Lack of mutations in the leptin receptor gene in severely obese children

Ausência de mutação no gene receptor de leptina em crianças gravemente obesas

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ABSTRACT

Objective: To analyze the *LEPR* gene in obese children and to investigate the associations between molecular findings and anthropometric and metabolic features. **Subjects and methods:** Thirty-two patients were evaluated regarding anthropometric characteristics, blood pressure, heart rate, serum glucose, insulin, leptin levels, and lipid profile. The molecular study consisted of the amplification and automatic sequencing of the coding region of *LEPR* in order to investigate new mutations. **Results:** We identified a high prevalence of metabolic disorders: impaired fasting glucose in 12.5% of the patients, elevated HOMA-IR in 85.7%, low HDL-cholesterol levels in 46.9%, high triglyceride levels in 40.6%, and hypertension in 58.6% of the patients. The molecular study identified 6 already described allelic variants: rs1137100 (exon-2), rs1137101 (exon-4), rs1805134 (exon-7), rs8179183 (exon-12), rs1805096 (exon-18), and the deletion/insertion of the pentanucleotide CTTTA at 3'untranslated region. **Conclusions:** The frequency of alleles observed in this cohort is similar to that described in the literature, and was not correlated with any clinical feature. The molecular findings in the analysis of the *LEPR* did not seem to be implicated in the etiology of obesity in these patients. Arq Bras Endocrinol Metab. 2012;56(3):178-83

Keywords
Obesity; childhood obesity; leptin; leptin receptor gene

RESUMO

Objetivo: Analisar o *LEPR* em crianças obesas e investigar associações entre achados moleculares e características antropométricas e metabólicas. **Sujeitos e métodos:** Foram avaliados 32 pacientes quanto às características antropométricas, à pressão arterial, à frequência cardíaca, às dosagens séricas de glicemia, à insulina, à leptina e ao perfil lipídico. O estudo molecular consistiu na amplificação e no sequenciamento automático da região codificadora do *LEPR* para pesquisar mutações. **Resultados:** Identificou-se uma alta prevalência de distúrbios metabólicos: glicemia de jejum alterada em 12,5%, HOMA-IR elevado em 85,7%, níveis de HDL-colesterol baixos em 46,9%, níveis de triglicérides elevados em 40,6% e hipertensão arterial em 58,6%. O estudo molecular identificou 6 variações alélicas já descritas na literatura: rs1137100 (éxon-2), rs1137101 (éxon-4), rs1805134 (éxon-7), rs8179183 (éxon-12), rs1805096 (éxon-18) e deleção/inserção do pentanucleotídeo CTTTA na região 3' não traduzida. **Conclusões:** A frequência das variações alélicas observada é semelhante à descrita na literatura e não se correlacionou com nenhuma característica clínica. Os resultados da análise molecular do *LEPR* não parecem estar implicados na etiologia da obesidade desses pacientes. Arq Bras Endocrinol Metab. 2012;56(3):178-83

Descritores
Obesidade; obesidade infantil; leptina; gene do receptor de leptina

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INTRODUCTION

Obesity in childhood and adolescence is following the wide-reaching epidemic of obesity in adulthood. It is estimated that at least 155 million children worldwide are overweight or obese (1). The main factor responsible for the obesity epidemic seems to be the environmental change that promotes excessive calorie intake and physical inactivity in a population that had their genes selected for survival during times of privation (2).

Classically, few syndromes associated with obesity have been identified, in which mental retardation and abnormal development are also commonly observed (3). Besides these syndromes, in monogenic disorders, mutations have been described in patients in which obesity results from the loss of hypothalamic leptin-melanocortin pathway signaling, which is responsible for regulating satiety and energy expenditure (4-9).

