Molecular analysis of the CART gene in overweight and obese Italian children using family-based association methods

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Abstract

Aim: In our study, we evaluated if CART gene A1475G and ΔA1457 polymorphisms could be associated with obesity.

 Patients and methods: We recruited 133 Italian trios from among 103 (50 males and 53 females) overweight children (mean age 10.5 years, range 6–14 years; mean BMI 26.1 ± 3.2 kg/m²), and 30 (16 males and 14 females) obese children (mean age 9.0 years, range 6–11 years; mean BMI 32.3 ± 2.0 kg/m²). We also selected 187 non-obese unrelated controls.

 Results: The allele frequencies of the A1475G single nucleotide polymorphism (SNP) were significantly higher in overweight children (0.07) than in control children (0.02) (p = 0.03) and control adults (0.02) (p = 0.02). Moreover, the allele frequencies were significantly different between obese children (0.08) and control children (0.02) (p = 0.03), and between obese children (0.08) and control adults (0.02) (p = 0.02). The ΔA1457 SNP showed any significant association with overweight/obesity. TDT statistic revealed a preferential transmission of the 1475G allele from heterozygous parents to overweight children (p < 0.01) and to obese children (p < 0.05). No statistically significant excess transmission of the ΔA1457 allele was found.

Conclusion: Our results supported the hypothesis that inherited variations of the CART gene could influence the development of obesity also in Italian children.

INTRODUCTION

Obesity is a typical common multifactorial disease in which environmental and genetic factors interact (1,2). Until recently, however, little precise information regarding genetic causes of human obesity has been available. In the past 10 years, five monogenic defects causing human morbid obesity have been identified, namely, mutations in the genes encoding leptin (3,4), leptin receptor (5), prohormone convertase-1 (6), pro-opiomelanocortin (7) and the melanocortin-4 receptor (8,9). In all of these syndromes, hyperphagia results from dysfunction of hypothalamic pathways controlling satiety. Genetic defects in these molecules are responsible for only a small fraction of human morbid obesity, and it is likely that the detailed elucidation of the molecular mechanisms of appetite control will provide further candidate genes that may be implicated in human obesity. One such molecule is cocaine- and amphetamine-regulated transcript (CART), a hypothalamic neuropeptide (10). The role of CART in the obesity was demonstrated when recombinant CART injected intracerebroventricularly into rats inhibited feeding (11,12).

The human CART gene is encoded in a 2.5-kb segment of genomic DNA on chromosome 5 (13). The 900-nucleotide CART transcript is contained within three exons and encodes a mature peptide 89 amino acids in length (13). Two common SNPs were studied within the 3'-untranslated region (3'-UTR) of exon 3 of the CART gene: the A1475G and the ΔA1457 genetic variants. The A1475G mutation occurred in 39 base pairs (bp) 3' from the stop codon. The ΔA1457 variant involved the deletion of an adenine at position 1457, 21 nucleotides 3' from the stop codon (14).

The aim of this study was to estimate the prevalence of the CART gene A1475G and ΔA1457 SNPs in a group of 103 Italian overweight children and 30 obese children and to assess whether these mutations cosegregate with obesity. We used a case-parent trio design to overcome potential confounding as a result of population stratification, and we evaluated allele transmission using the transmission disequilibrium test (TDT).

METHODS

Subjects

We used a family-based and a case-control study. A group consisting of 133 trios was recruited from among 103 (50 males and 53 females) overweight children and 30 obese children (16 males and 14 females) attending our Department of Pediatrics (University Hospital, Messina, Southern Italy). The mean age of the overweight patients was 10.5 (6–14) years, and the mean age of the obese children was 9.0 (6–11) years. The mean age at overweight onset was 7.5 ± 2.1 (5.0–10.0) years and the mean age at obesity onset.
was 7.8 ± 3.2 (4.0–11) years (Table 1). The children were recruited during the years 2007 and 2008.

