Clinical and molecular analysis of human reproductive disorders in Brazilian patients

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Abstract

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Several genes that influence the development and function of the hypothalamic-pituitary-gonadal-axis (HPG) have been identified. These genes encode an array of transcription factors, matrix proteins, hormones, receptors, and enzymes that are expressed at multiple levels of the HPG. We report the experience of a single Endocrinology Unit in the identification and characterization of naturally occurring mutations in families affected by HPG disorders, including forms of precocious puberty, hypogonadism and abnormal sexual development due to impaired gonadotropin function. Eight distinct genes implicated in HPG function were studied: *KAL*, *SF1*, *DAX1*, *GnRH*, *GnRHR*, *FSHß*, *FSHR*, and *LHR*. Most mutations identified in our cohort are described for the first time in literature. New mutations in *SF1*, *DAX1* and *GnRHR* genes were identified in three Brazilian patients with hypogonadism. Eight boys with luteinizing hormone- (LH) independent precocious puberty due to testotoxicosis were studied, and all have their LH receptor (LHR) defects elucidated. Among the identified LHR molecular defects, three were new activating mutations. In addition, these mutations were frequently associated with new clinical and hormonal aspects, contributing significantly to the knowledge of the molecular basis of reproductive disorders. In conclusion, the naturally occurring genetic mutations described in the Brazilian families studied provide important insights into the regulation of the HPG.

Introduction

Over the past decade, many genes that influence the development and function of the hypothalamic-pituitary-gonadal axis (HPG) have been identified (1). These genes encode an array of transcription factors, matrix proteins, hormones, receptors, and enzymes that are expressed at multiple levels of the HPG, and regulate the complex developmental, paracrine, and endocrine interactions that are necessary for normal reproduction (1).

We review here the extensive experience of a single Brazilian Endocrinology Unit in the identification and characterization of naturally occurring mutations in families affected by disorders of the HPG, including forms of

precocious puberty, hypogonadism and abnormal sexual development due to impaired gonadotropin function. Eight distinct genes implicated in HPG function were studied: *KAL*, *SF1*, *DAX1*, *GnRH*, *GnRHR*, *FSHß*, *FSHR and LHR* (Table 1). The mutations

identified in Brazilian patients in these genes were shown in Table 2.

KAL gene

Kallmann syndrome (KS) consists of con-

Table 1. Single gene disorders causing impaired gonadal development and function.

Gene	Locus	Inheritance	Gonadotropin levels	Phenotype
KAL	Xp22.3	X-linked	Low or normal	Hypogonadotropic hypogonadism and anosmia/hyposmia
SF1	9q33	AR or AD	High or normal	XY sex reversal, adrenal and gonadal agenesis
DAX1	Xp21	X-linked	Low	Hypogonadism and congenital adrenal hypoplasia
GnRH	8p11.2	Unknown	Prediction of low or normal	Prediction of isolated hypogonadotropic hypogonadism
GnRHR	4q13.2	AR	Low or normal	Isolated hypogonadotropic hypogonadism
FSHß	11p13	AR	High LH or low FSH	Congenital hypogonadism
FSHR	2p21	AR	Elevated FSH>LH	Hypergonadotropic hypogonadism Premature ovarian failure
LHR	2p21	AR	Elevated LH>FSH	Hypergonadotropic hypogonadism ambiguous genitalia in males

Table 2. Mutations identified in patients with reproductive disorders.

Gene	Mutations	Clinical features	Functional analysis	Ref.
KAL	R191X	HH and anosmia	–	4
SF1	8-bp deletion at position 27 resulting in a truncated protein	XY sex reversal and normal adrenal function	–	–
DAX1	G insertion at position 71 resulting in a truncated protein	Congenital adrenal hypoplasia and pseudoprecocious puberty in boys	–	21
	A300V	Adrenal insufficiency and HH	–	–
GnRHR	R139H	Complete isolated HH	Total impaired binding activity	30
	N10K/G106R	Partial isolated HH	Decreased binding activity	–
FSHß	Y76X	Primary amenorrhea and partial breast development (FSH ↓ and LH ↑)	No FSH activity	–
LHR	Activating mutations: A568V, L457R, T577I, L368P, and M571L	Gonadotropin-independent precocious puberty	Increasing cAMP levels in basal condition	37-40
	Inactivating mutations: R554X, ΔL608-V609	Leydig cell hypoplasia in males, amenorrhea and infertility in females	Non-stimulated cAMP levels after LH/hCG	41,42

HH = hypogonadotropic hypogonadism.

