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Molecular Analysis of the Adiponectin Gene in Severely Obese Patients from Southern Italy

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Key Words
Adiponectin · Polymorphisms · Obesity · Metabolic syndrome

Abstract
Background: Severe obesity is a major worldwide public health concern affecting 0.5–5% of the adult population. Adiponectin (Acpr30), an adipokine secreted from adipocytes, shows pleiotropic beneficial effects on obesity and related disorders. In this study, sequence analysis of Acpr30 gene (ACDC) was performed in a highly selected population of severely obese young adult patients from Southern Italy to investigate the associations between polymorphisms in the ACDC gene and the development of severe obesity concomitantly with other features of the metabolic syndrome. ***Methods:*** The ACDC gene was analyzed by direct sequencing in the severely obese patients (n = 220) and compared to healthy controls (n = 116). The associations between the ACDC gene single-nucleotide polymorphisms (SNPs) and the levels of serum Acpr30 as well as the correlation with the presence of severe obesity jointly associated with other features of the metabolic syndrome were also investigated. Total serum Acpr30 concentrations were measured by the ELISA method. ***Results:*** ACDC gene molecular screening revealed the presence of previously described SNPs and a new nucleotide alteration, c.355T>G, leading to a protein variant, p.L119V. Measurement of serum concentration of Acpr30 demonstrated lower levels of Acpr30 in the obese population compared to controls (30.5 ± 28.3 vs. 43.9 ± 35.7 μg/ml, $p < 0.01$); in particular, significantly lower Acpr30 concentrations were observed in obese patients bearing c.–11377C>G SNP CG+GG genotypes than in those with CC genotype (22.9 ± 20.5 vs. 33.1 ± 29.4 μg/ml, $p < 0.05$). ***Conclusions:*** Our results confirmed that low serum levels of Acpr30 are related to severe obesity and a difference in protein expression is associated with variants in ACDC gene promoter region.

Introduction

The adipose tissue is nowadays recognized as an important endocrine organ that controls the whole-body metabolism through a series of biologically active mole-

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cules, such as leptin, acylation-stimulating protein, tumor necrosis factor-α, plasminogen activator inhibitor-1, interleukin-6 and adiponectin (Acpr30) [1–2]. Acpr30 is abundantly secreted in serum, where its concentration is approximately 0.05% of total serum proteins [3]. In humans, Acpr30 concentration levels are inversely related to adipose tissue mass and positively associated with insulin sensitivity both in healthy subjects and diabetic patients [3]. In humans, as well as in animal models, serum Acpr30 levels decrease in some insulin-resistant states (IRS), such as obesity and type 2 diabetes mellitus (T2DM), as well as coronary artery disease [3–7]. Instead, chronic renal failure, type 1 diabetes and anorexia nervosa are associated with increased serum Acpr30 levels [8–9]. Serum Acpr30 concentrations correlate negatively with glucose, insulin, triglyceride levels and body mass index (BMI), and positively with high-density lipoprotein-cholesterol levels and insulin-stimulated glucose disposal [10]. Weight loss and therapy with thiazolidinediones increase endogenous Acpr30 production in humans [11].

Human Acpr30 is transcribed by adipocyte C1q and collagen domain-containing (ACDC) gene that is composed by 3 exons and 2 introns spanning 16 kb. ACDC maps to the 3q27 region, where genome-wide scans have revealed a susceptibility locus for type 2 diabetes, coronary heart disease and measures of adiposity [12, 13]. The ACDC gene encodes a 30-kDa protein (Acpr30), characterized by 2 domains: a collagen-like fibrous domain and a complement C1q-like globular domain. Acpr30 circulates in the blood as different molecular weight isoforms produced by multimerization of the monomer [14, 15]; oligomeric complex distribution is critical for the antidiabetic and antiatherogenic activity of this hormone [16].

Epidemiologic studies assess that single-nucleotide polymorphisms (SNPs) and some haplotypes present in the ACDC gene, promoter region included, are associated with obesity [3, 17–19], as well as with T2DM, metabolic syndrome (MS) and coronary artery disease in some populations [3, 20–22].