We also selected 187 non-obese unrelated controls. In this group, 90 children (41 boys and 49 girls) were studied and their median age was 11 (6–15) years. The median age of the remaining 97 adult controls (50 men and 47 women) was 38 (33–41) years. The mean BMI (SD) was 26.1 (3.2) kg/m² for the overweight children, 32.3 (2.0) kg/m² for the obese group and 18.2 (2.10) kg/m² for the control group (Table 1).

### Phenotypic studies

From all 133 probands, information on birth weight and length, follow-up weight and height, and medical history were obtained from the medical records provided by paediatricians. BMI was calculated as body weight in kilograms divided by the square of the height in metres. Overweight and obesity were assessed according to the International Obesity Task Force recommendations, by using the Cole's cutoffs for BMI (15). Body circumferences were measured by standard procedures. All families gave informed consent and participated in the study. The study was performed according to the Helsinki Declaration and approved by the local Ethics Committee.

### Genotyping

DNA was extracted from peripheral leucocytes. To detect the CART gene A1475G SNP, three primers were used, two allele-specific mutagenically separated PCR (MS-PCR) primers designed from the same DNA strand (5'-GGT GTC CTC TGG GGA ACG T-3' and 5'-GCC AAA CTC CAG GGA GGA ACC TGT GTT CCT CGG AAA CC-3') (mutagenic and allelic positions are underlined) and one primer with a sequence derived from the complementary strand (5'-AGC TGT GTG ACT GTC CCG A-3'). Forty cycles (30 s at 96°C, 30 s at 57°C; and 30 s at 72°C) were performed. Gel electrophoresis was performed using 4% agarose gels (Gibco BRL, Paisley, U.K.), and the products were subsequently detected with ethidium bromide under ultraviolet illumination.

The ΔA1457 deletion was examined by direct sequencing as previously described (16).

### Statistical analysis

Descriptive values are given as mean or standard error of the mean. Statistical significance was determined by χ²-test or Fisher’s exact test for differences in allele and genotype frequencies.

A TDT was used to analyse the pattern of allele transmission from heterozygous parents to all affected offspring calculating χ² and the corresponding p value (17). A value of p < 0.05 was considered statistically significant. Statistical analysis was performed through use of the Stata 9 program (StataCorp., College Station, TX, USA).

### RESULTS

The genotype and allele distributions of the CART gene A1475G and the ΔA1457 SNPs among the 103 overweight children, the 30 obese patients and the control groups are listed in Tables 3 and 4. The genotype distributions for the A1475G and the ΔA1457 polymorphisms were not different from those expected by Hardy–Weinberg equilibrium for all studied groups. Parental genotype distribution was also in Hardy–Weinberg equilibrium for the two SNPs.

### Genotype and allele frequencies for the A1475G SNP

A significant difference was observed in the genotype frequencies of the A1475G polymorphism between overweight children (13.5%) and control children (4.4%) (p = 0.03;

### Table 2 CART gene single nucleotide polymorphisms (SNPs) allele frequencies of overweight children vs. control groups

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Overweight children (n = 103)</th>
<th>Allele frequency</th>
<th>Heterozygous frequency</th>
<th>Control children (n = 90)</th>
<th>Allele frequency</th>
<th>Heterozygous frequency</th>
<th>p*</th>
<th>OR (95% CI)</th>
<th>Control adult (n = 97)</th>
<th>Allele frequency</th>
<th>Heterozygous frequency</th>
<th>p†</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1475G</td>
<td>Wild-type</td>
<td>89 (86.4%)</td>
<td>0.07</td>
<td>86 (95.6%)</td>
<td>0.02</td>
<td>0.03</td>
<td>3.38 (1.1–10.1)</td>
<td>93 (95.9%)</td>
<td>0.02</td>
<td>0.02</td>
<td>3.65 (1.2–10.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Heterozygous</td>
<td>14 (13.6%)</td>
<td>4 (4.4%)</td>
<td>5 (5.5%)</td>
<td>0.03</td>
<td>NS</td>
<td>NS</td>
<td>1.0 (0.3–3.6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔA1457</td>
<td>Wild-type</td>
<td>97 (94.2%)</td>
<td>0.03</td>
<td>85 (94.5%)</td>
<td>0.03</td>
<td>NS</td>
<td>NS</td>
<td>1.0 (0.3–3.6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Heterozygous</td>
<td>6 (5.8%)</td>
<td>5 (5.5%)</td>
<td>5 (5.5%)</td>
<td>0.03</td>
<td>NS</td>
<td>NS</td>
<td>1.0 (0.3–3.6)</td>
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</tr>
</tbody>
</table>

NS = no significance.

