasing the activity of the sympathetic nervous system, it is conceivable that the engagement of this novel pathway may contribute to the increased thermogenesis and energy expenditure characteristic of leptin action. The detection of CART-like immunoreactivity in sympathetic ganglia and adrenal glands of the rat has further supported the idea that the CART peptide(s) functions as a signaling molecule in the sympathetic nervous system (6–8). The

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CART, cocaine- and amphetamine-regulated transcript; SSCP, single-strand conformation polymorphism; UTR, untranslated region.



Wild:	AA GAA GTC TTG AAG AAG CTC AA
	32 33 34 35 36 37
	Glu Val Leu Lys Lys Leu
	Glu Val <u>Phe</u> Lys Lys Leu
Mutated:	AA GAA GTC <u>TTC</u> AAG AAG CTC AA
	32 33 34 35 36 37

FIG. 1. CART exon 2 partial double-stranded sequencing and deduced amino acid sequences of patient IV-1. The arrow indicates the position of the G/C mutation. The base substituted and the relative amino acid are underlined.

aim of this study was to investigate whether variation in the CART gene may contribute to the variation of the clinical phenotype in Italian children and adolescents with early onset obesity.

We examined 130 (72 girls) unrelated obese Italian children and adolescents aged (mean \pm SD [range]) 10.7 ± 3.1 (2–16) years. The mean of their Z-score relative to BMI percentiles (9) was 3.9 ± 1.8 (2.1–8.8), and the average age at obesity onset was 4.7 ± 2.6 (1–11) years.

The CART gene was screened by single-strand conformation polymorphism (SSCP). Automatic sequencing of PCR products showing an aberrant pattern of migration was performed. The amino acid numbering is in agreement with Kuhar and Dall Vechia (10), and nucleotide positions are numbered according to Douglas and Daoud (11). Two previously described silent polymorphisms were found in the 3' untranslated region (UTR): a deletion of A at position 1457 in 9 patients (allele frequency 0.035) and the change of A with G at position 1475 in 11 patients (allele frequency 0.042). A similar allelic frequency (0.041 for the mutation at position 1457 and 0.039 for the mutation at position 1475) was found by screening 100 unrelated lean control subjects.

A missense mutation of G729C, TTG→TTC, resulting in the substitution of Leu with Phe at the codon 34 (Fig. 1), within the NH₂-terminal CART region has been detected in the heterozygous state in a 10-year-old obese boy who has been obese since the age of 2 years (Fig. 2). His birth weight was normal. His BMI at age 10 years was 32 kg/m²

