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Mutations in *NR5A1* and *PIN1* associated with idiopathic hypogonadotropic hypogonadism

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ABSTRACT. We tested the hypothesis that mutations in *NR5A1* and *PIN1* cause disorders in gonadotropin-gonadal system development and function, throught direct DNA sequencing of the coding sequence and splice-sites of *NR5A1* and *PIN1* in 50 subjects with sporadic idiopathic hypogonadotropic hypogonadism. These patients were recruited from the Pediatrics section of Tongji Hospital, Tongji Medical College, in Wuhan, China. None of the affected subjects had clinical signs of adrenal insufficiency. The *NR5A1* and *PIN1* mutations were found in 7 of the 50 cases. These 7 individuals presented severely low serum concentrations of testosterone or of estradiol and gonadotropin. Adrenal insufficiency was not diagnosed in any of these patients. Consequently, *NR5A1* and *PIN1* mutations should be considered in idiopathic hypogonadotropic hypogonadism patients with normal karyotypes and without adrenal insufficiency.

Key words: Hypogonadotropic hypogonadism; Gene mutations; *NR5A1*; *PIN1*

INTRODUCTION

Idiopathic hypogonadotropic hypogonadism (IHH) can present anosmia, which is known as Kallmann syndrome (KS) and is characterized by total or partial loss of olfaction (Hardelin and Dode, 2008). Although autoimmune, environmental, and genetic factors contribute to IHH, there is no known cause in the majority of cases. In the past decades, a number of genes have been identified in the pathogenesis of IHH. Nevertheless, identification of genetic causes of IHH has been difficult elusive. For example, mutations in *GPR54*, *FGF8*, *FGFR*, *NELF*, *KAL1*, *GnRH*, *GnRHR*, *GnRH1*, *PROKR2*, *CHD7*, *LHRH*, *PROK2*, *TAC3*, *TACR3*, *SF1*, and *DAX-1* are associated with IHH. Still, these genetic defects account for only about 30% of all cases of IHH (Crowley et al., 2008). This suggests that there are additional unknown factors that remain to be identified. The orphan nuclear receptor steroidogenic factor 1 (*SF1*), also called Ad4BP, is encoded by the *NR5A1* gene and is an essential regulator of endocrine function and development. It regulates the expression of both gonadotropin hormones in the pituitary and steroidogenic enzymes in the gonad (Schimmer and White, 2010). Newborn mice lacking *NR5A1* exhibited a complex endocrine phenotype, including adrenal and gonadal agenesis, impaired expression of pituitary gonadotropins, and absence of the ventromedial hypothalamic nucleus (Ingraham et al., 1994; Shinoda et al., 1995; Achermann et al., 2001). PIN1 is a peptidyl-prolyl cis-trans isomerase that catalyzes the isomerization of phosphorylated Ser/Thr-Pro peptide bonds. *PIN1* knock-out mice show marked abnormalities in their reproductive development and function (Atchison and Means, 2003). Recently, the important developments of genetics have occurred in the disorder of sex development field.

MATERIAL AND METHODS

Laboratory assays

Evaluation of basal hormones in the hypothalamus-pituitary-gonad axis consists of measuring serum testosterone, estradiol, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) by enzyme-linked immunosorbent assay. When gonadal hormones or gonadotropins are low or inappropriately low in relation to the testosterone or estradiol levels, it indicates impairment of the hypothalamic-pituitary axis. In such case, magnetic resonance imaging of the pituitary should be considered.

Patients

Over a 2-year period (2008-2010), we studied 50 adolescent patients with sporadic primary amenorrhea and IHH from our pediatric endocrine clinic or genetics laboratory. Neither adrenal insufficiency nor syndromic features were reported in any of the patients. Patients with normal chromosomes, gonadal dysgenesis, and obvious defects in androgen and estrogen biosynthesis and action were included. The phenotypic spectrum of patients included: 1) prepubertal testicular volume <4 mL, absence of secondary sexual features (e.g., deepening of the voice, axillary and facial hair growth, and decreased muscle mass), 2) females with little or no breast development and primary amenorrhea, 3) absence of chronic disease in patients with

certain conditions (e.g., obesity, hypothyroidism). The patients' clinical data were collected, and the correlation between genotype and phenotype was analyzed.