No patients homozygous for CART gene A1475G and ΔA1457 SNPs were found in this study population.

*Overweight children vs. control children.

†Overweight children vs. control adult.
Transmission disequilibrium test

The TDT statistic and associated p-value for all heterozygous parents are shown in Tables 5 and 6. There was a preferential transmission of the 1475G allele of the CART gene from heterozygous parents to overweight children ($\chi^2 = 7.2$, p = 0.01), with a percentage of variant transmission of 65.0%. No statistically significant excess transmission of the A1457 allele was found. Moreover, there was a preferential transmission of the 1475G allele of the CART gene from heterozygous parents to obese children ($\chi^2 = 6.0$, p = 0.05), with a percentage of variant transmission of 75.0%. No statistically significant excess transmission of the A1457 allele was found.

DISCUSSION

CART mRNA was originally identified by PCR differential display techniques as a transcript acutely up-regulated in rat striatum after cocaine or amphetamine administration (10). Interestingly, CART maps to chromosome 5q13.2, 4.8 Mb from the D5S647 locus, a region previously linked to obesity and serum leptin levels in obese French Caucasian families (18). In obese animal models with disrupted leptin signalling, CART mRNA is almost absent from the arcuate nucleus (11). Peripheral administration of leptin to obese mice stimulates CART mRNA expression (11).

### Table 3
CART gene single nucleotide polymorphisms (SNPs) allele frequencies of obese children vs. control groups

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Obese children (n = 30)</th>
<th>Control children (n = 90)</th>
<th>Allele frequency</th>
<th>p*</th>
<th>OR (95% CI)</th>
<th>Control adult (n = 97)</th>
<th>Allele frequency</th>
<th>p†</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1475G</td>
<td>Wild-type 25 (83.3%)</td>
<td>86 (95.6%)</td>
<td>0.08</td>
<td>4.30 (1.1–15.9)</td>
<td>93 (95.9%)</td>
<td>0.02</td>
<td>4.60 (1.2–17.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Heterozygous 5 (16.6%)</td>
<td>4 (4.4%)</td>
<td>0.03</td>
<td>28 (93.4%)</td>
<td>85 (94.5%)</td>
<td>0.03</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1457</td>
<td>Wild-type 28 (93.3%)</td>
<td>85 (94.5%)</td>
<td>0.03</td>
<td>1.20 (0.2–5.8)</td>
<td>92 (94.9%)</td>
<td>0.02</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Heterozygous 2 (6.6%)</td>
<td>5 (5.5%)</td>
<td>0.02</td>
<td>65.0%</td>
<td>52.5%</td>
<td>0.2</td>
<td>NS</td>
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### Table 4
Transmission of the CART gene A1475G and A1457 single nucleotide polymorphisms in overweight children of 40 Italian trios

<table>
<thead>
<tr>
<th>Allele</th>
<th>Variant transmitted</th>
<th>Variant not transmitted</th>
<th>Total transmission</th>
<th>Variant transmission (%)</th>
<th>$\chi^2$</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1475G</td>
<td>52</td>
<td>28</td>
<td>80</td>
<td>65.0%</td>
<td>7.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>A1457</td>
<td>42</td>
<td>38</td>
<td>80</td>
<td>52.5%</td>
<td>0.2</td>
<td>NS</td>
</tr>
</tbody>
</table>

