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ORIGINAL

Novel C617Y mutation in the 7th transmembrane segment of luteinizing hormone/choriogonadotropin receptor in a Japanese boy with peripheral precocious puberty

Keisuke Nagasaki[1)], Noriyuki Katsumata[2)], Yohei Ogawa[1)], Toru Kikuchi[1)] and Makoto Uchiyama[1)]

[1)]*Division of Pediatrics, Department of Homeostatic Regulation and Development, Niigata University Graduate School of Medical and Dental Sciences, Niigata 951-8510, Japan*
[2)]*Department of Molecular Endocrinology, National Research Institute for Child Health and Development, Tokyo 157-8535, Japan*

Abstract. Testotoxicosis, also known as familial male-limited precocious puberty, is an autosomal dominant form of gonadotropin-independent precocious puberty caused by heterozygous constitutively activating mutations of the *LHCGR* gene encoding the luteinizing hormone/choriogonadotropin receptor (LH/CGR). The patient is an 8-year-old boy who started to develop pubic hair and penile enlargement at 6 years of age. The patient had elevated serum testosterone levels, but initially exhibited a prepubertal response of gonadotropins to GnRH, which was followed by central activation of the hypothalamo-pituitary-gonadal axis. The father reported having experienced precocious puberty, and is 158 cm tall. There is no history of short stature and precocious puberty in the family except for the father. The *LHCGR* gene was analyzed by direct DNA sequencing of amplified PCR products from the patient and his parents. The wild-type and mutant LH/CGRs were transiently expressed in COS-1 cells and cAMP levels in the cells were determined with or without hCG stimulation. Genetic analysis revealed a novel C617Y mutation of the *LHCGR* gene in the patient and his mother, while his father had no mutations. Functional expression study demonstrated around 15% increase in the basal intracellular cAMP level in cells expressing the mutant LH/CGR compared with that in cells expressing the wild-type receptor. We have reported the first missense C617Y mutation located in the 7th transmembrane segment of LH/CGR causing testotoxicosis. The modest phenotype of our patient may be explained, at least in part, by the modest increase in the intracellular cAMP level caused by the C617Y mutation.

Key words: Peripheral precocious puberty, Testotoxicosis, *LHCGR* gene, Activating mutation, Hypothalamo-pituitary-gonadal axis

TESTOTOXICOSIS, also known as familial male-limited precocious puberty, is an autosomal dominant form of gonadotropin-independent precocious puberty. This disorder results from heterozygous constitutively activating mutations of the *LHCGR* gene encoding the luteinizing hormone/choriogonadotropin receptor (LH/CGR), a member of the large family of G protein-coupled receptors with 7th transmembrane segments [1, 2]. To date, only 16 kinds of *LHCGR* mutations are reported to cause testotoxicosis, most of which are concentrated in the 6th transmembrane segment from the 3rd intracellular loop of the receptor, and none have been described in the 7th transmembrane segment [3, 4].

In the present study, we reported a Japanese boy with peripheral precocious puberty due to a novel C617Y mutation in the 7th transmembrane segment of LH/CGR, followed by central activation of the hypothalamo-pituitary-gonadal axis.

Case Report

The patient is a Japanese boy, who was first seen at the regional hospital at 6 years and 6 months of age because of pubic hair development and penile enlargement which had been noticed since 6 years of age. His height was 118.0 cm (+0.39 SD), and weight was 21.3 kg. Testes were 4 mL bilaterally, and pubic hair development was at Tanner stage 2. Endocrinological findings are summarized in Table 1. Serum testosterone level was elevated, but serum LH and FSH levels were

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Correspondence to: Keisuke Nagasaki, M.D., Division of Pediatrics, Department of Homeostatic Regulation and Development, Niigata University Graduate School of Medicine and Dental Sciences, 1-757, Asahimachi, Niigata 951-8510, Japan.
E-mail: nagasaki@med.niigata-u.ac.jp