In the present study, we performed an extensive mutational screening of the ACDC gene in a highly selected group of patients affected by severe obesity with a BMI ≥40 compared to a control population (BMI ≤25); both populations were from Southern Italy. We aimed at investigating whether SNPs in the ACDC gene were associated with levels of serum Acpr30; furthermore, we verified the impact of the various SNPs of the ACDC gene on the presence of severe obesity concomitantly with other features of the MS.

Methods

Patients and Controls

The patient group included 220 unrelated severely obese young adult individuals (39.9% males; mean age 32.2 ± 11 years; mean BMI 48.2 ± 7.1) recruited by the outpatient clinic of the Department of Clinical and Experimental Medicine, University Federico II, Naples, Italy; all participants were Caucasian and had lived in Southern Italy for at least 3 generations.

Inclusion criteria were absence of T2DM and no coronary heart disease; severe obesity was classified as BMI ≥40.0. The presence of MS was diagnosed according to Adult Treatment Panel III criteria [23]. Among the obese participants, 91.2% had a positive family history of metabolic diseases. Particularly, 52.2% had relatives suffering from obesity combined with hypertension and diabetes; relatives with uncomplicated obesity, hypertension, diabetes and hyperlipidemia were reported by 20.4, 10.2, 5.4 and 2.7% of the obese patients, respectively. As controls, for genetic analyses, 116 normal-weight healthy individuals without MS (BMI ≤25, 40 male and 76 female, age range 27–55 years), from the same geographic area, were recruited from the Federico II University Hospital staff. Written informed consent was obtained from all participants. The research protocol was approved by the Ethics Committee of the School of Medicine, University of Naples Federico II and was in accordance with the principles of the Helsinki II Declaration.

Anthropometric and Metabolic Measurements

Weight and height measurements were recorded. BMI was calculated as the body weight (kg) divided by the square of the height (m^2). Systolic and diastolic blood pressure were measured after 10 min rest in the sitting position. For laboratory studies, 20 ml of blood were obtained from an antecubital vein without compression after a >10-hour overnight fasting period. Blood samples were collected in serum tubes and immediately centrifuged at room temperature. Plasma glucose, total cholesterol, triglycerides and high-density lipoprotein-cholesterol were determined by standard enzymatic methods (Hitachi Modular; Roche) [24]. Serum insulin was measured by chemiluminescence method (Immunolight 2000; Medical System). Insulin resistance was estimated according to the homeostasis model assessment method from fasting glucose and insulin concentrations, according to the formula: insulin (μU/ml) × glucose (mmol/l)/22.5 [25].

Total serum Acpr30 concentrations were measured in duplicate in obese and control individuals by enzyme immunoassay (LINCO Research). This assay is a sandwich ELISA that measures total native human Acpr30 using monoclonal anti-human Acpr30 antibodies.

Screening of Mutations in the ACDC Gene

We sequenced the 3 exons, the exon-intron boundaries and the promoter region of the ACDC gene in the 2 groups of 220 unrelated Caucasian outpatients affected by severe obesity (BMI ≥40.0) and 116 control subjects. Blood samples (5 ml) were collected by venipuncture in EDTA from each subject and genomic DNA was extracted using a commercial kit (Nucleon BACC-2; Amersham Biosciences). We used a homemade primer set that enabled all exons and the promoter to be amplified by the same PCR protocol. The PCR primers were chosen by the Primer 3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.

