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Biallelic and triallelic approaches of *5-HTTLPR* polymorphism are associated with food intake and nutritional status in childhood☆,☆☆

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Abstract

Background: The 5-HTT gene contains polymorphisms in its promoter region, the insertion/deletion (*5-HTTLPR*) that creates long (L) or short (S) alleles (biallelic approach) and SNP (rs25531) in L allele (triallelic approach).
Objectives: The aim of this study is to investigate the association of the *5-HTTLPR* and rs25531 polymorphisms, using bi- and triallelic approach, with dietary intake and anthropometric parameters in children followed until 8 years old.
Methods: The sample were 303 children who were recruited at birth and examined at 1, 3 to 4 and 7 to 8 years old. The polymorphisms were analyzed by polymerase-chain-reaction-based methods.
Results: In the biallelic approach, children with the S/S genotype presented a higher body mass index *Z*-score in the three developmental stages and higher sum of skinfolds at 3 to 4 and 7 to 8 years old than carriers of the L allele. In the triallelic approach, S/S, Lg/S plus Lg/Lg genotypes were associated with higher energy intake daily at 1 year old and with waist circumference at 3 to 4 years old.
Conclusions: In the biallelic approach, the *5-HTTLPR* polymorphism is associated with food intake, body mass index *Z*-score and sum of skinfolds in children, reinforcing the role of the serotonin transporter in childhood obesity. Our data indicate that the biallelic approach is more sensible than the triallelic approach for detected associations with food intake and nutritional status in childhood. Identifying susceptibility genes in early life could provide the foundations for interventions in lifestyle to prevent children to become obese adults.

Keywords: Eating; Obesity; Serotonin; *5-HTTLPR*; Children

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is an important chemical neurotransmitter that can be found widely across the body and is associated with a large number of conditions, including obesity [1]. Some studies illustrate the inverse relationship between the level of brain serotonin signaling and food intake: when brain serotonin signaling is increased, food intake is reduced, and *vice versa*. The bioavailability of endogenous serotonin has been manipulated using drugs that affect serotonin release or serotonin reuptake through the serotonin transporter. D-fenfluramine, which promotes serotonin efflux from the intracellular compartment into the synapse through the serotonin transporter and blocks serotonin reuptake, produces hypophagia [2]. Serotonin reuptake inhibitors increase extracellular serotonin levels and reduce food intake [3,4]. Changes of endogenous serotonin synthesis, bioavailability and metabolism provide important evidence for the role of endogenous serotonin in coordinating food intake and body weight.

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Serotonin transporter has a polymorphism named 5-*HTTLPR* that is in the regulatory region of its gene (solute carrier family 6 member 4, *SLC6A4*; chromosome 17q11.1–q12), consisting of imperfect repetitive sequences of 20–23 bp rich in GC. An insertion/deletion in this region creates a long allele (L, insertion, 16 repetitions allele) or a short allele (S, deletion, allele of 14 repetitions) that results in a biallelic approach [5,6] that can alter protein expression [7]. Few studies have been performed to investigate association of this variant with obesity in adolescents and adults, and demonstrated an association between the short allele of *5-HTTLPR* and higher body mass index (BMI) [8,9]. However, to the best of our knowledge, no study evaluated association of this genetic variant with food intake and anthropometric parameters in early childhood. Another single nucleotide polymorphism (SNP) named rs25531 causes A>G change at position 6 of the first of two extra repeats of L allele [10], resulting in a triallelic approach: S, La and Lg alleles. The expression of the Lg allele has been described as similar to the expression of the S allele [11].

As the serotonergic system is involved in the control of energy intake and body weight [12], we hypothesize that 5-*HTTLPR* and rs25531 polymorphisms influence food intake and nutritional status in children. The purpose of this study was to investigate the possible association of these polymorphisms with food intake and nutritional status at three stages of early childhood in children followed up to 7 to 8 years of age.

2. Methods

2.1. Subjects

The sample consisted of 303 children recruited at birth at the Hospital Centenário (located in São Leopoldo, Brazil) between October 2001 and July 2002. Newborns eligible for the study had birth weights of 2500 g or more and a gestational age equal to or greater than 37 weeks. This a cohort study nested in a randomized trial. The trial aimed to evaluate the effectiveness of maternal dietary counseling during the first year of life, based on guidelines from the Brazilian Ministry of Health and the Pan American Health Organization, on infant feeding practices [13]. The newborns were randomized into the intervention or control group. The mothers from the intervention group received dietary advices during the child's first year of life. This dietary advice had aimed exclusive breastfeeding up to 6 months; after this, the mothers were encouraged to continue breastfeeding and gradually introduce foods. Both groups received visits at 6 and 12 months and routine follow-up by their pediatricians. The children from both groups were longitudinally evaluated at three time points: when they were 1 (mean=1), 3 to 4 (mean=3.9) and 7 to 8 (mean=7.7) years old. The intervention was not the primary objective of present study; our research group has performed some studies that evaluated the association of genetic variants with food intake and nutritional status in this sample [14–18].

