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Polymorphisms of β-Adrenergic Receptor Gene Appears to Be a Genetic Risk Factor for Obesity in Preschool Children with Partial Growth Hormone Deficiency

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Over the last years, several single nucleotide polymorphisms influencing early-onset obesity have been characterized. As candidate genes, recent studies have focused on the adrenergic receptor system. A descriptive study using the medical charts of 46 children (19 girls and 27 boys) were of short stature and treated by growth hormone (GH). Anthropometric data of the children were recorded longitudinally from 1 to 6 years of age prior to the commencement of GH treatment. The mean percent overweight increased linearly during the 5-year study period, from −5.3% to 17.7% in the group of children with the mutation of the β3-adrenergic receptor codon 64 polymorphism, and −5.0% to 1.3% in those without this mutation. In contrast, the differences among the subtypes of the β2-adrenergic receptor gene codon 16 polymorphism did not attain statistical significance at any time point during this study. Trp64Arg polymorphism of the β3-adrenergic receptor gene appears to be a genetic risk factor for obesity in Japanese preschool children.

Key words: β-adrenergic receptor gene, growth hormone, obesity, polymorphism, preschool children

Introduction

Obesity results from an imbalance between energy intake and energy expenditure. In humans, a low resting metabolic rate is a risk factor for weight gain and obesity. There is strong evidence from family studies that the body weight is regulated by genetic factors. Over the last ten years, several single nucleotide polymorphisms influencing early-onset obesity have been characterized[1]. As candidate genes, recent studies have focused on the adrenergic receptor (AR) system, because of the important role of these receptors in the stimulation of thermogenesis and in activating lipid mobilization from fat stores. In particular, genes of the β2- and β3-AR subtypes appear to be attractive candidate genes[2].

Few studies have been conducted on the effects of these polymorphisms on obesity in children. Obesity is influenced by multiple behavioral, environmental and genetic factors. Children of this age are not habituated to drinking, smoking or any particular sports activities. Preschool children in Japan get regular exercise at the nursery or kindergarten that they attend. Thus, in early childhood, environmental factors have little influence on the development of obesity, unlike in adolescents and adults. In the past report, we showed the appearances of the β2- and β3-AR subtypes in Japanese children[3].

This study was conducted to ascertain whether polymorphisms of the β2- and β3-AR subtypes influence the development of obesity over time in early childhood.

Subjects and Methods

Subjects

A total of 46 children (19 girls and 27 boys) who were treated for short stature under the aegis of the Foundation for Growth Science in Japan were investigated at the Tokyo Women's Medical Uni-

versity Medical Center East, Tokyo, Japan. The mean chronological age at the start of growth hormone (GH) treatment was 7.3 years and the mean height was −2.63 (Standard Deviation; SD) scores (SDS; range, −5.06 to −1.51 SDS) during 1988 and 2000.

GH deficiency was diagnosed on the basis of a peak GH concentration of less than 10 mU/*l* in two or more standard provocation tests using insulin hypoglycemia (0.1 U/kg), glucagon (0.03 mg/kg), arginine infusion (0.5 g/kg), L-dopa (10 mg/kg) or propranolol (0.1 mg/m^2). The GH deficiency cases that all peaks were below 5 mU/*l* were defined as complete, the other as partial. No subject had associated malformations, chromosomal abnormalities or structural abnormalities of the pituitary gland, as determined by magnetic resonance imaging studies. All the children enrolled in this study were diagnosed as having partial but not complete GH deficiency, and were well nourished. The thyroid, kidney and liver functions were normal, and none of the children had celiac disease.

Written informed consent was obtained from the parents after emxplaining in detail the objectives and investigational procedure of the study.

Anthropometric measurements

All of the children were evaluated at 1, 2, 3, 4, 5 and 6 years of age retrospectively. At each visit, the standing height and body weight were measured. During this period, the GH treatment had not yet been initiated. Height was measured with a portable standiometer and a digital scale, and expressed as the SD score for chronological age, based on recent Japanese growth references[4]. Percent overweight was determined on the basis of Japanese standard body weights for height by age and sex− (actual body weight−standard weight)/standard weight×100 (%)− and overweight exceeding 20% of ideal weight is defined as obesity, exceeding 50% as severe obesity, in Japan[5].