Table 1. General and biochemical features of the control group and the severely obese patients in dependence of presence or absence of MS

	Controls (n = 116)	All patients (n = 220)	MS+ (n = 124)	MS– (n = 96)	MS+ vs. MS– p
Sex, % males	34.5	39.9	39.5	40.6	–
Age, years	32.1 ± 7.8	32.4 ± 12.0	32.0 ± 12.0	32.5 ± 32.0	NS
BMI	22.6 ± 2.0	48.7 ± 7.8	49.4 ± 7.7	46.9 ± 6.1	<0.02
Glucose, mmol/l	4.7 ± 0.4	5.2 ± 1.2	5.3 ± 1.4	5.2 ± 0.8	NS
Total cholesterol, mmol/l	4.4 ± 0.8	4.8 ± 1.0	4.9 ± 1	4.7 ± 1.1	NS
HDL-cholesterol, mmol/l	NA	1.2 ± 0.3	1.0 ± 0.2	1.4 ± 0.3	<0.02
Triglycerides, mmol/l	0.9 ± 0.3	1.5 ± 0.8	1.7 ± 0.9	1.2 ± 0.3	<0.02
Insulin, pmol/l	NA	22.4 ± 10.8	23.6 ± 16.4	20.2 ± 10.8	NS
HOMA	NA	5.2 ± 2.8	5.4 ± 3.7	4.7 ± 2.6	NS
Adiponectin, μg/ml	43.9 ± 35.7	30.5 ± 28.3	31.0 ± 27.0	30.3 ± 30.6	NS
Systolic blood pressure, mm Hg	108.7 ± 13.4	126.8 ± 16.6	128.8 ± 16.7	123.9 ± 16.8	<0.05
Diastolic blood pressure, mm Hg	69.8 ± 9.7	81.0 ± 10.7	81.8 ± 10.4	79.9 ± 11.4	NS

In all obese patients, waist circumference was much above the Adult Treatment Panel III criteria (males: 102 cm; females: 88 cm). MS+ = MS present; MS– = MS absent; NS = not significant; HDL = high-density lipoprotein; HOMA = homeostasis model assessment; NA = not available.

cgi) and were used as follows: 5′GCTCTGTGTGGACTGTGGAG′3 and 5′CCACACCACTCCAGGAACTT′3 for promoter; 5′CAAGGCCTGGAAACACAAGT′3 and 5′CACCTGTATCCACTCCCACA′3 for exon 1; 5′TCTCTCCATGGCTGACAGTG′3 and 5′AGCTTTGCTTTCTCCCTGTG′3 for exon 2; 5′GGAGCCACAGGGATGGTAAT′3 and 5′ATTGACTTTGGGGCTGTTTG′3 for exon 3. The reaction was carried out in a final volume of 50 μl containing: 1× PCR buffer II (Applied Biosystems), 1.5 mM $MgCl_2$ (Applied Biosystems), 0.5 mM each of the deoxynucleotide triphosphates (Amersham Biosciences), 15 μM of each primer, 1 U/μl of Taq DNA polymerase (Applied Biosystems) and 100 ng/μl of DNA. The reaction mixture was first subjected to 1 cycle of 3 min of denaturation at 94°C, after which the DNA was amplified during 35 cycles, of which 14 cycles consisted of 20 s of denaturation at 94°C, 40 s of annealing at 62°C, decreasing 0.5°C each cycle, and 45 s of extension at 72°C; then 25 cycles of denaturation at 94°C for 20 s, 40 s of annealing at 55°C and 45 s of extension at 72°C. After amplification, the reaction mixture was subjected to a final cycle of 7 min of extension at 72°C. PCR products were electrophoresed on a 1% agarose gel and sequence analysis was performed on both strands with an automated procedure using the 3100 Genetic Analyzer (Applied Biosystems). PCR fragments were sequenced using the same primers used for PCR amplification.

Statistical Analysis

Allele frequencies were calculated by allele counting and the departure from Hardy-Weinberg expectation was evaluated by the χ^2 test using the Haploview 3.2 software with default p value cutoff [26]. The χ^2 test was also used to test any difference in allele SNP frequency between obese patients and controls. The unpaired t test was used to compare clinical and biochemical data in males and females as well as in MS+ or MS– patients. Differences were considered statistically significant when $p < 0.05$.

Binomial logistic analysis was performed to investigate the risk of MS associated with ACDC gene polymorphisms adjusted for age, sex and BMI. All statistical analyses were performed with the SPSS (version 13.0) for Windows software.