Table 1 Endocrinological findings of the patient

	Chronological age (years)			
	6 6/12	7 4/12	8 4/12	
Testosterone (ng/dL)	27.9	118.5	150.2	(<10)
GnRH test				
LH (mIU/mL)				
Basal	<0.6	<0.6	<0.6	(<0.6)
Peak		4.1	9.1	(<7)
FSH (mIU/mL)				
Basal	0.5	0.8	1.5	(0.4-1.1)
Peak		2.6	5.0	(4.4-9.5)
hCG-β (ng/mL)			<0.1	(<0.1)
hCG (mIU/mL)			<1.0	(<1.0)
17-OHP (ng/mL)			0.5	(<0.6)
DHEAS (ng/mL)			202	(77-1280)
ACTH (pg/mL)			25.6	(7.4-55.7)
Free T3 (pg/mL)			3.4	(2.1-3.8)
Free T4 (ng/mL)			1.3	(0.9-1.6)
TSH (μIU/mL)			2.33	(0.6-4.1)

In the parentheses, reference values for prepubertal boys are shown.

in the prepubertal range. Magnetic resonance imaging of the brain was normal. During the following 10 months, his somatic and penile growth continued to be accelerated. When the patient was 7 years and 4 months old, his height was 125.3 cm (+0.65 SD) and weight was 25.3 kg. Serum testosterone level was further elevated, but a GnRH stimulation test revealed a prepubertal response of gonadotropin. The patient was diagnosed as having peripheral precocious puberty, and referred to our hospital for further examination.

When referred at the age of 8 years and 4 months, his height was 132.9 cm (+1.26 SD), and weight was 27.7 kg. Penile length was 7.5 cm, and testicular volume was 6 mL bilaterally. Pubic hair was found only in the scrotal region, remaining at Tanner stage 2. His bone age was 10 years and 4 months. Serum testosterone level was elevated, and a GnRH stimulation test revealed a pubertal response to gonadotropin. Serum concentrations of 17α-hydroxyprogesterone, dehydroepiandrosterone sulfate, hCG-β and hCG were not elevated and thyroid function was within normal ranges. No tumor lesions were found on thoracic and abdominal computerized tomography.

His family history was negative for short stature and precocious puberty except for the father, who allegedly experienced precocious puberty, and is 158 cm tall (-2.2 SD). The mother is 160 cm tall (+0.36 SD). Based on these clinical and laboratory findings, the patient was diagnosed as suffering from peripheral precocious puberty followed by central activation of the hypothalamo-pituitary-gonadal axis. The patient was put on treatment with subcutaneous GnRH analogue injections and oral medroxyprogesterone acetate administration. Serum testosterone level decreased from 150.2 to 34 ng/dL over 3 months of treatment.

Materials and Methods

Genetic analysis of the LHCGR and CYP21A2 genes

The genetic study was approved by the Institutional Ethical Review Board at the National Center for Child Health and Development. Written informed consent for the genetic analysis was obtained from the parents.

The entire exon 11 of the *LHCGR* gene was amplified as previously reported [5]. The amplified PCR products were fractionated and isolated on a 1% agarose gel (Bio-Rad Laboratories, Richmond, CA, USA), and directly sequenced using a Thermo Sequenase II Dye Terminator Cycle Sequencing Premix kit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) and ABI PRIM 310 Genetic Analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan). Southern blot and sequencing analyses of the *CYP21A2* gene were performed as described previously [6].

In vitro expression of the wild-type and mutant LH/CGR

The pcDNA3-hLHR plasmid in which the wild-type human *LHCGR* complementary DNA (cDNA) is ligated to a mammalian expression plasmid pcDNA3 was provided by Dr. T. Minegishi [7]. To create the C617Y mutant *LHCGR* cDNA, a *Bst*X I-*Dra* III (New England Biolabs, Inc., Beverly, MA, USA) segment of pcDNA3-hLHR corresponding to c.1554-c.2093 of the *LHCGR* cDNA was replaced by a DNA fragment created by the *Bst*X I-*Dra* III cleavage of the above described PCR products from the patient's DNA. The resultant constructs were sequenced to confirm the validity, and the construct harboring the C617Y mutation but no other mutations was designated as the pcDNA-C617Y mutant.