DNA amplification and sequence analysis

Informed consent was obtained from participants in the study and their parents. The Ethics Committee of Tongji Hospital, affiliated with Tongji Medical College, Huazhong University of Science and Technology approved this study. Genomic DNA was extracted from peripheral blood leukocytes using a Tiangen Biotech RelaxGene Blood DNA System. The entire exons and splice sites of *NR5A1* and *PIN1* were amplified by polymerase chain reaction (PCR). Oligonucleotide primers were designed with the Primer 5.0 software (Table 1). After amplification, the PCR products were purified using Shrimp and Alkaline Phosphatase and Exonuclease (Fermentas Company). Sequencing of these genes was performed on PCR-amplified target regions using the ABI Prism Dye Terminator Cycle Sequencing Kit and an ABI 96-capillary 3730xl DNA analyzer.

Table 1. Exon and splice sites of the *NR5A1* (*SF1*) and *PIN1* genes PCR primer and annealing temperature as follow.

Exon	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temperature (°C)	Length (bp)
SF1-Exon2-3	gggcacagagaggggatta	aagagtcatctccatagggttca	60.7	626
SF1-Exon4	tgaaagggtgttgagcagg	tgaagccagtgggaaggat	60.8	849
SF1-Exon5	gtagatgggcacagagaggtta	ggctgctcacagagggttt	60.8	431
SF1-Exon6	cctgcacctccaatccatg	gccctcagacctttgttcact	62.9	420
SF1-Exon7-1	tggtgacgatggtggtgtt	ccaagcagcaggcaagtg	62.6	699
SF1-Exon7-2	ctgcccctgagttctgacac	aatgacccagcaccaccc	63.0	821
SF1-Exon7-3	gcttggggattgccactaa	aaagagggtctgcgtgcc	60.7	765
PIN1-Exon1	ccagcctttcttctacccaat	agcaggggtaggttgtggac	61.5	657
PIN1-Exon2	ggatttgttgaatgaaggcg	gctcaccctcaaatgccag	60.8	533
PIN1-Exon3	cttgggtgtgggtgaggac	gatgccaggaagaaagtgct	64.0	532
PIN1-Exon4	cgctcagcactggcaataa	ccctccccacgacatctt	60.3	818

RESULTS

Mutational analysis of *NR5A1* and *PIN1*

Direct sequencing of *PIN1* from DNA of patient 1 identified a heterozygous c.94A>T mutation in exon 2 (Figure 1), which resulted in a serine (Ser) to cysteine (Cys) substitution in codon 32 (p.Ser32Cys). Patient 2 showed c.734T>G variant (Figure 2). This mutation led to a leucine (Leu) to Tryptophan (Trp) substitution at codon 245(p.leu245Trp). We identified 4 new heterozygous *NR5A1* mutations in patients 3, 4, 5, 6, and 7. Among them, 2 were nucleotide substitutions, c.437G>C (p.Gly146Ala), in patients 4 and 5 (Figure 3); 1 was a synonymous mutation, c.351C>G (p.Gly117Gly), in patient 3 (Figure 4). The other 2 *NR5A1* mutations were in non-coding regions: at position 1655 C>T of patient 6 (Figure 5) and 2973 T>C of patient 7 (Figure 6) in exon 7. Although DNA was not obtained from these patients' siblings and parents, they had apparently normal fertility and reproductive function. Restriction analysis of DNA from 100 racially matched control subjects did not identify any of these variants.