Results

The general and biochemical characterization of obese patients is shown in table 1. The frequency of MS+ obese patients represents 55.6% of the total. The prevalence of MS+ was very similar in males and females (55.7 vs. 57.3%). BMI, high-density lipoprotein-cholesterol, triglycerides and systolic blood pressure were significantly different in the 2 MS groups, as expected (table 1). The allele and genotype of ACDC variants detected by sequence analysis in control subjects and in severe obese patients are reported in table 2.

Molecular analysis revealed 9 variants previously reported in Caucasian populations [3] and a new nucleotide alteration c.355T>G, in exon 3, identified in a control subject, that causes an amino acid change in the codon 119 (p.L119V; fig. 1; table 2). The p.G90S, the p.Y111H and the p.R112C variants (all located in exon 3) were detected, in heterozigous state, with a very low frequency: p.G90S was found in 2 control subjects and in 9 patients; p.Y111H mutation was detected in 2 controls and in 7 obese patients; p.R112C mutation was detected only in 2 obese patients.

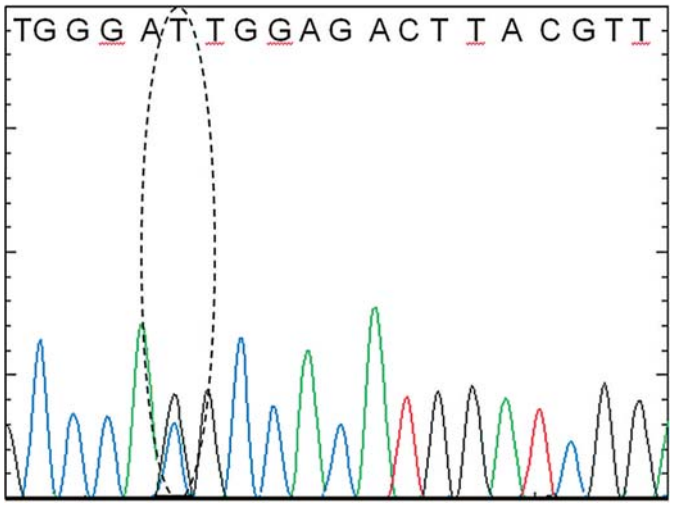

Fig. 1. Sequence electropherogram showing the exon 3 region of the ACDC gene bearing the nucleotide alteration c.355T>G (L119V) detected at heterozygous state in a control subject.

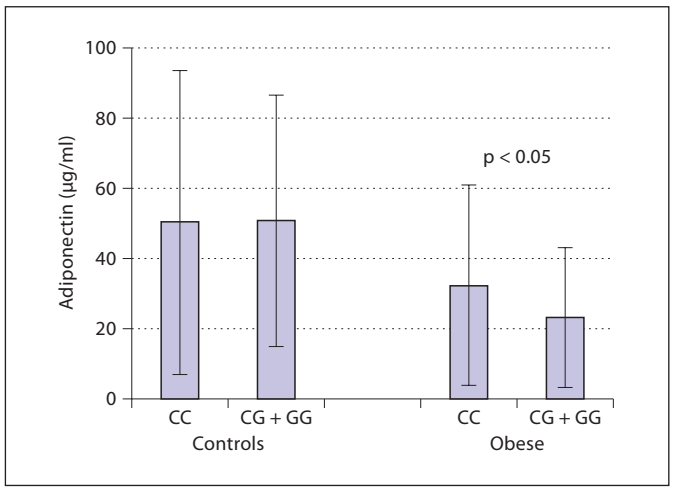

Fig. 2. Comparison of gene ACDC polymorphism c.–11377C>G and Acpr30 serum concentrations in control subjects (left) and obese patients (right). With the t test, statistically significantly higher Acpr30 levels were detected in obese patients with CC genotype vs. CG+GG genotypes ($p < 0.05$).