Leptin is a hormone specifically produced by adipocytes, and its serum concentration is proportional to body fat mass which, in turn, has its amount regulated by the hypothalamic effects of leptin. Intravenous administration of leptin reduces appetite, while its deficiency increases food intake (10). Its action occurs by means of the leptin receptor, encoded by the *LEPR* gene. Which is located on the short arm of chromosome 1, at position 1p31, and it is composed of 18 exons and 17 introns. It encodes a protein of 1,172 amino acids (816 amino acids in the extracellular domain, 303 in the intracellular domain, and 23 in the transmembrane domain), expressed mainly in the hypothalamus, and known as the OB-RL isoform (OB-large receptor). Three other minor isoforms are known, and the smallest one is OBRS (short OB-receptor), which is expressed in various tissues, such as the choroid plexus, lungs and kidneys (11).

LEPR mutations have been described in patients with early-onset severe obesity, although the prevalence of these mutations has not been systematically studied. Clément and cols. (6) documented the intronic mutation IVS16 +1 G> A in *LEPR,* which causes early-onset severe obesity and pituitary dysfunction. Other mutations in *LEPR* have been described by Farooqi and cols. (12), in 3% of patients with severe obesity starting in childhood, and hyperphagic eating behavior. The sample in the study by Farooqi and cols. (12) showed BMI z scores of 5.1 ± 1.6 and body fat percentage of 52.8% ± 3.2%.

Thus, congenital *LEPR* deficiency should be considered in all patients with morbid obesity and hyperphagia in the absence of developmental delay and dysmorphic features, since this diagnosis may significantly alter the course of treatment, the choice of therapy, and allow genetic counseling for affected families.

Regarding polymorphisms in *LEPR*, Rosmond and cols. (13) identified the protective influence of two polymorphisms (Lys109Arg and Gln223Arg) against higher blood pressure levels. Men carrying arginine alleles had lower blood pressure levels; the difference was higher when subjects carried arginine in both codons. In the same group, hypertensive subjects had higher BMI, leptin levels, and frequency of Lys109 allele (14).

Moreover, polymorphisms in *LEPR* also seem to influence carbohydrate metabolism in women. Insulin response after an oral glucose load was significantly affected by Lys109Arg and Gln223Arg polymorphisms in post-menopausal women with impaired glucose tolerance. Insulin levels, adjusted for fat mass (fasting, 2h and area under the curve of insulin) were about twofold higher in Lys109 and Gln223 homozygotes compared with Arg carriers (13).

Our goals in this study were to analyze the coding region of the leptin receptor gene in Brazilian children with early-onset severe obesity, and to investigate the associations between molecular findings and anthropometric and metabolic features.

SUBJECTS AND METHODS

Patients and parents or guardians received information about the research and signed a consent form. The inclusion criteria were: onset of obesity before the age of 4 years, inappropriately high leptin levels, and/or a BMI Z score ≥ 2.5 according to the CDC growth charts (15) (this cutoff value was arbitrary). The exclusion criteria were: presence or suggestive features of genetic syndrome, obesity related to endocrine disease (Cushing's syndrome or hypothyroidism), and/or presence of chronic diseases, such as liver, kidney or heart failure.

Clinical evaluation

The sample consisted of 32 Brazilian patients with early-onset severe obesity (17 females, and 15 males) with mean age of 11 ± 3.9 years. Clinical evaluation included anthropometric measures (weight and height for BMI calculation), heart rate, and sitting blood pressure that was measured on the right arm after at least 5 minutes of rest. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) percentiles were calculated (Na-

tional High Blood Pressure Education Program Working Group, 2004) (16). Laboratory tests performed with blood samples were: serum glucose, total cholesterol, HDL-cholesterol (HDL-c), LDL-cholesterol, triglyceride, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyltransferase (GGT), thyroid stimulating hormone (TSH), free thyroxine (FT4), and leptin levels.

We adopted dyslipidemia cut-off values established by the First Brazilian Guideline for Atherosclerosis Prevention in Children and Adolescents, namely triglyceride levels ≥ 130 mg/dL and/or HDL-c ≤ 45 mg/dL (17); and the American Diabetes Association criteria for diagnosis of impaired fasting glucose, namely glucose levels > 100 mg/dL (18). A homeostatic model assessment index (HOMA-IR) > 2.5 was adopted as an index of insulin resistance (19).