No significant difference was observed in the genotype frequencies between overweight children (16.6%) and control children (6.6%) and control adults (5.1%) (p = 0.6; OR: 1.31; 95% CI: 0.28–6.27). The allele frequencies of the A1457 polymorphism were significantly different neither between the obese children (0.03) and control children (0.03) (p = 0.8; OR: 4.10; 95% CI: 1.25–17.27) nor between overweight children (5.8%) and control adults (5.1%) (p = 0.8; OR: 1.20; 95% CI: 0.26–5.57) nor between overweight children (0.02) (p = 0.7; OR: 4.60; 95% CI: 1.20–17.27). The allele frequencies of the A1475G SNP were significantly different neither between the obese children (0.07) and control children (0.02) (p = 0.02; OR: 4.65; 95% CI: 1.08–17.27) nor between overweight children (0.07) and control adults (0.02) (p = 0.7; OR: 4.65; 95% CI: 1.08–17.27). No statistically significant excess transmission of the CART gene A1475G was observed. Moreover, there was a preferential transmission of the 1475G allele of the CART gene from heterozygous parents to overweight children ($\chi^2 = 7.2$, p = 0.01), with a percentage of variant transmission of 65.0%. No statistically significant excess transmission of the A1457 allele was found. Moreover, there was a preferential transmission of the 1475G allele of the CART gene from heterozygous parents to obese children ($\chi^2 = 6.0$, p = 0.05), with a percentage of variant transmission of 75.0%. No statistically significant excess transmission of the A1457 allele was found.

No significant difference was observed in the genotype frequencies between overweight children (16.6%) and control children (6.6%) and control adults (5.1%) (p = 0.6; OR: 1.31; 95% CI: 0.28–6.27). The allele frequencies of the A1457 polymorphism were significantly different neither between the obese children (0.03) and control children (0.03) (p = 0.8; OR: 4.10; 95% CI: 1.25–17.27) nor between overweight children (5.8%) and control adults (5.1%) (p = 0.8; OR: 1.20; 95% CI: 0.26–5.57) nor between overweight children (0.02) (p = 0.7; OR: 4.60; 95% CI: 1.20–17.27). The allele frequencies of the A1475G SNP were significantly different neither between the obese children (0.07) and control children (0.02) (p = 0.02; OR: 4.65; 95% CI: 1.08–17.27) nor between overweight children (0.07) and control adults (0.02) (p = 0.7; OR: 4.65; 95% CI: 1.08–17.27). No statistically significant excess transmission of the CART gene A1475G was observed. Moreover, there was a preferential transmission of the 1475G allele of the CART gene from heterozygous parents to overweight children ($\chi^2 = 7.2$, p = 0.01), with a percentage of variant transmission of 65.0%. No statistically significant excess transmission of the A1457 allele was found. Moreover, there was a preferential transmission of the 1475G allele of the CART gene from heterozygous parents to obese children ($\chi^2 = 6.0$, p = 0.05), with a percentage of variant transmission of 75.0%. No statistically significant excess transmission of the A1457 allele was found.

### Table 5
Transmission of the CART gene A1475G and A1457 single nucleotide polymorphisms in obese children of 12 Italian trios

<table>
<thead>
<tr>
<th>Allele</th>
<th>Variant transmitted</th>
<th>Variant not transmitted</th>
<th>Total transmission</th>
<th>Variant transmission (%)</th>
<th>$\chi^2$</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1475G</td>
<td>18</td>
<td>6</td>
<td>24</td>
<td>75.0%</td>
<td>6.0</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>A1457</td>
<td>14</td>
<td>10</td>
<td>24</td>
<td>58.3%</td>
<td>0.6</td>
<td>NS</td>
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NS = no significance.
Moreover, CART has been found in a population of leptin-activated cells located in the lateral arcuate nucleus. These cells directly innervate the sympathetic preganglionic neurons in the thoracic spinal cord. These neurons regulate interscapular brown adipose tissue, which plays an integral role in regulating body temperature, energy expenditure, and diet-induced thermogenesis (19).