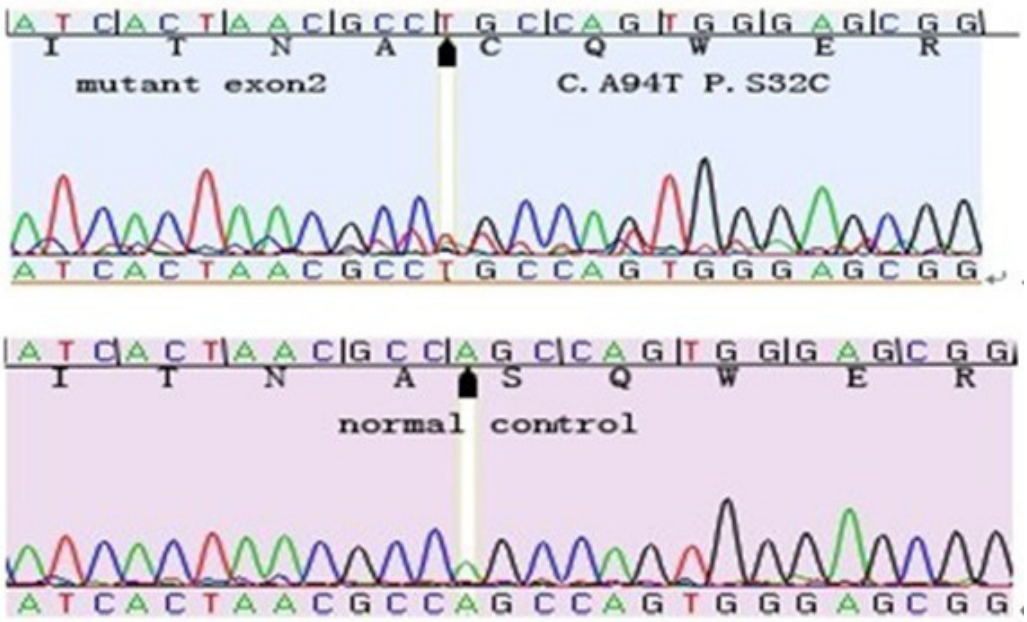

Figure 1. Patient 1 c.94AT(p.S32C).

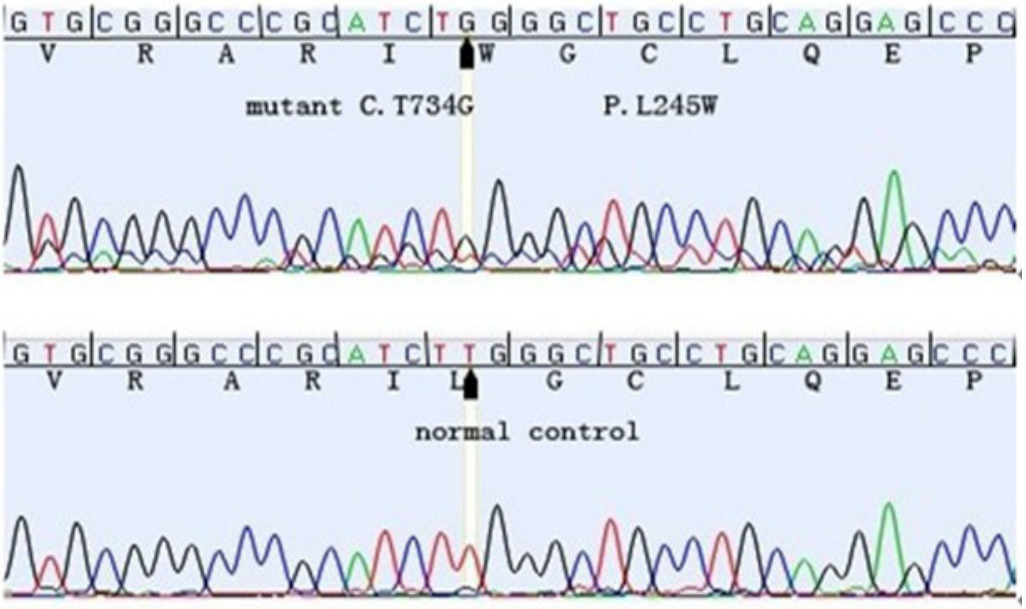

Figure 2. Patient 2 c.734TG(p.L245W).