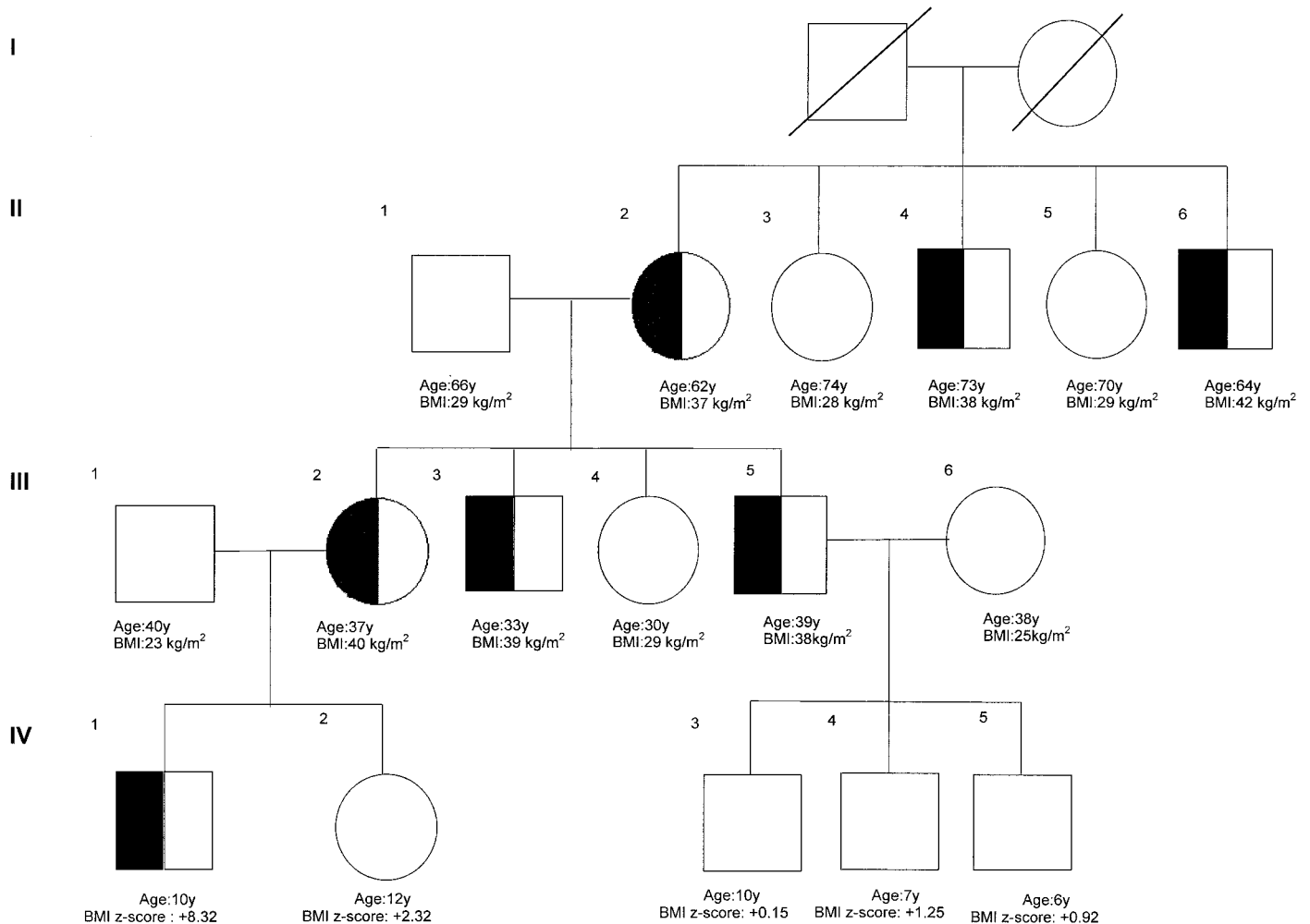


FIG. 2. Pedigree and clinical characteristics of the family members of the patient (IV-1) showing the G729C mutation. Age and BMI are indicated for each individual (BMI Z-score only for children). Half-filled symbols indicate the subjects heterozygous for this mutation.

TABLE 1

Some characteristics of the phenotype of 110 obese children homozygous for the CART wild allele, 11 patients heterozygous for the A1475G variant, and 9 obese children heterozygous for the CART allele with the deletion of adenine at position 1457

	Homo- zygotes (wild allele)	Hetero- zygotes A1475G	Hetero- zygotes Adel1457	<i>P</i>
<i>n</i>	110	11	9	
Obesity onset (years)	4.6 ± 2.6	3.8 ± 1.9	5.5 ± 3	NS
BMI (Z-score)	3.7 ± 2.06	3.8 ± 1.9	4.1 ± 2.4	NS
Leptin (Z-score)	0.7 ± 2.4	1.1 ± 1.9	0.9 ± 2.2	NS

Data are means ± SD. Both groups of heterozygotes were compared with homozygotes by unpaired *t*-test.

(weight 60 kg, height 142 cm, Z-score 8.3). Serum leptin concentration (23 ng/ml) was adequate for BMI, sex, and pubertal development (12) (leptin Z-score 0.5). The patient belongs to a large family of obese subjects (Fig. 2). His mother and many components of the maternal kindred had a very similar history of early onset obesity. His father, on the contrary, was not obese. To test for the inheritance of this missense mutation in the maternal proband's family, exon 2 of the CART gene was sequenced. The mutation cosegregated with the severe obesity phenotype over three generations. Subjects II-2, -4, and -6 have type 2 diabetes. The mutation was not found in the control population. Comparing resting metabolic rates with those expected after adjusting energy expenditure for age, sex, weight, height, and an age-adjusted value for urinary nitrogen, we found a difference of -230 Kcal/24 h (-14%) and -240 Kcal/24 h (-16%) for the patients III-2 and IV-1. We also found differences of +50 Kcal/24 h (+4%), -40 Kcal/24 h (-4%), and +50 Kcal/24 h (+6%) for the normal subjects IV-2, IV-3, and IV-4, respectively.

Although the CART gene has recently been localized in the 5q13-14 locus (13), a known human obesity locus (14), three mutational screenings performed in Danes with early onset obesity (13), in a group of obese Pima Indians (15), and in 91 severely obese Caucasian children (16) failed to detect genetic variations clearly contributing to the obesity phenotype. The only mutation found in the coding region, Ser39Thr, did not cosegregate with obesity in family studies (16). Three polymorphisms in the 3' UTR region have been identified (A1475G, 1457delA, and, among Pima Indians, C1442G). The prevalence of these variants was determined among obese and control subjects, but no associations between these alleles and variations of BMI were demonstrated. However, it is noteworthy that in one study, the A1475G variant has been associated with a lower waist-to-hip ratio in male A/G heterozygotes (16). When comparing the age of obesity onset, BMI Z-scores, and leptin levels adjusted for BMI, sex, and pubertal stage (leptin Z-scores), we found no difference between the obese patients heterozygous for one of these polymorphisms and those homozygous for the wild allele (Table 1), further eliminating the possibility that these mutations are a significant source of obesity variation.