COS-1 cells (2×10^6) (RIKEN Cell Bank, Tsukuba, Japan) were transfected by electroporation (Gene Pulser II, Bio-Rad Laboratories, Hercules, CA, USA) with 2 μg of pcDNA3-hLHR, pcDNA3-C617Y mutant, or the empty pcDNA3 plasmid. In addition, 1 μg of pRK-GH1, a human growth hormone (hGH) expression plasmid, was included in the transfection mix-

ture, and used as an internal control for transfection efficiency. The cells were suspended in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corp., San Diego, CA, USA) containing 10% fetal calf serum and transferred to 35 × 10 mm FALCON tissue culture dishes (Nippon Becton Dickinson Co., Ltd., Tokyo, Japan). At 48 h after transfection, the media were collected, and the amount of hCG was determined by an immunoradiometric assay kit (Fujifilm RI Pharma Co., Ltd., Tokyo, Japan). The cells were incubated with 0, 1, 10 or 100 mU hCG (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in 1 mL of DMEM containing 0.1% bovine serum albumin (Sigma-Aldrich Corp., St. Louis, MO, USA), 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer, pH 7.5 (Nakalai Tesque, Inc., Ltd., Kyoto, Japan) and 1 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich Corp., St. Louis, MO, USA). After 1 h of incubation, the media were removed, and cAMP was extracted from the cells with 1 mL of 0.1 N HCl. The amount of cAMP was determined by a radioimmunoassay kit (Yamasa Corp., Ltd., Chiba, Japan). The experiments, each performed in duplicate, were repeated four times, and the results were presented as the means ± SEM (n=4). Dose-response curves fitted to the 4-parameter logistic equation were created using the SoftMax Pro software (Molecular Devices Corp., Sunnyvale, CA, USA). Statistical analysis was performed using unpaired *t* test.

Results

Genetic analysis of the LHCGR and CYP21A2 genes

Sequencing analysis of the *LHCGR* gene revealed a heterozygous G to A transition at the second nucleotide of codon 617 (c.1850G>A) in the patient (Fig. 1). This transition substituted tyrosine for cysteine at codon 617 (p.C617Y). The mother was heterozygous for the p.C617Y mutation, while the father did not carry the mutation.

Southern blot analysis of genomic DNA from the patient and the parents revealed neither large conversions nor large deletions of *CYP21A2* gene. Sequencing analysis of the entire *CYP21A2* gene revealed no mutations in either the patient or the parents.

Functional analysis of the mutant LH/CGR

COS-1 cells expressing the p.C617Y mutant LH/CGR exhibited a significantly higher basal cAMP level

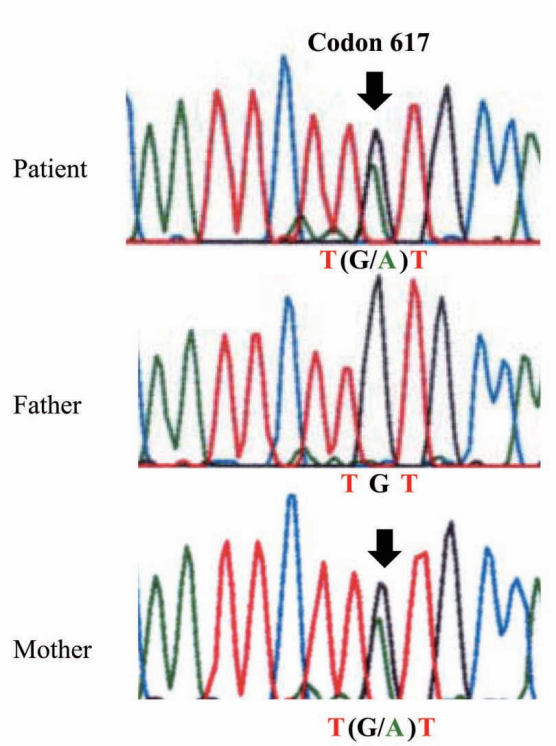

Fig. 1 Mutation analysis of the *LHCGR* gene. Electrochromatograms show a heterozygous mutation (c.1850G>A, p.C617Y) in the patient and his mother as indicated by arrows. His father does not have the mutation.

than those expressing the wild-type LH/CGR (30.7 ± 2.3 *vs.* 26.7 ± 1.1 pmol/dish, p=0.0081) (Fig. 2). Cells expressing the wild-type LH/CGR increased intracellular cAMP levels dose-dependently in response to hCG stimulation, as did those expressing the mutant LH/CGR (Fig. 2). Despite the higher basal cAMP level in cells expressing the mutant LH/CGR, the hCG-stimulated cAMP levels were significantly lower than those in cells expressing the wild-type LH/CGR (p=0.0487, 0.0002, and 0.0002 at 1, 10, and 100 mIU/mL hCG, respectively). EC_{50} values of cells expressing the wild-type and mutant LH/CGR were 15.4 and 18.7 mU/mL, respectively.