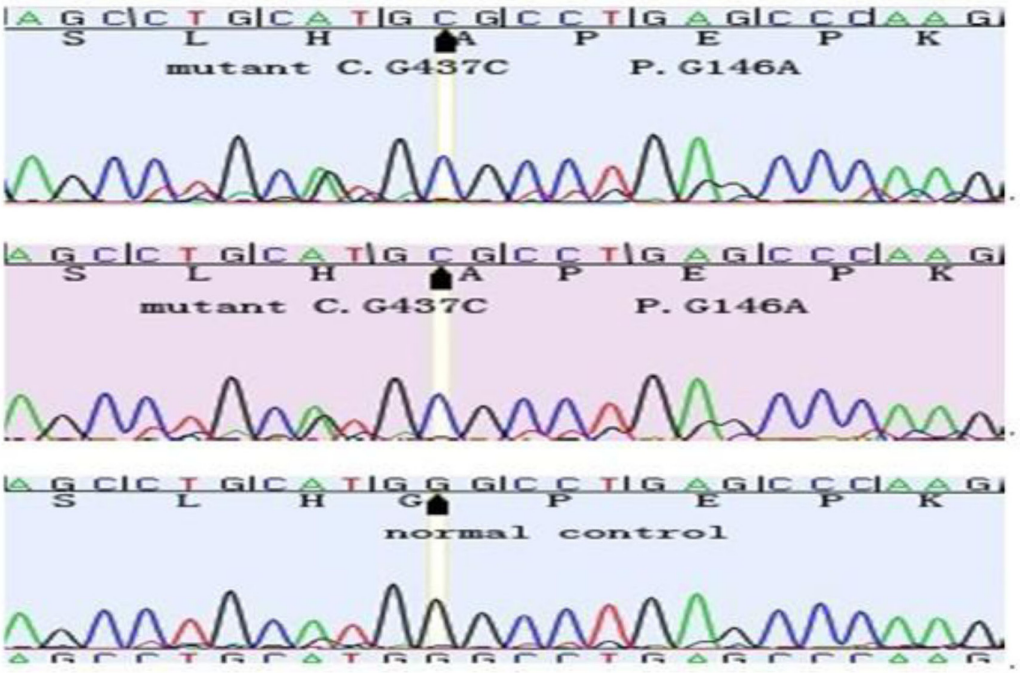

Figure 3. Patients 4 and 5 c.437GC(p.G146A).

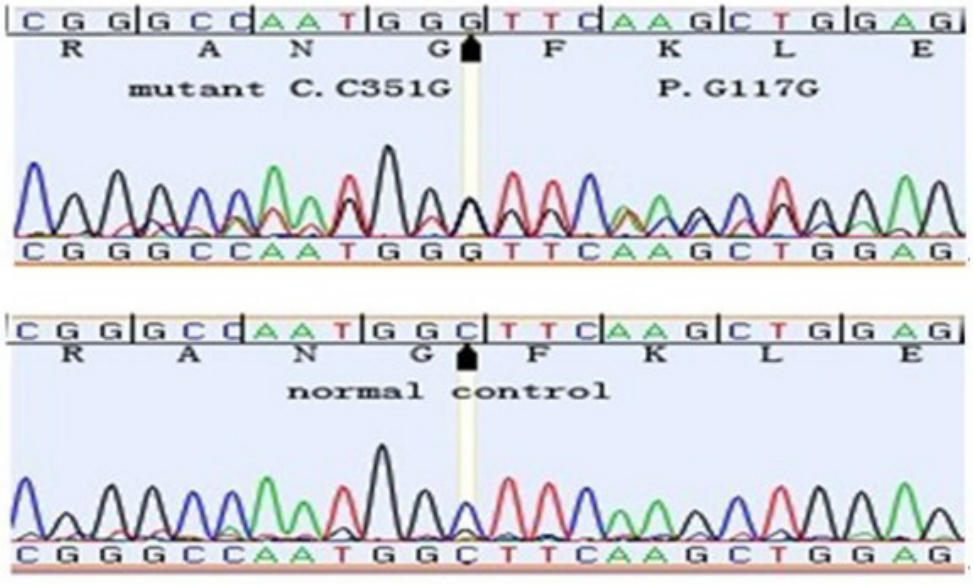

Figure 4. Patient 3 c.351CG(p.G117G).