CART is a neuropeptide that is secreted and processed. This idea is based on the identification of a hydrophobic leader sequence at the NH₂-terminus, which suggests involvement in a secretion pathway, and on the presence

of several pairs of basic amino acids commonly found in propeptides that are processed before their subsequent use (10,11). In the rat, the CART precursor has been found to be processed differently in central and peripheral sites. The CART long-form peptides (1-89 and 10-89) were isolated from the adrenal gland, and they most likely represent the CART peptides present in the adrenal medulla, in contrast to the hypothalamus, from which the shorter-form peptides (42-89 and 49-89) were purified (17,18). Although the involvement of central CART in the regulation of energy expenditure is possible (CART is also found in the hypothalamic neurons innervating the sympathetic preganglionic neurons), this tissue-specific processing suggests that different CART peptides may have different biological functions in the periphery (energy expenditure increase) and in the central nervous system (food intake inhibition). However, because of the limitations of the techniques used in these studies (i.e., antibody against the COOH-terminal part of the CART precursor), it has not been possible to determine whether further processing of the NH₂-terminal part of CART (1-39) takes place in the periphery. Leucine at codon 34, conserved in this position in the human and rat sequences, immediately precedes a couple of lysine residues that may well represent a dibasic processing site. The substitution of a phenylalanine for the wild-type leucine in this position might alter the susceptibility to proteolysis of a potential processing site. Alternatively, this mutation might produce a perturbation of the protein's three-dimensional structure large enough to increase the sensitivity to proteolysis; this increase in sensitivity might even occur at sites distant from the mutation itself, also affecting, in this case, the activity of central CART peptides.

In agreement with results from resting metabolic rates in the proband and his mother, we suggest that the Leu34Phe mutation may play a role in contributing to the obese phenotype by altering the CART mediated leptin effect on thermogenesis and energy expenditure.

RESEARCH DESIGN AND METHODS

A blood sample was drawn for each patient after an overnight fast. The serum was frozen at -10°C until analyzed. A double-antibody radioimmunoassay (Human Leptin RIA Kit; Linco Research, St. Charles, MO) for serum leptin measurement was used. The sensitivity cutoff for the leptin assay was 0.5 ng/ml; the intra- and interassay variation coefficients were 10.8 and 7%, respectively. Serum leptin levels were adjusted for BMI using reference ranges stratified according to sex and pubertal development (12). The deviation from the mean reference value was evaluated by calculating Z-scores.

Genomic DNA was collected from nucleated white blood cells. CART exons 1 and 2 were PCR-amplified with two pairs of primers placed within the flanking intronic regions: CART1 F (5'-ACTATAAAAGGTGGGAGCGC-3') and CART1 R (5'-ACACGTCAAGGGGGTTCGAGA-3') were used to amplify a 248-bp fragment; CART2 F (5'-AGCGCAACTTCAGGCTCCG-3') and CART2 R (5'-GGGTGTACTTCTCTGGA-3') were used to amplify a 173-bp fragment; exon 3 was PCR-amplified with a sense intronic primer (CART3 F: 5'-TGGGTTTGTTCATCTGCAT-3') and an antisense primer within the 3' UTR (CART3 R: 5'-TGCTTAAGCCAACTCCAGG-3') to generate a 237-bp fragment.

PCRs were carried out using the following conditions: denaturation at 95°C for 5 min followed by 35 cycles of 30 s at 94°C, 30 s at 58°C (CART1) or 54°C (CART2 and CART3), and 30 s at 72°C. PCR products were analyzed for the SSCP; after a 5-min denaturing, PCR products were loaded into Hydrolink MDE gel (BioWhittaker Molecular Applications, Rockland, ME). Gels were silver-stained, and those PCR products showing an aberrant SSCP pattern were subjected to bi-directional sequencing using Big Dye terminator and electrophoresed on an automatic sequencer (ABI Prism 310; Perkin Elmer, Foster City, CA).

Resting energy expenditure was measured in the proband, his mother, and

three family members without the mutation (patients IV-1 and III-2 and subjects IV-2, IV-3, and IV-4) after an overnight fast by open-circuit indirect calorimetry (Deltatrac Metabolic Monitor; Sormometrics, Yaba Hinde, CA) and expressed as kilocalories per 24 h. Resting metabolic rates were measured using the same machine, calibrated before each measurements with calibration gas, and the same protocol, and the results were compared with the expected metabolic rates predicted by the Schofield equations (19). The percentages of deviation were calculated dividing the measured metabolic rate by the predicted one.