genital hypogonadotropic hypogonadism (HH) associated with anosmia (absent sense of smell). During fetal development, GnRH-releasing neurons originate in the olfactory placode and migrate with olfactory neurons through the cribform plate to the olfactory bulb and into the fetal hypothalamus. Abnormalities in these processes explain the association of HH with anosmia in patients with KS (2). The gene known to be responsible for the X-linked form of KS, *KAL*, encodes a protein, anosmin, that plays a key role in the migration of GnRH neurons and olfactory nerves to the hypothalamus (1). Anosmin is also expressed in the development of Purkinje cells located in the cerebellum, meso-, and meta-nephros, oculomotor nucleus, and facial mesenchyme, explaining the association of X-linked KS with synkinesia, renal agenesis, visual abnormalities and midline facial defects (3). In addition to X-linked pedigrees, autosomal dominant and recessive kindreds with KS have been reported (4). The frequency of *KAL* deletions or mutations is currently unknown because these are rare disorders and large series are unusual. We examined 23 patients with KS and determined their modes of inheritance and incidence of deletions and mutations in the coding sequence of the *KAL* gene. The diagnosis of HH was based on absent or incomplete sexual development after 17 years of age associated with low or normal levels of luteinizing hormone (LH) in both sexes and low levels of testosterone in males and estradiol in females. All patients had anosmia or hyposmia which was confirmed by an olfactory specific test, i.e., "The Smell Identification Test" (5). No associated features were observed in our patients. Of the 23 KS patients, 12 were familial, whereas 11 were sporadic cases. No deletion in the coding sequence of *KAL* was found and a point mutation, R191X, was identified for the first time in a patient with a familial history that suggested X-linked inheritance without other associated abnor-

malities. This variable penetrance of features is common in families with *KAL* mutations, suggesting that modifier genes or epigenetic phenomena influence phenotypic expression (6). We also confirmed that mutations in the coding sequence of the *KAL* gene occur in a minority of KS cases, as previously reported by Oliveira et al. (4) who demonstrated that only 14% of familial cases and 11% of sporadic cases of KS had mutations identified in the *KAL* gene. These findings suggest that autosomal genes not yet identified account for the majority of familial cases of KS.

SF1 gene

The orphan nuclear receptor steroidogenic factor 1 (*SF1*) gene, officially designated NR5A1, was initially identified as a regulator of the tissue-specific expression of the cytochrome P450 steroid hydroxylases (7,8). Subsequent studies have shown that *SF1* is a key regulator of endocrine function within the HPG axis and adrenal cortex (9). Disruption of the mouse *Nr5a1* gene encoding *SF1* causes adrenal and gonadal agenesis, XY sex reversal, structural abnormalities of the ventromedial hypothalamic nucleus, and altered gonadotropin secretion by the pituitary gonadotrope.

To date, only three mutations in *SF1* have been described in humans (10-12). In each of these patients, the *SF1* mutation caused adrenal insufficiency with varying degrees of gonadal dysfunction, supporting the importance of the role of *SF1* in human adrenal development. We identified a novel 8-bp microdeletion of the *SF1* gene in a 46,XY Brazilian patient with primary amenorrhea and HH and unexpected normal adrenal function. This microdeletion resulted in a premature termination upstream of sequences encoding the activation function 2 domain, suggesting that this defect is the cause of loss of function of the *SF1* leading the XY sex reversal in this patient (Correa

RV, Domenice S, Bingham NC, Billerbeck AEC, Rainey WE, Parker KL and Mendonca BB, unpublished data).

DAX1 gene

The *DAX1* (DSS locus-dosage sensitive sex reversal-adrenal insufficiency, X chromosome gene 1) gene has been mapped to the short arm of the X chromosome (Xp21) and encodes a transcriptional repressor factor that is classified as an orphan nuclear receptor (13,14). *DAX1* is expressed in adrenal cortex, gonads, hypothalamus and pituitary and plays a key role in the development of the adrenal gland and HPG axis (15,16). Mutations in this gene in humans cause an X-linked adrenal cytomegalic form of adrenal hypoplasia and HH (17,18). Recently, a unique case extended the clinical spectrum of the disease including mild forms of HH, delayed-onset of adrenal insufficiency and abnormal spermatogenesis (19). The majority of *DAX1* mutations reported are frameshift or nonsense mutations. These mutations cause truncation of the functionally important carboxyl-terminal region of the protein and impair *DAX1* interaction with other factors and genes (20).