Genetic DNA analysis

Genomic DNA was extracted from peripheral blood leukocytes by digestion with proteinase K, followed by phenol/chloroform extraction. Amplification of the β2- adrenergic receptor gene sequences was done by PCR in a 40-μl volume of a mixture containing 0.1 U of AmpliTaq gold DNA polymerase, using the following oligonucleotide primers: 5′-CTTCTTGCTGGCACGCAAT-3′ and 5′CCAGTGAAGTGATGAAGTAGTTGG-3′ for codon 16. The annealing temperature for codon 16 was 56℃. After 30 cycles of amplification, 2-μl aliquots of the PCR products were analyzed on 2% agarose gels to confirm proper amplification. The amplified PCR products were then digested with BsrD1. After incubation for 1h, the digested samples were separated by electrophoresis on 4% agarose gel and visualized by staining with ethidium bromide[6]. Trp 64Arg of the β3-adrenergic receptor was amplified using the primers 5′-CGCCCAATACCGCCAACAC-3′ and 5′-CCACCAGGAGTCCCATCACC-3′.PCR was performed as described by Wieden et al with denaturation at 95℃ for 60s, annealing at 60℃ for 60s, and extension at 72℃ for 120s for 35 cycles. The PCR products were digested with 10 U of BstNI for 1 h at 37℃. The resultant fragments were analyzed on 2.5% agarose gels[7].

Statistical analysis

Results were expressed as means, with standard error of the mean (Standard error of means; SEM) indicated in parentheses, unless otherwise stated. Contingency table chi-square tests were used to compare the genotype and allele frequencies in the children of this study and control subjects from a previous study[8][9]. As the distributions of the samples were somewhat skewed, the Wilcoxon's rank sum test was applied to examine the significance of differences between the genotype groups. The differences among three genotype groups were determined by performing one-way analysis of variance (ANOVA), followed by Fisher's protected Least Significant Difference (LSD). Differences were considered significant when the p value was 0.05 or less.

Results

Genotype and allele frequencies of the β2- and β3-AR gene

The results of the β2- and β3-AR genotyping are shown in Table. The genotype distribution of the β2-AR Arg16Gly polymorphism in the 46 children with partial GH deficiency was as follows: 17% (n=

Table Frequency of the β2,3-adrenergic receptor genotypes in short children with partial growth hormone deficiency

		β3-adrenergic receptor genotypes Codon64		
		Trp64Trp	Trp64Arg	Arg64Arg
β2-adrenergic receptor genotypes				
Codon16	Arg16Arg	4	3	1
	Arg16Gly	11	11	1
	Gly16Gly	12	3	0

No. of genotypes

8), 50% (n=23) and 33% (n=15) had Arg/Arg, Arg/Gly and Gly/Gly, respectively. The frequencies of this polymorphism did not differ significantly from those reported in non-obese Japanese subjects by Ishiyama-Shigemoto et al[8]. Among the study children, two (4%) had the Trp64Arg mutation in a homozygous form, 17 (37%) were heterozygous for the mutation, and the remaining 27 (59%) did not have any mutation of the β3-AR gene (Trp64Trp homozygote, wild type). No difference in the frequency distribution was observed as compared with that in Japanese non-diabetic subjects reported by Fujisawa et al[9].

Effect of the β2- and β3-AR gene on obesity

To evaluate the relative importance of the β2-AR gene polymorphism at codon 16, we conducted a stratified analysis for the three genotype groups. The mean percent overweight increased from −6.7% to 0.5% in children with Gly/Gly during the study period of 5 years. In contrast, it increased to a significantly greater extent, from −3.2 to 7.4% in children with Arg/Gly, and from −6.1% to 17.3% in children with Arg/Arg, although the differences among the three groups did not attain statistical significance in three groups at any time point during the 5 years.

To determine if the percent overweight was modulated by mutation of the Trp64Arg polymorphism of the β3-AR gene, the children were divided into those with mutation (Trp/Arg and Arg/Arg, n=19) and those without mutation (Trp/Trp, n=27, because of the limited number of Arg/Arg subjects). The mean percent overweight increased linearly during the 5 years, from −5.3% to 17.7% in the group of children with mutation, and from −5.0% to 1.3% in the group without the mutation. These differences attained statistical significance 4, 5 and 6 years of age (Fig. 1).

Effect of the β2- and β3-AR gene on height

In relation to the Arg16Gly polymorphism of the β2-AR gene, the mean SDS for height decreased significantly at the age of 6 years only in children with Arg/Arg among the three genotypes. On the other hand, there were no significant differences in the SDS for height at any given time-point between the groups with and without the Trp64Arg mutation of the β3-AR gene (Fig. 2).

Discussion

In this study, we analyzed the influence of two common polymorphisms of the human β2- and β3-AR genes on longitudinal changes in the body weight and height of preschool children with partial GH deficiency. We found that only the Trp64Arg polymorphism of the β3-AR modified the risk of developing obesity. Subjects with the Arg16Gly and Gly16Gly genotypes of the β2- AR gene tended to have higher SD scores for height than those with Arg16Arg, with the difference becoming statistically significant at 6 years of age.