Discussion

In the present study we describe a C617Y mutation in the *LHCGR* gene in a Japanese boy with peripheral precocious puberty. The p.C617Y mutation has not been previously reported according to "The Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff" (http://www.hgmd.cf.ac.uk) and PubMed search (http://www.ncbi.nlm.nih.gov/

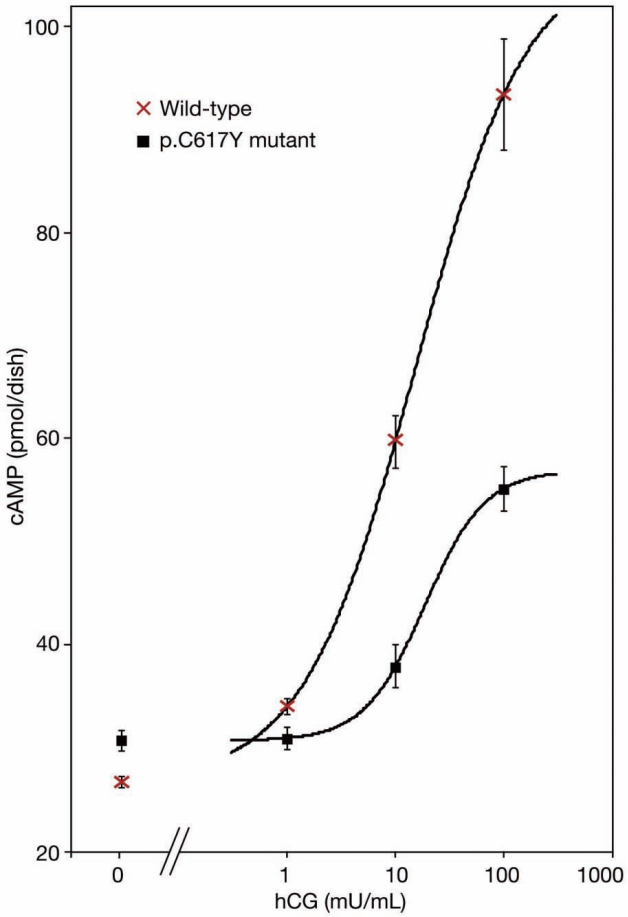

Fig. 2 Basal and hCG-stimulated levels of cAMP in COS-1 cells expressing the wild-type or p.C617Y mutant LH/CGR. Cells expressing the p.C617Y mutant LH/CGR exhibits a significantly higher basal cAMP level, but lower hCG-stimulated cAMP levels than those expressing the wild-type LH/CGR.

Human_LH/CGR	I	N	S	C	A	N	P	(617)
Pig_LH/CGR	V	N	S	C	A	N	P	(617)
Mouse_LH/CGR	V	N	S	C	A	N	P	(621)
Rat_LH/CGR	V	N	S	C	A	N	P	(621)
Zebrafish_LH/CGR	I	N	S	C	A	N	P	(627)
Human_TSHR	L	N	S	C	A	N	P	(672)
Pig_TSHR	L	N	S	C	A	N	P	(672)
Mouse_TSHR	L	N	S	C	A	N	P	(672)
Rat_TSHR	L	N	S	C	A	N	P	(672)
Zebrafish_TSHR	L	N	S	C	A	N	P	(445)
Human_FSHR	I	N	S	C	A	N	P	(620)
Pig_FSHR	I	N	S	C	A	N	P	(620)
Mouse_FSHR	I	N	S	C	A	N	P	(619)
Rat_FSHR	I	N	S	C	A	N	P	(619)
Zebrafish_FSHR	I	N	S	C	S	N	P	(601)

Fig. 3 Comparison of amino acid sequences surrounding C617 of human LH/CGR. C617 of human LH/CGR is a highly conserved amino acid beyond species and also conserved among the glycoprotein receptor family as shown by the box. C617 of human LH/CGR corresponds to C672 of human TSHR. In the parentheses amino acid positions of the conserved cysteine residue are shown.

pubmed/), thus this mutation appears to be a novel mutation. Furthermore, the p.C617Y mutation is the first activating mutation reported to be located in the 7th transmembrane segment of LH/CGR. Cys617 of LH/CGR is a highly conserved residue showing complete identity in all the vertebrate glycoprotein hormone receptors (Fig. 3). Of note, a p.C672Y mutation in the *TSHR* gene, equivalent to the p.C617Y mutation in the *LH/CGR* gene, is reported to constitutively activate adenylyl cyclase and to cause non-autoimmune autosomal dominant hyperthyroidism [8]. Thus, the p.C617Y mutant LH/CGR is presumed constitutively active.