Leptin mediates many of its physiological effects by increasing the activity of the sympathetic nervous system. Therefore the engagement of this pathway may contribute to the increased thermogenesis and energy expenditure characteristic of leptin action (19). Further some studies support the idea that the CART peptide(s) functions as a signalling molecule in the sympathetic nervous system (20).

Although CART has therefore been shown to be involved in control of feeding behaviour (20), a direct relationship with obesity has not yet been established. Contradictory findings have been reported about the association between the CART gene SNPs and obesity phenotypes. Echwald et al. (14) identified two polymorphisms in the coding region of the CART gene: an A1475G substitution and a ΔA1457 deletion. By screening in Danish subjects with early onset obesity, they found that neither of these variants was associated with obesity. Moreover, a mutational screening performed in a group of obese Pima Indians failed to detect genetic variations (21). Subsequently, a study was performed in a large Caucasian population in the U.K. The A1475G and ΔA1457 SNPs did not show any significant relationship with obesity; however, the A1475G SNP was associated with a lower waist-to-hip, fasting plasma insulin, and fasting triglycerides (16). The ΔA1457 deletion was found also in a Japanese population, but the variant allele frequency was equivalent between lean subjects and obese subjects (0.12 for both) (22). In 292 morbidly obese French subjects, the A1475G mutation was significantly associated with obesity. A weak association was observed for the ΔA1457 deletion (23). Del Giudice et al. (24) studied 130 unrelated obese Italian children and adolescents, but they failed to detect any association between the CART gene A1475G and ΔA1457 SNPs and obesity. To our knowledge, this is the first time the familial study of CART gene A1475G and ΔA1457 SNPs has been undertaken in overweight and obese children originated from Southern Italy. In our overweight and obese children, the allele frequencies of the two SNPs were higher than those found in a previous Italian study (24). They were similar to the allele frequencies described in a large Caucasian population (23). Moreover, we found that the allele frequency of the A1475G SNP was significantly higher in overweight (0.07) and obese (0.08) children to respect the control children (0.02) and adults (0.02), but no significant association was found between the ΔA1457 deletion and overweight/obesity. The TDT showed a preferential transmission of the A1475G polymorphism from the heterozygous parents to overweight and obese offspring, with a high percentage variant transmission (65.0% and 75.0% respectively). If parents who are heterozygous for the allele transmit it to affected children on >50% of occasions, this is evidence for both linkage and linkage disequilibrium between the marker and disease loci (17). By contrast, we failed to detect this linkage disequilibrium in the overweight/obese children when we studied the ΔA1457 SNP. Our study of the genetic variability of the CART gene in overweight and obese Italian children suggests a possible association with obesity which is mainly attributable to the effect of A1475G SNP. It is possible that this polymorphism influence the CART expression, either directly through influences on mRNA stability or indirectly through linkage disequilibrium with other functional variants in or near the CART gene. Certainly, our results are in contrast with some previous studies, but it may be explained by ethnic differences or a particular environmental influence. In the obese children of our study, we found an association between obesity and CART gene polymorphisms. These data have not been demonstrated in obese adults (14). It is possible that the obese and overweight children of our study represent a different cohort to adults who may have not had early onset obesity recruited in different countries. Moreover, these differences could be explained by the research design used in other studies (24). In Italian obese children without familial history of obesity, Del Giudice et al. (24) showed no association of the CART gene polymorphisms and obesity. However, they found a Leu34Phe mutation of the CART gene in a 10-year-old obese boy who belongs to a large family of obese subjects. The mutation was not found in the control population. Therefore, it is possible that the familial studies could permit a better identification and characterization of susceptibility genes underlying obesity. It will contribute to a greater understanding of the pathogenesis of obesity and ultimately will assist in the development of better strategies for prevention and therapeutic intervention.

ACKNOWLEDGEMENTS
We thank the doctors Maria Amorini, Giuseppina Lo Giudice, Petronilla Romeo, Francesca Pugliatti, Rosario Alberto Caruso and Giuseppe Finocchiaro for their excellent collaboration.

References


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