The interviewer classified race or ethnicity by skin color as white or nonwhite (black and brown). The study protocol was approved by the Ethics Committee of the Universidade Federal de Ciências da Saúde de Porto Alegre, and parents/guardians of all participants provided written informed consent before beginning of the study.

2.2. Nutritional status and dietary data assessed

Measurements of anthropometric and dietary data were collected at the three times cited above. Anthropometric measurements were taken in children wearing light clothing and unshod using a digital balance to weight (Filizola, Campo Grande, MS, Brazil), a stadiometer to height (Seca, Mexico, D.F.), an inflexible measuring tape to waist circumference and a skinfold caliper to measure the triceps and subscapular skinfold thickness (Lange, Santa Cruz, CA, USA). The sum of the two individual skinfold thicknesses was computed. BMI was calculated [weight (kg)/height^2 (m^2)], and the values were transformed into *Z*-scores. The BMI variation percent of each child was computed by the formula $[(BMI_a - BMI_b)/BMI_b] \times 100$ (a = BMI final and b = BMI initial).

Dietary data were collected from questionnaires also including socioeconomic and family factors. Two 24-h dietary recalls were collected, performed with an interval of 15 to 30 days, to obtain a view of the child's usual intake in the diet by calculating the average of the parameters of both recalls. The interviewers asked detailed questions about the types of foods, quantities, brands and preparation methods. Portion sizes were confirmed with the aid of an album, specifically designed for this study, containing photographs of utensils and foods and based on domestic measures, such as cups, tablespoons and teaspoons.

2.3. DNA analysis

Genomic DNA was extracted from peripheral whole blood by a standard salting-out procedure [19]. The *5-HTTLPR* polymorphism was genotyped by polymerase chain reaction (PCR) using 10 pmol of each primer (forward 5′-CGCTCCTGCATCCCCCATTA-3′ and reverse 5′-GGGATGCGGGGGAATACTGGT-3′ [20]), 200 μM of each deoxynucleotide, reaction buffer [7.5 mM Tris–HCl, 5 mM KCl and 2 mM $(NH_4)_2SO_4$], 2.5 mM $MgCl_2$ and 1.0 U Taq DNA Polymerase. Thermal cycling consisted of 5 min of initial denaturation at 94°C followed by 35 cycles of 94°C (40 s), 57°C (40 s) and 72°C (60 s) each with a final extension step of 5 min at 72°C. PCR products containing 253 bp for the short allele and 297 bp for the long allele were analyzed after electrophoresis on the 2.5% agarose gel containing ethidium bromide. The different genotypes were visualized due to the difference between the sizes of the fragments by comparison with a 50-bp DNA ladder. For triallelic genotyping (rs25531), polymorphism was detected by PCR-RFLP analysis. The same PCR products of *5-HTTLPR* polymorphism were digested with 1 μl of FastDigest *Msp*I restriction enzyme (Fermentas), FastDigest GreenBuffer 1× (Fermentas) and 10 μl of amplified product for 5 min at 37°C according to the manufacturer's instructions. Genotypes were determined after electrophoresis on 2.5% agarose gels stained with ethidium bromide by comparison with a 50-bp DNA ladder. These are the fragments according to genotypes: LaLa: 233 and 63 bp; LaLg: 233, 174, 63 and 59 bp; LgLg: 174, 63 and 59 bp; LaS: 233, 189, 64 and 63 bp; LgS: 189, 174, 64, 63 and 59 bp; SS: 189 and 64 bp.

2.4. Quality control

Undergraduate nutrition students were trained to collect the data, and the data from 5% of the participants were confirmed by telephone. In the DNA analysis, two samples of each genotype were confirmed by sequencing. The bands corresponding to the size of the allele fragments were excised from a gel, and the DNA was extracted by purification using Wizard SV gel and PCR Clean-Up System (Promega, Madison, WI, USA), according to the manufacturer's protocol. After sequencing, the fragments were analyzed using an automatic sequencer ABI Prism 3130 with BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, USA). The sequences were analyzed and compared with sequences available in the GenBank nucleotide sequence database with the help of the programs Chromas (version 2.33) and Generuner. In addition, 5% of the samples were randomly selected and regenotyped for each polymorphism, and 100% cross-validation was indicated.

2.5. Statistical analysis

The normality of the variables was verified with Kolmogorov–Smirnov test and by the evaluation of the histogram distribution. Due