Our functional expression study indicates that cells expressing the p.C617Y mutant LH/CGR have a higher intracellular cAMP level at basal state than those expressing the wild-type LH/CGR, thus the p.C617Y mutant LH/CGR is confirmed to be constitutively active. Because increased production of intracellular cAMP mediates LH effects *via* LH/CGR, including testosterone production, we postulate that intracellular cAMP accumulation in Leydig cells triggered by the mutant allele is responsible for the gonadotropin-independent production of testosterone in our patient. Cells expressing the mutant LH/CGR respond to hCG with an increase in intracellular cAMP levels, as do those expressing the wild-type receptor. Since the EC_{50} values are similar, there seems to be no significant difference in the sensitivity to hCG between the wild-type and mutant receptors. By contrast, cells expressing the mutant receptor clearly displayed a blunted maximum response to hCG as compared to those expressing the wild-type receptor. This may be caused by the decreased number of plasma membrane binding sites in cells expressing the mutant receptor, as shown in cells expressing the p.C672Y mutant TSHR which display a decrease in TSH binding as well as a decrease in cAMP production in response to TSH [8].

Typically, patients with testotoxicosis present by age 1-4 years with signs of puberty, rapid virilization, growth acceleration and skeletal advancement [5, 9-12]. By contrast, our patient presented at age 6 years with pubic hair development and penile enlargement,

and the tempo of their development was not rapid progression. These modest phenotypes may reflect unique characteristics of the mutant LH/CGR of the patient. The p.C617Y mutation results in 15% increase in basal intracellular cAMP over the wild-type receptor, while the p.D578A mutation in the 6th transmembrane segment, accounting for majority of testotoxicosis cases, leads to 3-5 fold increases in basal intracellular cAMP [1, 9]. Obviously other genetic and environmental factors might also be responsible for the modest phenotype of our patient since significant phenotypic heterogeneity is well recognized in testotoxicosis patients with *LHCGR* mutations especially in those with the p.M398T and p.D564G mutation [5, 10-12].

Another clinical feature to be pointed out in our patient is that he showed a pubertal response to GnRH when he was first examined at our hospital at 8 years of age. If he had not been endocrinologically examined in the regional hospital, he would have been diagnosed as suffering from idiopathic central precocious puberty. Therefore, testotoxicosis should be taken into account when seeing male patients with sexual precocity even if the age at onset is atypical and gonadotropin levels are not in the prepubertal range.

Regarding the treatment of testotoxicosis, different pharmacological regimens have been used. Some reports describe the achievement of normal adult height despite the lack of therapy in affected males [5, 12]. Recently, a combination therapy consisting of anastrozole, a third generation aromatase inhibitor, and bicalutamide, a nonsteroidal anti-androgen, has been reported to be efficacious in decreasing growth rates and skeletal maturation while controlling signs of virilization [13]. Regrettably, these medications are not indicated for testotoxicosis in Japan. Since our patient has already developed secondary gonadotropin-dependent precocious puberty, he is treated with the GnRH analogue and medroxyprogesterone acetate administration, to which he responds well although the observation period is limited. The prognosis of adult height may not be poor in our case, since he has a mild clinical phenotype and the mutant LH/CGR exhibits a modest increase in basal intracellular cAMP *in vitro*. Long-term follow-up is required regarding final adult height in our patient.

The mother harboring the heterozygous p.C617Y mutation in *LHCGR* has no phenotype as previously reported females with other heterozygous activating *LHCGR* mutations, which may be explained by low or absent LH receptor expression in gonads of prepubertal girls [3]. By contrast, the father who presented with sexual precocity has no mutations in *LHCGR*, excluding testotoxicosis as a cause of sexual precocity. The father as well as the patient has no mutations in *CYP21A2*, which rules out nonclassic or simple virilizing steroid 21-hydroxylase as a cause of sexual precocity in the father as well as in patient. We suspect the father as having suffered from idiopathic precocious puberty.

In summary, we have described a novel mutation in the 7th transmembrane segment of LH/CGR in a Japanese boy with peripheral precocious puberty.

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