In Japan, it is difficult to obtain blood samples from healthy children. We therefore selected patients with GH therapy as the subjects of this study, and obtained detailed anthropometric data. We also selected percent overweight over the Body Mass Index (BMI) for estimating the severity of obesity. BMI is a commonly used index for obesity in western countries, especially for adults; however, it has been reported that the BMI is influenced by age and gender. In Japan, there is no established cutoff value of BMI for childhood obesity, and percent

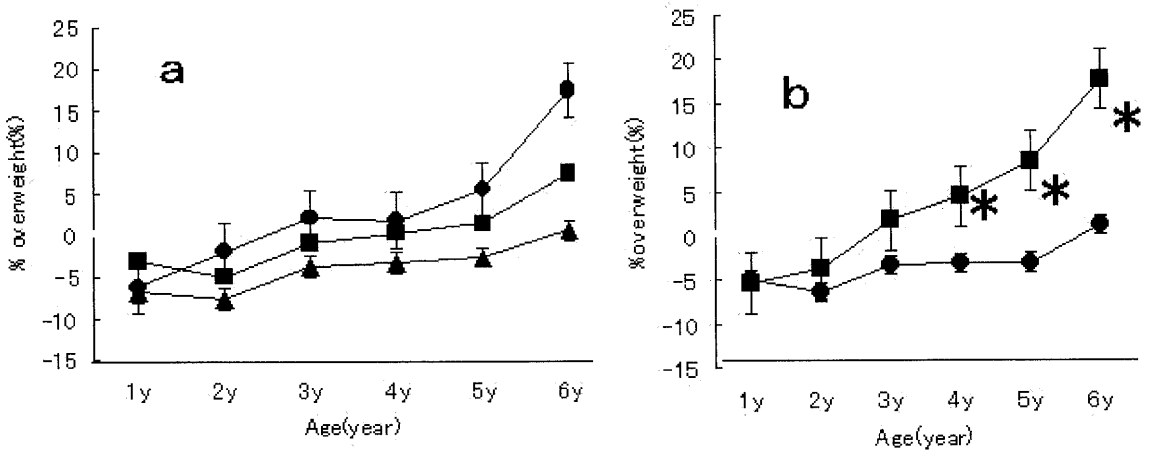

Fig. 1 Changes in percent overweight in preschool children with partial growth hormone deficiency between two genotype groups as determined by Wilcoxon's rank sum test.
a: β2-adrenergic receptor polymorphism (●: Arg16Arg, ■: Arg16Gly, ▲: Gly16Gly), b: β3-adrenergic receptor polymorphism (●: Trp64Trp, ■: Trp64Arg & Arg64Arg), *: $p < 0.05$.

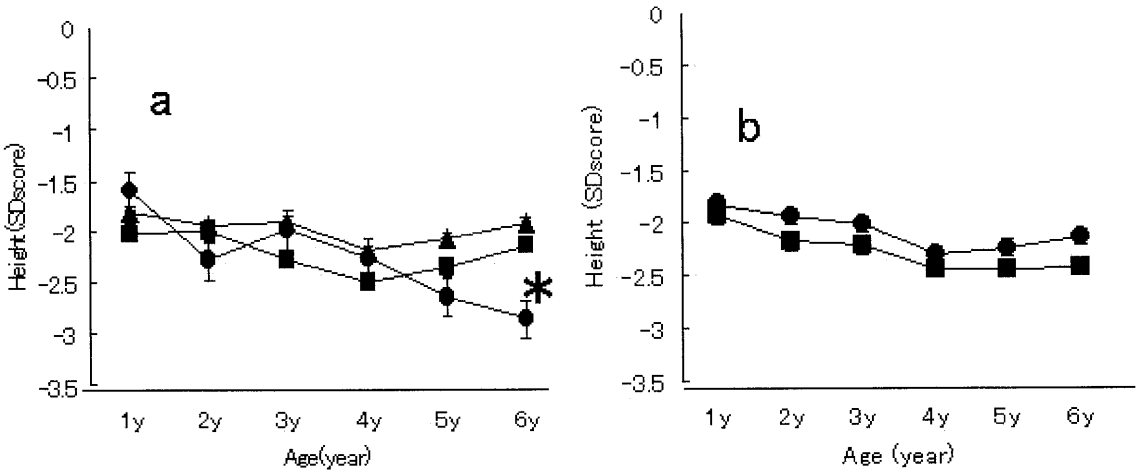

Fig. 2 Changes in the SD score for height in preschool children with partial growth hormone deficiency among 3 genotypes of each polymorphism as determined by one-way analysis of variance (ANOVA), followed by Fisher's protected least significant difference (LSD).
a: β2-adrenergic receptor polymorphisms (●: Arg16Arg, ■: Arg16Gly, ▲: Gly16Gly), b: β3-adrenergic receptor polymorphisms (●: Trp64Trp, ■: Trp64Arg & Arg64Arg), *: $p < 0.05$.

overweight is the most commonly used index to measure obesity in childhood. Some reports have indicated that percent overweight has a good association with the severity of complications of obesity[10].

