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Frequency of Mutations in The *GnRH* Receptor Gene in Pakistani Patients With Hypogonadotropic Hypogonadism

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Abstract.- The hypothalamic gonadotropic releasing hormone (GnRH) is a key regulator in normal puberty, sexual development and function. The proper binding of GnRH to its receptor, *GNRHR*, is necessary for normal secretion of gonadotropins. The deficiency in release or action of GnRH leads to hypogonadotropic hypogonadism (HH) characterized by low follicle stimulating hormone (FSH), luteinizing hormone (LH) and estradiol (E_2) and results in absent or impaired sexual development at puberty. There are about 20 genes identified as possible regulator of puberty. The mutations in *GNRH1* and *GNRHR* are possible causes of HH. The present study was designed to identify mutations in *GNRH1* and *GNRHR* genes and their correlation with HH in Pakistani girls. Fifty two HH patients and fifty two age matched controls were included in the study. Genomic DNA was extracted and amplified by PCR using specific primers for *GNRH1* and *GNRHR* exons. Mutations were analyzed by Sanger sequencing. No mutation was identified in *GNRH1* gene, while two mutations in *GNRHR* gene were observed in one sporadic case of isolated HH. The first was a synonymous substitution mutation of T to C at nucleotide position 221, which does not result in the alteration of coded amino acid residue, histidine. The other was missense mutation determined at nucleotide position 101, which results in the substitution of serine with phenylalanine at 34th position of the extracellular domain of *GNRHR*. In conclusion, the present study demonstrates that mutations in *GNRHR* may play an important role in delaying puberty in the local population.

Key words: Hypogonadotropic hypogonadism, *GNRH1, GNRHR.*

INTRODUCTION

Puberty is the developmental phase that is characterized by the appearance of secondary sexual characteristics, pubertal growth spurt and ultimate attainment of fertility which is manifested in the menarche. The process of puberty starts as a result of the re-activation of hypothalamo-pituitary-gonadal (HPG) axis, which remains dormant after first 6 months of life. The increased nocturnal and pulsatile secretion of gonadotrophin-releasing hormone (GnRH) from hypothalamic neurons results in initiation of sexual maturation. The anterior pituitary secretes luteinizing hormone (LH) and follicle-stimulating hormone (FSH) under the influence of GnRH which results in ovarian growth and sex hormones secretion including estrogen. The growth and development of the female reproductive tract takes place in a predictable sequence under the sustained supply of estrogen. Menarche (the first menstrual cycle) initiates due to gradual increase in the thickness of endometrium under the influence of fluctuating but raised concentration of estrogen. The menarche usually occurs about 2 years after the onset of breast development (Patton and Viner, 2007).

The puberty is considered as delayed, if the signs of sexual development are absent in the girls by the age of 13.4 years (2 SD above the mean of chronological age for the onset of puberty) or no menarche by the age of 16 years (Traggiai and Stanhope, 2003). Hypogonadotropic hypogonadism (HH) is a leading cause of pubertal delay, which is characterized by failure of initiation of puberty due to insufficient gonadotropin and sex steroids release which results in failure of development of secondary sexual characteristics and maturation of reproductive system (Seminara *et al*., 1998; Styne and Grumbach, 2011; Skałba and Guz, 2011). HH may be presented with normal (normosmic HH) (Quinton *et al*., 2001; Bhagavath *et al*., 2006) or decreased (hyposmia) or absent (anosmia) (Kallman Syndrome) sense of smell (Seminara *et al*., 1998; Fechner and McGovern, 2008).

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The pubertal and reproductive deficiencies in humans are caused by a number of genetic mutations in HPG axis. HH is generally caused by mutations in the genes expressed specifically in the hypothalamus, while mutations in pituitary-specific genes causing delayed puberty result either in deficiency of some or of all pituitary hormones like FSH and LH, thyroid stimulating hormones (TSH), prolactin (PRL) and growth hormone (GH). Some genetic mutations are also well known to be specific for gonads (Layman, 2002).

The hypothalamic GnRH is a key regulator in normal puberty, and sexual development and function (Beate *et al*., 2011). The proper binding of GnRH to its receptor, *GNRHR*, expressed on the pituitary gonadotropes is necessary for normal secretion of gonadotropins. The decapeptide GnRH binds to the *GNRHR*, initiating a cell signaling cascade involving G proteins, typically $G_{\alpha q}$, activates phospholipase Cβ resulting in the production of second messengers inositol triphosphate (IP_3), diacylglycerol, and calcium with subsequent secretion of both FSH and LH (Kim *et al.,* 2010). It has been demonstrated that the *GNRHR* plays a major role in normal gonadotropins secretion during the time of puberty (Beate *et al*., 2011), whereas defects in *GNRHR* impair the proper binding of GnRH with its receptor and causes low secretion of FSH, LH and sex steroids (Noel and Kaiser, 2011). Thus, *GNRHR* has significance in establishing the timing of the onset or delay of puberty.