Table 2. Allele and genotype frequencies of ACDC variants in obese patients (n = 220) and control subjects (n = 116)

SNP/ genotypes	Location	Control frequencies, %	Obese frequencies, %	p value[1]
c.–11426A>G				
AA		93.3 (108)	93.8 (206)	
AG	promoter	6.7 (8)	4.9 (11)	0.642
GG		0	1.3 (3)	
c.–11391G>A				
GG		81.3 (94)	87.9 (193)	
GA	promoter	18.7 (22)	10.7 (24)	0.107
AA		0	1.3 (3)	
c.–11377C>G				
CC		64.0 (74)	71.9 (158)	
CG	promoter	30.7 (36)	21.0 (46)	0.215
GG		5.3 (6)	7.1 (16)	
c.–11156insCA				
WT		93.3 (108)	85.3 (188)	
INS/WT	promoter	6.7 (8)	13.8 (30)	0.140
INS/INS		0	0.9 (2)	
c.45T>G (p.G15G)				
TT		69 (80)	74.9 (165)	
TG	exon 2	26.7 (31)	23.7 (52)	0.1331
GG		4.3 (5)	1.4 (3)	
c.214+62 G>T (276G>T)				
GG		50.9 (59)	60 (132)	
GT	intron 2	44.8 (52)	34 (75)	0.3048
TT		4.3 (5)	6 (13)	
c.268G>A (p.G90S)				
GG		98.3 (114)	95.8 (211)	
GA	exon 3	1.7 (2)	4.2 (9)	0.2345
AA		0	0	
c.331T>C (p.Y111H)				
TT		98.3 (114)	97.2 (214)	
TC	exon 3	1.7 (2)	2.8 (6)	0.5451
CC		0	0	
c.334C>T (p.R112C)				
CC		100 (116)	99.1 (218)	
CT	exon 3	0	0.9 (2)	0.2970
TT		0	0	
c.355T>G (p.L119V)				
TT		99.1 (115)	100 (220)	
TG	exon 3	0.9 (1)	0	0.1741
GG		0	0	

Figures in parentheses are numbers. WT = Wild type; INS = insertion. [1] χ^2 test.

In the control population, the distributions of all SNPs were in the Hardy-Weinberg equilibrium ($p < 0.05$); this was also observed in the obese population except for the c.–11426A>G, –11391G>A and c.–11377C>G SNPs.

The binomial logistic regression analysis showed a statistically significant relationship between the patients bearing exon 3 mutations (p.G90S, p.Y111H and p.R112C) and MS presence (OR = 5.6, 95% CI 1.5–20.3, $p < 0.01$).

The obese patients showed significantly decreased serum Acpr30 levels in comparison to the control subjects (30.5 ± 28.3 vs. 43.9 ± 35.7 µg/ml, $p < 0.01$). Furthermore, obese subjects bearing a wild-type ACDC genotype showed slightly higher, even if not statistically significant, Acpr30 levels compared to those bearing 1 or more out of

10 polymorphic variants (32.7 ± 27.8 vs. 29.9 ± 28.5 μg/ml). With the t test, statistically significant lower Acpr30 concentrations ($p < 0.05$) were only observed in obese patients bearing the polymorphic c.-11377C>G allele (CC vs. CG+GG genotypes). In figure 2, the Acpr30 values detected both in obese patients and in control subjects for this polymorphism are reported. This latter difference remained statistically significant when we divided obese patients by sex (CC vs. CG+GG: females, 36 vs. 27 μg/ml $p < 0.05$; males, 28 vs. 18 μg/ml, $p < 0.05$).

Discussion

The increasing prevalence of obesity and MS has attracted considerable interest as both represent risk factors for cardiovascular and other degenerative diseases, actually the most important causes of morbidity and mortality in developed and in developing countries [27]. Different evidences suggest that Acpr30 possesses antihyperglycemic, antiatherogenic and anti-inflammatory properties, so it can be speculated that low Acpr30 levels might be considered an early marker of metabolic risk in obesity [3, 22]. Several common SNPs have been identified in the promoter region and in the coding sequence of the ACDC gene in different populations [17–21, 28–29]. In some cases, but not all, these SNPs are associated with lower Acpr30 levels and/or T2DM and obesity [19, 29–30]. Today, severe obesity (BMI >40) is associated with high premature mortality and represents a major public health concern, representing a variable but significant percentage (0.5–5%) of the adult population [27]. Since medical treatment of severely obese patients is frequently unsuccessful even in the case of sufficient compliance to treatment, a genetic component is advocated. Furthermore, genetic polymorphisms have been repeatedly considered as a predisposing genetic background to develop obesity and its complications. We, therefore, have analyzed the ACDC gene in a population from Southern Italy to verify if the ACDC SNPs may affect serum Acpr30 levels in severe obesity and influence its metabolic complications. For these reasons we selected a population of severely obese young adult patients. Furthermore, it is of note that the population of Southern Italy, from a genetic viewpoint, is highly heterogenous showing differences from other Italian and European regions [31].