Values are given as the means \pm SD. Student's unpaired *t* test was used for statistical comparisons.

REFERENCES

- Barsh GS, Farooqui IS, O'Rahilly S: Genetics of body-weight regulation. *Nature* 404:644–651, 2000
- Schwartz MW, Woods SC, Porte D Jr, Seeley RJ, Baskin DG: Central nervous system control of food intake. *Nature* 404:661–671, 2000
- Vrang N, Larsen PJ, Kristensen P, Tang-Christensen M: Central administration of cocaine-amphetamine-regulated transcript activates hypothalamic neuroendocrine neurons in the rat. *Endocrinology* 141:794–801, 2000
- Kristensen P, Judge ME, Thim L, Ribel U, Christjansen KN, Wulff BS, Clausen JT, Jensen BB, Madsen OD, Vrang N, Larsen PJ, Hastrup S: Hypothalamic CART is a new anorectic peptide regulated by leptin. *Nature* 393:72–76, 1998
- Elias CF, Lee C, Kelly J, Aschkenasi C, Ahima RS, Couceyro PR, Kuhar MJ, Saper CB, Elmquist JK: Leptin activates hypothalamic CART neurons projecting to the spinal cord. *Neuron* 21:1375–1385, 1998
- Koylu EO, Couceyro PR, Lambert PD, Ling NC, DeSouza EB, Kuhar MJ: Immunohistochemical localization of novel CART peptides in rat hypothalamus, pituitary and adrenal gland. *J Neuroendocrinol* 9:823–833, 1997
- Dun NJ, Dun SL, Kwok EH, Yang J, Chang J: Cocaine- and amphetamine-regulated transcript-immunoreactivity in the rat sympatho-adrenal axis. *Neurosci Lett* 283:97–100, 2000
- Bray GA: Reciprocal relation of food intake and sympathetic activity: experimental observations and clinical implications. *Int J Obes Relat Metab Disord* 24 (Suppl. 2):S8–S17, 2000
- Frisancho AR: *Anthropometric Standards for the Assessment of Growth and Nutritional Status*. Ann Arbor, MI, The University of Michigan Press, 1990
- Kuhar MJ, Dall Vechia SE: CART peptides: novel addiction- and feeding-related neuropeptides. *Trends Neurosci* 22:316–320, 1999
- Douglas J, Daoud S: Characterization of the human cDNA and genomic DNA encoding CART: a cocaine- and amphetamine-regulated transcript. *Gene* 169:241–245, 1996
- Blum WF, Englaro P, Hanitsch S, Juul A, Hertel NT, Muller J, Skakkebaek NE, Heiman ML, Birkett M, Attanasio AM, Kiess W, Rascher W: Plasma leptin levels in healthy children and adolescents: dependence on body mass index, body fat mass, gender, pubertal stage and testosterone. *J Clin Endocrinol Metab* 82:2904–2910, 1997
- Echwald SM, Sorensen TIA, Andersen T, Hansen C, Tommerup N, Pedersen O: Sequence variants in the human cocaine and amphetamine-regulated transcript (CART) gene in subjects with early onset obesity. *Obes Res* 7:532–536, 1999
- Hager J, Dina C, Francke S, Dubois S, Houari M, Vatin V, Vaillant E, Lorentz N, Basdevant A, Clement K, Guy-Grand B, Froguel P: A genome-wide scan for human obesity genes reveals a major susceptibility locus of chromosome 10. *Nat Genet* 20:304–308, 1998
- Walder K, Morris C, Ravussin E: A polymorphism in the gene encoding CART is not associated with obesity in Pima Indians. *Int J Obes Relat Metab Disord* 24:520–521, 2000
- Challis BG, Yeo GSH, Farooqi S, Luan J, Aminian S, Halsall DJ, Keogh JM, Wareham NJ, O'Rahilly S: The CART gene and human obesity: mutational analysis and population genetics. *Diabetes* 49:872–875, 2000
- Thim L, Kristensen P, Nielsen PF, Wulff BS, Clausen JT: Tissue-specific processing of cocaine- and amphetamine-regulated transcript peptides in the rat. *Proc Natl Acad Sci U S A* 96:2722–2727, 1999
- Kuhar MJ, Yoho LL: CART peptide analysis by western blotting. *Synapse* 33:163–171, 1999
- Schofield WN: Predicting basal metabolic rate, new standards and review of previous work. *Hum Nutr Clin Nutr* 39 (Suppl. 1):5–42, 1985