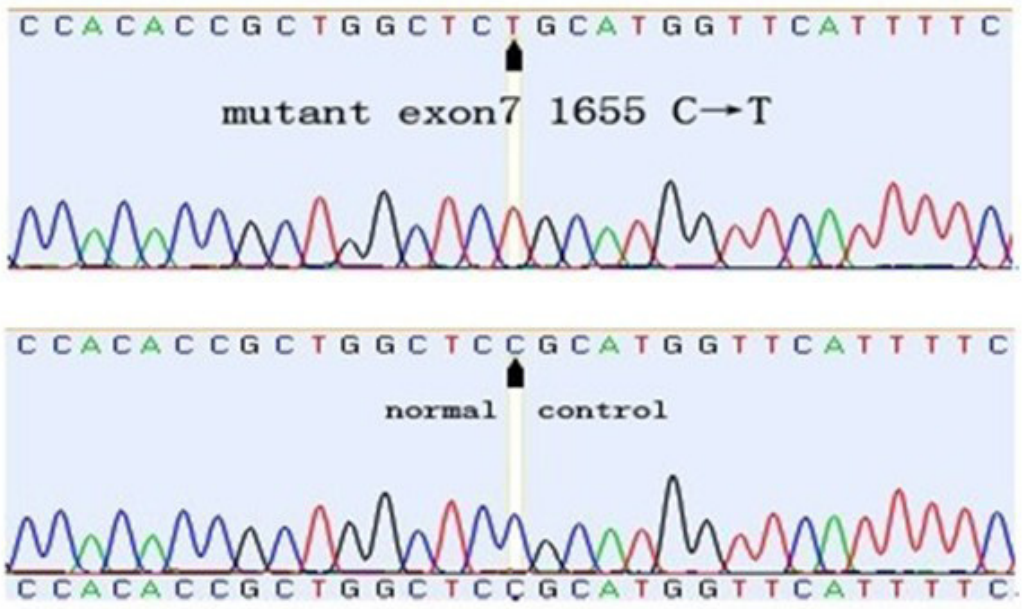

Figure 5. Patient 6 *NR5A1* exon 7 noncoding region 1655 C→T.

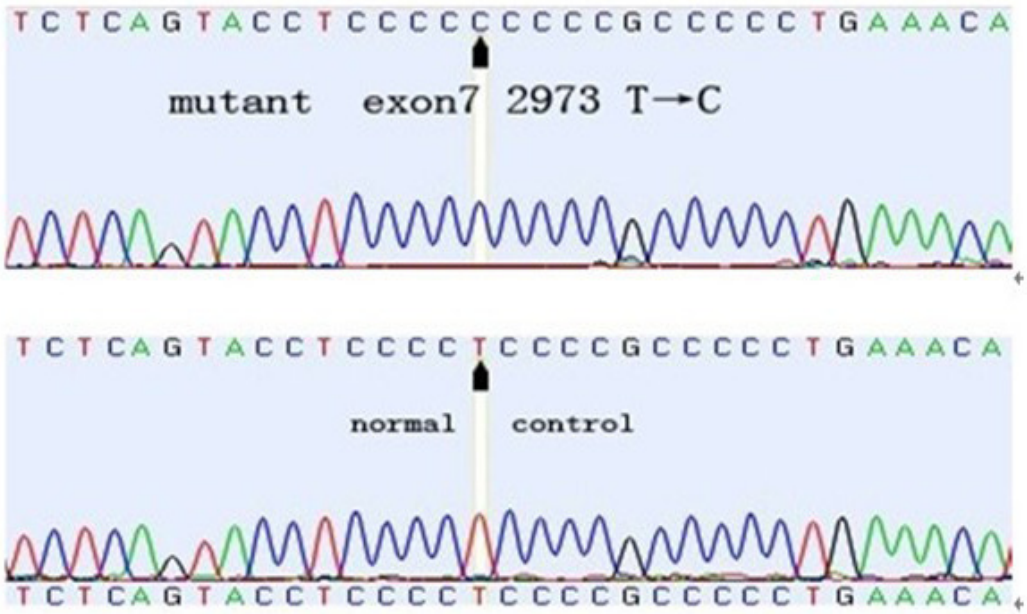

Figure 6. Patient 7 *NR5A1* exon 7 noncoding region 2973 T→C.