We described a 2-year-old boy with X-linked adrenal hypoplasia congenita due to a nucleotide G insertion between nucleotides 430 and 431 in exon 1, resulting in a frameshift mutation and premature stop codon at position 71 of *DAX1* (21). This boy showed isosexual gonadotropin-independent precocious puberty as the first clinical manifestation (21). He presented pubic hair, enlarged penis and testes, and advanced bone age. His testosterone levels were elevated, whereas basal and GnRH-stimulated LH levels were compatible with a prepubertal pattern. Chronic GnRH agonist therapy did not reduce his testosterone levels, supporting the diagnosis of gonadotropin-independent precocious puberty. In this patient, steroid replacement therapy resulted in a decrease of

testicular size and testosterone levels to the prepubertal range indicating an ACTH-dependent precocious puberty of testicular origin (21). These findings strongly suggest that chronic excessive ACTH levels resulting from adrenal insufficiency may stimulate Leydig cells and lead to gonadotropin-independent precocious puberty in boys with *DAX1* gene mutations.

More recently, another boy who presented adrenal insufficiency at 2 years of age was referred to us for clinical and molecular analysis. He was successfully treated with glucocorticoid and mineralocorticoid replacement and at the age of 16 years he was re-evaluated due to delayed puberty. Hormonal data confirmed adrenal insufficiency and HH. DNA analysis revealed a C>T transition at nucleotide 1133 in exon 1 of *DAX1* which caused an alanine (GCC) to valine (GTC) substitution at codon 300 in this boy. This missense mutation was not detected in his mother. Therefore, the *de novo* missense mutation A300V in the *DAX1* gene, located at the carboxy-terminal end the of DAX1 protein, caused a severe phenotype in this boy characterized by adrenal insufficiency and HH, suggesting that this region is fundamental for *DAX1* action.

GnRH gene

GnRH is a peptide that exerts an important regulatory control over the neuroendocrine axis by stimulating the production and release of LH and FSH. In patients with HH, both gonadotropins are low and GnRH replacement restores the reproductive potential in most of them. Based on these data, GnRH deficiency has been considered to be the etiologic defect in isolated HH and the *GnRH* gene would be an obvious candidate for mutations in patients with isolated HH (22). This idea was also supported by a naturally occurring animal model of human isolated HH, i.e., mice with autosomal recessive HH caused by a *GnRH* gene deletion

(23,24). However, no human *GnRH* gene deletions or mutations were identified in humans with HH (25-27). We have investigated the *GnRH* gene in four Brazilian patients with isolated HH and to date no mutations or deletions were found in this gene.

GnRHR gene

Several investigators have characterized *GnRH* receptor (*GnRHR*) gene mutations as the first identifiable cause of autosomal recessive isolated HH (28). de Roux et al. (28) hypothesized that partial-loss-of-function mutations similar to those described for the *LH/hCG* receptor gene could occur in patients with incomplete isolated HH. They studied a family with an affected brother and sister and found a compound heterozygous mutation, G106R/R262G, in the *GnRHR* gene. An increasing number of point mutations in the *GnRHR* gene have been described in congenital isolated HH. It currently appears that up to 20% of isolated HH patients may have mutations in this receptor (28,29). The clinical features of patients with *GnRHR* gene mutations are highly variable, even in the same kindred.

We investigated 17 patients from 14 different families, with HH and normal olfaction confirmed in all patients by the specific olfactory test (30). A homozygous missense mutation, Arg139His, located in the conserved DRS motif at the junction of the third transmembrane and the second intracellular loop of the GnRH receptor, was identified in one female with complete isolated HH. The Arg139His mutation completely eliminated detectable GnRH binding activity and prevented GnRH-induced stimulation of inositol phosphate accumulation *in vitro*. In another family, a compound heterozygous mutation (Asn10Lys and Gln106Arg) was identified in four Brazilian siblings (two males and two females) with partial isolated HH. The Gln106Arg mutation, located in the first extracellular loop of the *GnRHR* gene, was

previously described and *in vitro* analysis indicated that the mutant receptor was able to bind GnRH, but with a reduced affinity. The Asn10Lys mutation in the extracellular amino-terminal domain of the receptor also reduced the affinity for GnRH *in vitro*. A good correlation between genotype and phenotype was found in the Brazilian patients: the female, who was homozygous for the completely inactivating Arg139His mutation, had complete HH with undetectable basal serum LH and FSH levels which failed to respond to GnRH stimulation. In addition, the affected patients, who were compound heterozygotes for the Asn10Lys/Gln106Arg mutations, had partial HH with low basal serum LH levels which were responsive to GnRH stimulation. No clinical or hormonal differences were found between HH patients with and without mutations in the *GnRHR* gene, indicating that these data do not contribute to the identification of HH patients with *GnRHR* mutations (30).