Molecular study

Genomic DNA was extracted from peripheral blood leukocytes using the standardized protocols. DNA was amplified by PCR, using a set of primers to cover all exons and exon-intron boundaries regions of *LEPR* (Table 1). The samples were submitted to the following program for exon amplification: 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds; annealing temperature specific for each primer (50-56°C) for 30 seconds; and 72°C for 30 seconds, followed by a final extension at 72°C for 10 minutes.

All reactions were carried out together with a negative control. Reading of the samples was carried out on agarose gel 2% with ethidium bromide 0.5 mg/mL, and observed under ultraviolet transillumination.

All amplified products were purified using ExoSAP-IT (U.S. Biochemical, Cleveland, OH) according to the manufacturer's instructions, and sequenced using a 3130 Analyzer (Applied Biosystems, Foster City, CA, USA). The sequence plots were compared with a normal sequence and database plots (ENSG00000116678).

Statistics

Clinical data of patients distributed as continuous variables are presented as means and standard deviations. Variables with nonparametric distribution are presented as medians and interquartile ranges. The association between specific allelic variant and metabolic disturbances (impaired fasting glucose, insulin resistance, hypertension, hypertriglyceridemia, and low HDL cholesterol) was assessed using the chi-square or Fisher's exact test. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using SPSS software16.0 (Statistical Package for Social Sciences Inc., USA).

Table 1. Sequence of primers, annealing temperature and amplicon size for PCR reactions

Exon	Primer sense (5'-3')	Primer anti-sense (5'-3')	T(ºC)	Amplicon (pb)
1	CCTTTTCCAGGTGTACTTCT	CTCTACCATGTTTAAGGGC	55	129
2	GAGCACTACATGGTTTAATC	AATCATAGCCATAAGACATC	50	510
3	TTCACTGAGTTGTTCAGATGG	CTACTTCCGTATATGAAAGC	52	175
4	CCTGCTTTAAAAGCCTATCC	GCCACTCTTAATACCCCCAG	55	472
5	GTCCTTGGATAAAGTCACCT	TGCTATGGGACTTAAGAGGG	55	349
6	CCACATCAACTTGATGTTCTG	CCAGTAGAAGTGGCTATTAC	55	219
7,8	ATCTGATATCCTTTCTTCCC	TTTTTATCTCACTGTGCCCA	52	550
9	TCCCTGGTGCCAAAAAGGTT	GACACAACGCAGCTTGACAT	56	338
10	GCTTGATGAATACAGATGTATG	GAGGAAAGCTGAAGTTCTAAG	56	277
11,12	GCCCTTTAGATACATATGTG	CAGGATTATGGACCATGAAG	52	374
13	GCACTGCAGCCCTTAAACTA	GGATTACCACTCTGTACCTC	56	287
14	GTCTTCTCTTCCTTATTCCC	AGGATGGTAGTATCCTCTAT	52	406
15	GTATAAATGAGCCTTTTACG	TTAGGCACACACATTGGTG	50	178
16	CCTCAAGTTTCTGAGTTGTG	CGCGTAAGGACTTTTGCCGT	54	153
17	GGCATAGTTGATCTGGTGGA	TGGCGTCTAGATTGCAATCG	56	242
18A	CACACTTCCATTTCTGCCAGT	CAGCGTGGCGTATTTAACAA	55	394
18B	TCTCTGAGGCTGAGGGTACTG	TCTCTCTCTTTTTGATTGAGGTGA	55	420
18C	TGGATGAACTTTTGAAATTGGA	CAAACAGACAACATTCATTTGGA	55	633

RESULTS

Clinical, biochemical and hormonal characteristics of patients are shown in table 2. In this group of children, we found a high prevalence of metabolic abnormalities. Impaired fasting glucose was present in 12.5% of the patients, elevated HOMA-IR in 85.7%, low HDL-cholesterol in 46.9%, high triglyceride levels in 40.6%, and hypertension was present in 58.6% of patients.

No mutations or new polymorphisms were observed in the molecular analysis of this gene in 32 DNA samples. Allelic variants previously described in a database were observed. SNPs were located in the 3' untranslated region (3'UTR), and in exons 2, 4, 7, 12 and 18 (Table 3). No association between allelic variant and metabolic disturbances were identified.