Cohort analysis

PIN1 and *NR5A1* mutations were identified in 2 and 5 of the 50 patients studied, respectively. An overview of these changes and associated clinical phenotypes is provided in Table 2. Notably, these changes were found only in patients with inappropriately low serum concentrations of LH and FSH and testosterone or estradiol. *NR5A1* mutations were found in the cohort of patients with primary amenorrhea and uterine or ovarian hypoplasia.

Table 2. Genetic, clinical, and biochemical features of patients with *SF1* and *PIN1* mutations reported here.

	Patient						
	1	2	3	4	5	6	7
Amino acid change	Ser32Cys	Leu245Trp	Gly117Gly	Gly146Ala	Gly146Ala		
Exon or intron	Pin1exon2	sf1exon4	sf1exon4	sf1exon4	sf1exon4	sf1exon7	sf1exon7
Nucleotide change	c. 94 A>T	c.734T>G	c.351C>G	c.437G>C	c.437G>C	e.1655 C>T	e.2973T>C
Karyotype	XY	XY	XY	XX	XY	XX	XX
Phenotype	Male	Male	Male	Female	Male	Female	Female
Age (years)	19	20	22	20	18	22	17
Additional feature						No uterus and ovary	
LH (mu/mL)							
Basal	0.135	0.45	0.24	0.29	0.112	0.278	<0.10
Peak	1.45	5.21	6.86	0.68	2.22	0.697	2.18
FSH (mu/mL)							
Basal	0.32	1.16	1.12	0.59	0.466	0.944	0.18
Peak	3.22	4.14	6.68	1.18	3.25	1.84	2.91
Testosterone (nM)	2.73	<0.69	1.15	<0.69	<0.69	1.24	<0.69
Estradiol (pg/mL)	<20.0	<20.0	<20.0	20.0	<20.0	25.1	<20.0
ACTH (pg/mL)	35	11.9	23.9	45.6	21.1	38.1	0.99
Cortisol (nM)	372	195	317	91	317	328	<10.0
An (nM)	7.49	3.08	4.8	<0.10	1.68	3.75	237
DHEAS (nM)	6270	4070	3040	470	2560	1380	2050
17-OH-P (nM)	3.48	0.97	1.06	0.39	0.94	0.91	4.45

Case histories

All 7 patients were underwent hormone measurement, instrumental evaluation, medical history, and physical exam. Patients 1, 2, 3, and 5 presented a small phallus (<3 cm), small pre-pubertal testicular volume (<4 mL), abnormal sexual maturation, and incomplete development of secondary sexual characteristics (e.g., facial and axillary hair growth and deepening of the voice). KS is diagnosed in patient 3 whose low serum gonadotropins and gonadal steroids were coupled with a compromised sense of smell. Patients 4, 6, and 7 presented as a young adult with primary amenorrhea, who had normal female external genital. Patients 4 and 7 showed normal development of breasts, but patient 6 showed no breast development and uterus and ovary were not identified on ultrasound. The karyotype was 46,XY in male patients and 46,XX in female patients. Endocrine evaluation at diagnosis showed low-basal testosterone levels in all male patients and subnormal LH and FSH concentrations in all patients. Of note, 17-hydroxyprogesterone, DHEAS, and basal cortisol were within normal ranges in each patient and no symptoms or signs of adrenal insufficiency have emerged in any of these patients during follow up. Further investigation of the hypothalamic-pituitary-gonadal (HPG) endocrine axis in these patients suggested hypogonadotropic hypogonadism in additional to a primary gonadotropic defect. Testosterone supplementation was given, which resulted in a

good response in penile growth. The goal of estrogen treatment includes the development of breast tissue, attainment of appropriate stature, induction of menses, growth of the uterus for possible reproductive function, and maintenance of skeletal health.

