Molecular analysis of the neuropeptide Y1 receptor gene in human idiopathic gonadotropin-dependent precocious puberty and isolated hypogonadotropic hypogonadism

Karina Cocco Monteiro Freitas, M.D.,[a] *Ginny Ryan, M.D.,*[b] *Vinicius Nahime Brito, M.D,*[a] *Ya-Xiong Tao, Ph.D.,*[b] *Elaine Maria Frade Costa, M.D.,*[a] *Berenice Bilharinho Mendonca, M.D.,*[a] *Deborah Segaloff, Ph.D.,*[b] *and Ana Claudia Latronico, M.D.*[a]

[a] Developmental Endocrinology Unit, Hormone and Molecular Genetics Laboratory, Clinical Hospital, São Paulo University Medical School, São Paulo, Brazil, and [b] Department of Physiology and Biophysics, The University of Iowa Roy J. and Lucille A. Carver College of Medicine, Iowa City, Iowa

Objective: To investigate the role of mutations or polymorphisms in the *NPY-Y1R* gene in human idiopathic central pubertal disorders.
Design: Molecular studies.
Setting: University hospital.
Patient(s): Thirty-three patients with gonadotropin-dependent precocious puberty, 22 with hypogonadotropic hypogonadism, and 50 controls.
Intervention(s): Genomic DNA extraction, *NPY-Y1R* gene sequence analysis, cell-surface expression, and functional activity of an identified receptor variant.
Main Outcome Measure(s): Results of sequencing, cell-surface receptor expression, and receptor function.
Result(s): A heterozygous substitution of lysine (K) by threonine (T) at position 374 in the carboxyl terminal region of NPY-Y1R was identified in a girl with familial GDPP. Her mother, who had pubertal developmental at appropriate age, carried the same genetic variant. Introduction of the K374T variant into an expression vector containing the human *NPY-Y1R* complementary DNA led to a partial reduction in cell-surface expression of NPY-Y1R in transiently transfected HEK293 cells. This mutation did not lead to a significant reduction in NPY-stimulated activity of the receptor in this heterologous expression system. No other allelic variants of the *NPY-Y1R* gene were identified in patients or controls.
Conclusion(s): We have identified an inherited heterozygous variant of the *NPY-Y1R* gene in a girl with precocious puberty; however, this most likely did not contribute to her phenotype. Mutations of the highly conserved *NPY-Y1R* gene do not appear to represent a frequent mechanism underlying human idiopathic central pubertal disorders. (Fertil Steril® 2007;87:627–34. ©2007 by American Society for Reproductive Medicine.)

Key Words: GnRH secretion, neuropeptide Y (NPY), precocious puberty, hypogonadotropic hypogonadism

Normal human puberty begins at the end of the first decade of life and is characterized by the development of secondary sexual characteristics, growth acceleration, and the achievement of reproductive function (1). The activation of the ovary and testis during puberty leads to a dramatic increase in gonadal steroid production and completion of gametogenesis (1).

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Reprint requests: Karina Cocco Monteiro Freitas, M.D., Hospital das Clínicas, Faculdade de Medicina da Universidade de São Paulo–Disciplina de Endocrinologia e Metabologia, Av Dr Eneas de Carvalho Aguiar, 155-2° andar Bloco 6, 05403900, São Paulo, SP, Brasil (FAX: 55-11-306-9-7519; E-mail: karinamf@brturbo.com.br).

The pulsatile secretion of GnRH by a network of hypothalamic neurons is critical for pituitary gonadotropin secretion and, consequently, to gonadal steroid production during normal puberty (2, 3). The ability of hypothalamic GnRH neurons to generate a pulsatile discharge of GnRH originates during early fetal development and is subsequently restrained during childhood by several neurotransmitters (3, 4).

Neuropeptide Y (NPY) is considered one of the major components in the inhibition of the pulsatile GnRH secretion during the prepubertal period in nonhuman primates (5). It has been shown, for example, that the central administration of NPY to adult female monkeys causes inhibition of pulsatile GnRH release (6). More recently, El Majdoubi et al. (7) reported that the postnatal pattern of GnRH pulse generator activity was inversely related to the NPY gene and protein expression in the mediobasal hypothalamus of male rhesus monkeys. These findings suggest that NPY is a funda-

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mental component of the neurobiological brake-restraining pubertal onset in primates.

NPY mediates its effects through the activation of at least six different receptor subtypes (8, 9). The Y1 subtype, an inhibitory G-protein–coupled receptor, has been the subtype most frequently implicated in the effects of NPY on GnRH secretion (10). However, the role of NPY and its receptors in human puberty remains unclear.