Table 2. Clinical, biochemical and hormonal characteristics of patients

Parameter	Mean ± SD* / Median (Interquartile range)**
Gender	17F:15M
Age (years)	11.0 ± 3.9*
Weight (kg)	97.5 ± 37.7*
BMI (kg/m²)	42.57 ± 10.3*
BMI Z-score	+2.8 (0.25)**
SBP percentile	79.1 ± 28.6*
DBP percentile	85.4 ± 16.7*
Heart rate (bpm)	87.7 ± 13.7*
Fasting glucose (mg/dL)	85.8 ± 14.1*
Fasting insulin (mUI/L)	29.4 ± 22.2*
Total cholesterol (mg/dL)	157.6 ± 35.3*
HDL cholesterol (mg/dL)	40.1 ± 8.4*
LDL cholesterol (mg/dL)	91.1 ± 25.8*
Triglycerides (mg/dL)	115.6 ± 45.2*
AST (UI/l)	27.7 ± 9.2*
ALT (UI/l)	32.6 ± 19.7*
TSH (mUI/l)	2.9 ± 1.2*
Free T4 (ng/dL)	1.1 ± 0.2*
Leptin (ng/mL)	48.4 (40.3)**

BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma glutamyltransferase; TSH: thyrotropin stimulating hormone; T4: thyroxine.

DISCUSSION

The findings reported here show high prevalence of metabolic disorders such as insulin resistance, dyslipidemia and hypertension related to severe obesity in a small sample of severely obese Brazilian children and adolescents.

Table 3. Frequency of allelic variations found in patients

SNP	Exon	Replacement	Frequency
rs1137100	2	A**A**G>A**G**G K109R	A 81% G 19%
rs1137101	4	C**A**G>C**G**G Q223R	A 68% G 32%
rs1805134	7	AG**T**>AG**C** S343S	T 14% C 86%
rs8179183	12	AA**G**>AA**C** K656N	G 25% C 75%
rs1805096	18	CC**A**>CC**G** P1019P	A 62% G 38%
Del/Ins* 3'UTR		 -/CTTTA	-74% 26%

* -: deletion; +: insertion.

Clinical features

Fasting glucose levels were elevated in 12.5% of the subjects, and HOMA-IR was increased in 85.7% of them. This information is in agreement with several studies, including a recent one by Pastucha and cols. (20) that demonstrated the validity of using the HOMA index for children and adolescents, and reported HOMA-IR of 4.58 ± 1.92 for obese children (BMI > p95), compared with 1.8 ± 1.36 for non-obese children, suggesting greater insulin resistance in obese children and adolescents. Another study conducted with 122 adolescents (21) also found that the obese group was more insulin-resistant compared with the non-obese group.

Systemic hypertension, a multifactorial disease, was found in 58.6% of patients in our study. Considerable evidence indicates that hypertension is more common in obese children and adolescents. Recently, a Spanish study reported hypertension in 25% of cases, in a group of children and adolescents with a mean age similar to our cohort (respectively, 11.3 ± 2.8 and 11.0 ± 3.9). However, mean BMI of our population was 42.57 ± 10.3 kg/m², while the average BMI of the Spanish cohort (22) was 27.9 ± 3.6 kg/m².

Besides the influence of adiposity on blood pressure (23,24) the Bogalusa Heart Study (25) consolidated the relationship between blood pressure and insulin levels, even after adjusting it for BMI. Mean insulin levels in individuals of our study were 29.4 ± 22.2 mIU/L, while in the Spanish sample they were 12 ± 6 mIU/L.

The association between obesity and dyslipidemia was found in numerous studies. The Bogalusa Heart Study (25) found that overweight and obese students were 2.4 to 7.1 times more likely to have hypercholesterolemia, with elevated levels of LDL-cholesterol and

triglycerides, and the relationship between hyperinsulinemia and dyslipidemia was also demonstrated (21).