Molecular screening detected, in the ACDC promoter region, 4 SNPs of this, only the c.–11377C>G variant was linked to decreased levels of serum Acpr30 in our population of severely obese young adults; previously, in different populations, c.–11377C>G SNP has been associated with low serum Acpr30 as well as with T2DM [32]. This variant was associated with a higher prevalence of cardiovascular disease in patients with end-stage renal disease [33]. More recently, the c.–11377C>G variant has been correlated not only with the presence of coronary atherosclerosis, but it has also been demonstrated to be predictive of vascular events among men undergoing coronary angiography [17].

Another promoter region variant, c.–12823G>A, previously associated with variations in serum Acpr30 in Pima Indians [29], has not been detected in our population.

The exon 3 missense mutations, p.G90S, p.Y111H and p.R112C, have been detected in our population only in heterozygous status. They were found with a very low frequency both in control and in obese subjects; in particular, p.R112C mutation was detected only in 2 obese patients. The 2 mutations p.G90S and p.Y111H have been associated with a genetic risk of T2DM in French Caucasians [19]. Moreover, the p.Y111H genotype was not associated with body weight in a sample of Swedish obese subjects [20]. The p.R112C alteration has been described to alter serum Acpr30 concentrations in a Japanese population [29]. The association of this rare mutation with T2DM was recently shown in French Caucasians, but not confirmed in a Swedish population [19–20].

Other mutations, such as p.G84R, p.I164T and p.R221S, reported to contribute to the development of insulin resistance [3, 34], were not found in our patients.

We also detected the c.45T>G (p.G15G) and c.214+62G>T [276G>T] SNPs; these SNPs, in our population, did not present an association with severe obesity, which was in agreement with recent studies where no association was found between obesity or IRS and the c.45T>G polymorphism [21, 29, 35, 36]. Nevertheless, significant associations between these highly prevalent polymorphisms and obesity or IRS have also been reported [3]. Our results support the hypothesis that the c.45T>G and c.214+62G>T polymorphisms in exon 2 and intron 2 of the ACDC gene are not directly linked to severe obesity and related metabolic changes.

We also detected a new mutation, p.L119V, in the ACDC exon 3, in a young female of the control population. This mutation, placed at the beginning of the globular domain of Acpr30, affects residue 119 located in a phylogenetically highly preserved region; the change, however, should probably determine little effects into the

Acpr30 protein because the apolar, aliphatic amino acid leucine is replaced by valine, an amino acid which presents similar biochemical features.

In this study, severely obese patients have been examined for the presence of MS that represents an important summary of cardiovascular risk factors and the obese males show a higher, though statistically not significant, prevalence of MS; these results were in accordance with previous observations from our group [37, 38].

We have also observed in our severely obese patients a slight association between the sum of the observed ACDC exon 3 mutations and the presence of MS; these mutations have also been described in association with T2DM [19, 36].

In conclusion, molecular analysis of the ACDC gene revealed 9 previously reported polymorphisms and a new genetic alteration. Our study demonstrated that the nucleotide variant c.–11377C>G present in the ACDC gene promoter region is correlated to Acpr30 serum concentration in severely obese young adults. In fact, significantly lower concentrations of serum Acpr30 were found in obese bearing SNP c.–11377C>G. Further evaluations on the long term are required to clarify the role of this cytokine as related to the development of metabolic and cardiovascular complications in severely obese patients.

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