FSHß gene

The gonadotropins are heterodimers consisting of specific ß-subunits that are noncovalently bound to a common α-subunit. Selective FSH deficiency due to *FSHß*-subunit gene mutations causing hypogonadism and infertility is a very rare condition. To date, three distinct *FSHß*-subunit gene mutations have been described in four unrelated females and two males with hypogonadism (1). These mutations often affect the seatbelt region of the protein, and interfere with the synthesis and stability of the heterodimer complex. Consequently, patients have been found to have undetectable serum FSH and elevated serum LH. Recently, we identified an *FSHß*-subunit gene mutation in a woman with delayed puberty, who presented partial breast development and primary amenorrhea. Her basal and GnRH-stimulated LH levels were elevated, whereas her FSH levels were undetectable in both conditions.

Direct sequencing revealed a mutation at codon 76 (Tyr76X) in homozygous state in this patient. This nonsense mutation was previously described in another apparently unrelated woman with a partial phenotype of FSH deficiency (31). These findings, taken together, indicate that Tyr76X, the mutation of the *FSHβ* subunit, is associated with a partial phenotype of FSH deficiency, indicating a good phenotype-genotype correlation in this rare condition.

FSHR gene

The *FSH* receptor (*FSHR*) gene exerts its action by binding to a specific membrane receptor which belongs to the G-protein coupled receptor superfamily (32). An inactivating mutation (C566T) in exon 7 of the *FSHR* gene was first described in highly inbred Finnish families with autosomal recessive premature ovarian failure and normal karyotype (33). This mutation was associated with variable pubertal failure and primary or secondary amenorrhea (34). When we investigated the presence of abnormalities in the *FSHR* gene in 15 women with familial or sporadic premature ovarian failure we did not identify any inactivating mutations in exons 7-10 of these patients (35). Although the number of Brazilian patients evaluated was small, these findings support the hypothesis that C566T mutation is probably restricted to Finland. We demonstrated a high allelic frequency of two different nucleotide substitutions in exon 10 of the *FSHR* gene, G919A and G2039A, in patients with familial and sporadic premature ovarian failure (35). These polymorphisms also were found at similar frequencies in healthy women, indicating that these substitutions are of no biological significance.

LHR gene

The human *LH* receptor (*LHR*) gene is a member of the G protein-coupled receptor family with seven transmembrane helices (36). Distinct activating and inactivating mutations in the *LHR* have been described in males with gonadotropin-independent precocious puberty and in a rare form of pseudohermaphroditism (Leydig cell hypoplasia), respectively (32,36).

Familial male-limited precocious puberty, also known as testitoxicosis, is an autosomal dominant condition. Affected males develop rapid virilization, growth acceleration, and skeletal advancement between 2-4 years of age with elevated levels of testosterone, despite prepubertal LH levels. We reported the clinical and molecular aspects of 8 boys with testitoxicosis belonging to 7 unrelated families (37). Sequencing of exon 11 of the *LHR* gene revealed five different mutations, Ala568Val, Leu457Arg, Thr577Ile, Leu368Pro and Met571Leu (37-40). These mutations lead to high basal cAMP production in transfected COS-7 cells, reflecting an intrinsic ability of the mutant receptors to couple with and stimulate Gs in the absence of the ligand (36). The Ala568Val in the third intracellular loop was the most frequent defect in Brazilian boys with testitoxicosis (37). Interestingly, one boy had this mutation in homozygous state due the maternal isodisomy, which was demonstrated by microsatellite analysis. In addition, the common ancestral origin of the Ala568Val mutation was demonstrated by the high frequency of a polymorphic allele (D2S123) located within the short arm of chromosome 2 in three families studied. Female patients who were carriers of *LHR*-activating mutations did not have any clinical or hormonal abnormalities, indicating the lack of phenotypic expression of these mutations in females (37).

Leydig cell hypoplasia is characterized by failure of fetal testicular Leydig cell differentiation. Affected 46,XY individuals display female external genitalia or a micropenis associated or not with hypospadias (36). We reported the first occurrence of micrope-

nis and of amenorrhea and infertility due to inactivating mutations in *LHR* in a Puerto Rican boy and in a 46,XX Brazilian woman, respectively (41). This female patient had a homozygous Arg554Stop which truncated the *LHR* within the third transmembrane domain. A distinct homozygous deletion of two consecutive amino acids (Leu-608 and Val-609) in the seventh transmembrane helix of the *LHR* was also found in another family (42). In both families, the affected 46,XY patients had female external genitalia, whereas their sisters had irregular menses (oligoamenorrhea) or secondary amenorrhea and infertility.

In conclusion, identification of naturally occurring genetic mutations has led to significant benefits for the patients, such as appropriate and educated counseling, as well as specific treatment.

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