Our series showed low HDL-cholesterol levels (mean levels, 40.1 ± 8.4 mg/dL) in 46.9% of cases, and elevated triglycerides (mean levels, 115.6 ± 45.2 mg/dL) in 40.6% of them. A Japanese cohort of 319 overweight children aged 6 to 12 years, found HDL-cholesterol levels of 57 ± 12 mg/dL for boys, and 55 ± 11 for girls, and mean triglycerides of 107 (95% CI 99 – 115) for boys, and 112 (95% CI 100-125) for girls. However, both mean BMI (24.8 ± 2.1 kg/m^2 in boys and 24.7 ± 2.6 kg/m^2 in girls) and mean serum insulin levels (12.6 mIU/L in boys and 16.6 mIU/L in girls) were lower in Japanese children when compared to our cohort (26). Another population studied (290 children, mean age 11.3 ± 2.8 years, BMI 27.9 ± 3,6 kg/m^2 and insulin levels 12 ± 6 mUI/L) also showed better HDL-cholesterol (47 ± 11 mg/dL) and triglyceride (80 ± 44 mg/dL) levels than our group (22).

Diseases associated to obesity are common in childhood patients. In our cohort, these morbidities were more severe probably because of the higher degree of adiposity.

Molecular features

Yiannakouris and cols. (27) showed that the rs1137101 polymorphism is more prevalent in obese than in non-obese patients (20.7% *versus* 4.5%), and that it is a predictor of obesity and body composition. This finding was also observed in several studies, by Mattevi and cols. (28) and Duarte and cols. (29), carried out in Brazil; by Furusawa and cols. (30,31), in two studies conducted in Japan; and in another study by Duarte and cols. (32), in which they described that this polymorphism increased the risk of obesity by 58%. Chiu and cols. (33) also showed that the rs1137101 polymorphism is associated with increased serum LDL-cholesterol levels, and higher rates of insulin resistance. However, a study conducted in Turkey with a group of 232 obese patients found no relationship between this polymorphism and BMI (34).

Among the studies conducted with children, Riestra and cols. (35) showed that the rs1137101 polymorphism is associated with higher serum leptin levels and BMI in girls, but this finding was not seen in boys. A Mexican study (36) conducted with 103 adolescents (55 obese and 48 non-obese) found that the rs1137101 polymorphism was more prevalent among individuals with higher levels of insulin, who also had a higher percentage of fat in body composition and higher serum leptin levels. Okada and cols. (37) who studied 136 obese children between 5 and 17 years found no relationship between this polymorphism and serum lipids.

The rs1137100 polymorphism was not related to BMI, risk of obesity and body composition in several studies in the literature (27,30,31). The rs81789183 polymorphism was not associated with BMI and obesity (27) nor to insulin resistance (38). However, a Spanish study (39) carried out with 67 obese patients showed that the rs81789183 polymorphism was associated with decreased response to leptin, and lower weight loss secondary to changes in lifestyle behavior (hypocaloric diet and exercise). Among the studies involving children and adolescents, the results are also controversial, since Riestra and cols. (35) found no relationship between and rs81789183 and BMI or obesity, while Okada and cols. (37) associated the rs1137100 polymorphism with higher serum lipid levels.

In our study, the prevalence of polymorphisms was similar to that described in the literature in obese patients. However, no causal relationship between these polymorphisms and metabolic disturbances (impaired fasting glucose, insulin resistance, hypertension, hypertriglyceridemia, hepatic transaminases, and low HDL-cholesterol) was identified.

We did not find mutations in *LEPR*, confirming that these mutations are extremely rare causes of obesity. However, with the methodology we used, we cannot rule out modifications in the promoter region or in some transcription factor or post-translational defects that could lead to changes in the receptor.

In conclusion, *LEPR* point mutations are rare causes of obesity, even in individuals with early-onset and severe disease. The frequency of SNPs rs1137100, rs1137101, rs1805134, rs8179183, rs1805096, and deletion or insertion in the 3'UTR pentanucleotide CTTTA in our cohort was similar to that already described, and these mutations, isolated or in association, did not correlate with metabolic abnormalities. However, we found that the frequency of metabolic abnormalities increased with the degree of fatness.

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