The β2-AR regulates energy expenditure by stimulating the metabolism (breakdown) of fat in adipose tissue, and therefore, may play an important role in the development of human obesity. Because of the limited number of Gln/Glu and Glu/Glu genotypes of the Gln27Glu polymorphism in Japanese subjects, we studied the Arg16Gly polymorphism of the β2-AR gene. The Arg16Gly polymorphism occurs in the extracellular N-terminus and is believed to regulate the β2 receptor function. The Gly16 form has been associated with lower receptor density, and hence reduced efficiency, as compared with the Arg16 form, which may influence the propensity to gain weight in early childhood. The Arg16Gly polymorphism has not been widely studied in relation to obesity, but available evidence suggests

that it is not associated with obesity in European women and Japanese men, although limited evidence suggests a possible association with obesity in Japanese women[11)12)].

Recently, molecular mechanisms underlying the Trp64Arg polymorphism of the β3-AR gene were studied using an in-vitro transfection technique, and it was revealed that the response to various β3-adrenergic agonists (ie maximal cAMP accumulation) of the cell with the mutated receptor was significantly reduced[13)]. In addition, Umekawa et al reported that the Trp64Arg polymorphism of the β3-AR gene was associated with lower lipolytic activity induced by a β3-adrenergic agonist[14)]. These data suggest that the weaker response to β3-adrenergic stimulation in cases with the Trp64Arg mutation of the β3-AR gene was an important key for the association of this mutation with obesity. Previous reports suggested that the allele frequency of the Trp 64Arg mutation of the β3-AR gene was higher in Japanese than in Caucasians, African-Americans and Mexican-Americans, although the frequency was the highest in Pima Indians[15)]. Some studies attempted to clarify the association between this polymorphism and obesity in Japanese subjects. Kadowaki et al reported that the BMI of subjects with the Arg/Arg genotype was significantly higher than that of those with the Trp/Trp genotype among non-diabetic and obese subjects[16)]. Fujisawa et al also reported that the Arg/Arg genotype was associated with a significantly higher BMI than the Arg/Trp and Trp/Trp genotypes. These reports are consistent with our findings that polymorphism of the β3-AR gene is associated with the propensity to gain weight in preschool children.

GH has many effects on metabolism in addition to promoting growth. Lipolysis of fat cells could be stimulated by GH. Children with GH deficiency usually present with total obesity, especially in the trunk. In this study, we excluded patients with serious GH deficiency and attempted to make the subject population as homogeneous as possible, to ensure absence of differences in GH response to stimuli among subjects with different genotypes of the β3-AR gene.

A question that arises from the results of this study is why were the differences in the anthropometric data not observed in the lower aged children during this study. According to the Infancy-Childhood-Puberty growth model proposed by Karlberg and Albertsson-Wikland, the growth in infants is influenced by fetal growth and nutrition[17)]. The exact age at which GH begins to regulate linear growth significantly is not known, although it has been argued that it takes place toward the end of the first year of life. The contribution of the Trp 64Arg polymorphism to obesity may be masked by fetal growth, which controls the growth from the fetal stage to 3 years of age.

Differences in the response to GH treatment among children with various genotypes of the β2-AR gene were reported, while no difference was found in the SDS for height between cases with various Gly 64 Arg polymorphisms of the β3-AR gene[18)]. The present data are consistent with the aforementioned findings. Abdenur et al reported an inverse correlation between GH secretion and the indices of adiposity in children with idiopathic short stature[19)]. We speculate that the Arg16Gly variant could have an effect on the β2-AR gene function in GH deficiency subjects by lowering the β2-AR mediated lipolytic rates. Therefore, further studies will be needed to clarify the relationship between these polymorphisms and the sensitivity to GH.

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部分型成長ホルモン分泌不全を伴う乳幼児肥満の原因としてのβアドレナリン受容体遺伝子多型

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成人領域では，肥満に影響を及ぼす様々な遺伝子多型が近年発見され，βアドレナリン受容体もその1つである．今回，東医療センター小児科に成長ホルモン治療のため通院している男児27人，女子19人を対象として，1～6歳までの身体所見（身長，体重）縦断的データをβ2, 3アドレナリン両遺伝子多型群間で比較検討を行った．日本人に多いとされるβ3アドレナリン受容体遺伝子コドン64の変異群では，非変異群と比較して6歳時までの肥満度（%）が+17.7対+1.3と有意に大きく推移していた．β2コドン16では同様の遺伝子変異による差を認めなかった．就学前という低年齢時より肥満度の差を呈していることから，β3アドレナリン受容体遺伝子多型は本邦における小児肥満の原因の1つであることが示唆された．