DISCUSSION

Although puberty is thought to be controlled by a network of transcriptional modules, the mechanisms controlling puberty remain largely unknown. Once normal puberty is initiated, it is generally completed in 3 to 4 years through a progression of events (Layman, 2007). IHH is a rare disease that is defined by reduced synthesis and secretion of steroid hormones due to low LH and FSH secretion, resulting in the failed induction of puberty. The HPG axis differs from other endocrine axes involving the anterior pituitary gland, because gonadotropin-releasing hormone (GnRH)-producing neurons originate outside the central nervous system in the olfactory placode and migrate to the mediobasal hypothalamus. Further, the HPG axis is complex, featuring both negative and positive feedback by sex steroids (Ojeda et al., 2010). Therefore, this sophisticated system is inherently susceptible to genetic defects, resulting in pubertal and/or reproductive failure. Mutations in a number of genes have been identified in patients with primary genetic IHH. However, currently known genetic defects account for only 30% of all IHH cases. Despite recent advances in the field, the genetic and molecular mechanisms of mutation genes remain unknown, and there is room for further discovery. These genes encode proteins that regulate GnRH neuronal development, migration from the nasal placode to the hypothalamus, GnRH secretion, or GnRH action (Wray et al., 1989; Schwanzel-Fukuda, 1999; Balasubramanian et al., 2010). Some genetic mutations are sufficient to cause IHH; in other cases, IHH results from the combination of more than one genetic abnormality.

SF1 regulates the transcription of several enzymes involved in steroid/androgen biosynthesis. *SF1* is also a transcription factor that plays a pivotal role in adrenal and reproductive function, and it influences gene transcription at multiple levels and at different stages of development. In mice, complete loss of *SF1* function results in apoptosis of the developing adrenal gland and gonad during early embryogenesis (Luo et al., 1994; Sadovsky et al., 1995). *SF1* plays an important role in multiple aspects of testicular and ovarian development, integrity and function, as it regulates a number of critical genes involved in these processes. Indeed, a milder loss-of-function change in *SF1* has been associated with micropenis, undescended testes, and anomalies of ovarian development and function (Wada et al., 2005, 2006; Philibert et al., 2007). The initial search for changes in *SF1* among humans focused on patients with combined gonadal and adrenal failure (Achermann et al., 2002); however, more recent studies have shown that heterozygous *SF1* mutations are relatively common in 46,XY patients with mild or partial gonadal dysgenesis, impaired androgenization, and apparently normal adrenal function (Lin et al., 2007; Kohler et al., 2008). These mutations are also common in 46,XX girls or women with ovarian insufficiency (Coutant et al., 2007; Lourenco et al., 2009).

PIN1 plays a role in transcription of gonadotropin-subunit genes. Through a phosphorylation-regulated pathway, it also promotes *SF1* ubiquitination, which facilitates the interaction between *SF1* and *Pitx1* and results in an enhancement of *SF1* transcriptional activity (Zhao et al., 2001). In gonadotropic cells, sufficient levels of activated *PIN1* are maintained through transcriptional and post-translational regulation by GnRH-induced signaling cas-

cades. PIN1 activity is regulated by *GnRH*, indicating that it is a novel player in the GnRH-signaling pathway and gonadotropin gene expression (Lu et al., 2002). *PIN1* is involved in transcription of the gonadotropin β-subunit by modulating the activity of various transcription factors. *PIN1* has been found at the promoters of both gonadotropin β-subunit genes and is presumably recruited by its interaction with *SF1*, *Pitx1*, and/or *Egr-1* (Chen et al., 2007; Benayoun and Veitia, 2009). In some cases, there is functional synergy, although this appears to be promoter-context specific. The functional interaction with *SF1* on the *LHβ* gene was most striking (Walsh and Shupnik, 2009).

In conclusion, we identified a series of missense and synonymous mutations in the *NR5A1* and *PIN1* genes that are associated with primary amenorrhea and male IHH with apparently normal adrenal function. Whether these patients will develop adrenal insufficiency over time remains to be investigated. Thus, determining the genetic cause in patients with IHH may be useful for future research, counseling families of affected patients, long-term follow-up, developing a diagnostic genetic test, and future treatment of IHH.

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