Idiopathic forms of precocious and delayed puberty are not infrequent in humans, and genetic defects may be responsible for a significant proportion of these conditions. In the present study, we hypothesize that mutations of the human *NPY-Y1R* gene may be associated with idiopathic central pubertal disorders such as idiopathic gonadotropin-dependent precocious puberty (GDPP) and hypogonadotropic hypogonadism.

MATERIALS AND METHODS

Patients

We selected 55 Brazilian patients belonging to 52 families with idiopathic pubertal disorders who were willing to participate in this study. Thirty-three of these patients had GDPP, and 22 had permanent isolated hypogonadotropic hypogonadism (HH) without olfactory abnormalities.

Written informed consent was obtained from the subjects and/or their parents, depending on the subject's age. Patient recruitment and DNA sequencing protocols were approved by the Ethical Committee of the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil.

The sense of smell was tested in all patients with HH by using the Smell Identification Test, which was developed at the University of Pennsylvania (www.smelltest.com). Other forms of HH, such as transitory gonadotropin deficiency or HH caused by chronic or functional diseases were excluded from this study. GnRH receptor mutations had previously been ruled out in these patients (11). Fifty unrelated Brazilian subjects (age ranged from 18–40 years, 28 females and 22 males) who experienced normal pubertal development at the appropriate chronological age and normal fertility were used as controls. Chronic diseases were ruled out in these subjects.

Pertinent demographic, clinical, and family history data on each of the subjects were gathered. Family history revealed 6 patients with familial GDPP and 6 with familial HH. Basal and/or GnRH-stimulated gonadotropin levels and estradiol or testosterone levels were obtained for all patients with GDPP and HH.

Clinical and hormonal data from the patients are shown in Tables 1 and 2. Thirty-one patients with GDPP were females, and two were males. Chronological age at the onset of pubertal development ranged from 0.66–7.0 years, and age at first evaluation ranged from 1.6–10.2 years. Basal and/or GnRH-stimulated gonadotropin levels were within the pubertal range for all GDPP patients (basal LH $>$0.6 U/L and/or after GnRH-stimulation test $\geq$6.9 UI/L in girls and $\geq$9.6 UI/L in boys by immunofluorometric assays [IFMA]), and a satisfactory response to GnRH analog therapy was shown by all of these patients.

There were 14 males and 8 females among the subjects with HH. The chronological age in this group ranged from 18–30 years at first evaluation. All of them were followed up for a long period (range from 5–10 years). Basal and GnRH-stimulated gonadotropin levels were within the prepubertal or normal range in all patients. In the male subjects with HH, testosterone ranged from 14–54 ng/dL (486–1,875 pmol/L), whereas estradiol levels ranged from $<$13–15 pg/mL ($<$47–55 pmol/L) in the females with HH. All patients had a normal magnetic resonance imaging of the central nervous system, indicating an idiopathic cause of their pubertal disorders.

Hormonal Assays

Serum LH, FSH, estradiol, and testosterone were measured by immunofluorometric assays (Delfia; Wallac, Inc., Turku, Finland). The coefficient of variation was 5% or less for all assays. The lower limit of detection was 0.6 IU/L for LH, 1.0 IU/L for FSH, 13 pg/mL (47 pmol/L) for estradiol, and 14 ng/dL (0.6 nmol/L) for testosterone (12). Serum LH and FSH were also measured at $-$15, 0, 15, 30, 45, and 60 minutes after 100 μg IV of GnRH. The results were compared with normal values established in our population and previously published (12, 13).

DNA Analysis

Genomic DNA was extracted from peripheral blood leukocytes using standard procedures. The *NPY-Y1R* gene, located at chromosome 4q(31.3-32), is composed of 3 exons (www.ncbi.nml.nhi.gov, NM 000909). Exons 2 and 3, the translated region of this gene, were amplified by using 30 pmol of each PCR primer, 200 μmol of each deoxynucleotide triphosphate, 2.5 U *Taq* polymerase (Pharmacia, Upsala, Sweden), 10 mmol/L Tris-hydrochloride (Tric-HCl) (pH 8.3), and 200 ng genomic DNA. The amplified 1,240-bp fragment included exon 2 (698 bp), intron 2 (97 bp), and exon 3 (445 bp).

The primers used for amplification of exons 2 and 3 were as follows: 5′-GCTGAACAGTTGACCTGCTTTG-3′ and 5′-GGAGAACAGGT AATCAAAGTATGTTGCAGG-3′, whereas an additional inner primer, 5′-GTGAGGCGATGTGTAAG-3′, was used for sequencing. A negative control was used in each PCR.

Amplification was performed in a Gene Amp 9600 system (Perkin-Elmer Corp., Foster City, CA) using 40 cycles. DNA was denatured at 95°C for 5 minutes in the first cycle and for 30 seconds in all subsequent cycles. Annealing was per-

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