The human *GNRHR* gene spans 18.7 kb of sequence on chromosome 4q13.2. It consists of three exons and encodes a hepta-helical transmembrane domain G protein-coupled receptor that is expressed in the pituitary. This gene is also expressed in many other tissues including brain, ovaries, testes, prostrate, kidney and liver. In 1997, *GNRHR* inactivating mutations were first recognized as a monogenic cause of HH (de Roux *et al.,* 1997). Although, activating *GNRHR* mutations have not yet been identified, inactivating mutations of the *GNRHR* are the most frequent cause of HH, especially in familial cases (Beate *et al.,* 2003; Sykiotis *et al.,* 2010; Beate *et al.,* 2011). A large-scale screening has shown that *GNRHR* mutations account for about 3.5 to 16 percent of the sporadic cases of HH and up to 40 percent of familial cases of HH (Beranova *et al.,* 2001).

The current literature indicates the presence of twenty one different mutations in *GNRHR* (Noel and Kaiser, 2011**).** Of these confirmed mutant alleles, 17 were missense mutations, one was a nonsense mutation, and one was a splice site mutation (Kim *et al.,* 2010). Ten mutations have been identified in exon 1, two have been found in exon 2, and six have been observed in exon 3. The Gln106Arg and Arg262Gln mutations are about 50 percent of the total identified mutations (Kim *et al.,* 2010). Gln106Arg represents 27.1 percent mutant alleles and Arg262Gln constitute 17.4 percent mutant alleles (Kim *et al.,* 2010). These are mostly compound heterozygous missense mutations that affect the binding or signal communication (Chevrier *et al.,* 2011). The present study was undertaken to examined probable mutations in *GNRH1* and *GNRHR* genes causing HH in a local population.

MATERIALS AND METHODS

Subjects

Fifty two female patients, who failed to attain growth and signs of sexual maturation as late as 14 or 15 years and menarche by 16 years of age visiting public sector hospitals of Islamabad, Rawalpindi and Lahore were included in the present study. HH was defined as inappropriately low concentrations of estradiol in the setting of inadequately low, below or around the lowest limit of the normal range of gonadotropin levels (LH and FSH). The other diagnostic criteria included late or absent spontaneous pubertal maturation. The control group consisted of 52 age-matched girls with normal hormone levels and pubertal hallmarks as described by Tanner and Whitehouse **(**1976**)**.

Genetic screening

Genomic DNA was extracted from the peripheral blood leukocytes of the subjects using Fermentas Genomic DNA Purification Kit (#K0512) according to instruction manual. Primers designed for the exons of *GNRH1* and *GNRHR* genes are shown in Tables I and II. PCR reaction mixture, 25 µL contained 13.90µl H_2O, 5µl genomic

DNA template (10ng/µl), 1µl each primer (10µM), 1µl dNTPs (5mM), 0.5µl $MgCl_2$ (25mM), 2.5µl buffer A with 1.5mM $MgCl_2$ (10X) and 0.1µl KAPA Taq (5U/µl) (KAPA Biosystems, Boston, Massachusetts, United States). Amplification was done at 94°C for 3 min, followed by 35 cycles, each of 94°C for 45 s, 60°C for 30 s and 72°C for 45 s. It was followed by extension at 72°C for 3 min.

The probable genetic mutations in the *GNRH1* and *GNRHR* were determined by Sanger Sequencing.

Table I.- Primers for *GNRH1*

Primers	Sequences	T_m	Product size
GNRH1 Ex_1-F	ctgactctgacttccatcttctgcagg	60°C	396
GNRH1 Ex_1-R	gccttatctcacctggagcatctagg		
GNRH1 Ex_2-F	ctaatcctgcaactttcccaatctcccc	60°C	353
GNRH1 Ex_2-R	cagaggagtcaggaatgtaagcccc		
GNRH1 Ex_3-F	catgtctccctagcactaactagagcac	60°C	356
GNRH1 Ex_3-R	ggtatgccacttcattcacaacacagc		

Ex, Exon; F, Forward; R, Reverse.

Table II.- Primers for *GNRHR*

Primers	Sequences	T_m	Product size
GNRHR Ex_1-F	acctgtgacgtttccatctaaagaaggc	60°C	759
GNRHR Ex_1-R	tgacttccagaacccaagctcttaaagg		
GNRHR Ex_2-F	agaacagtatctgtcacatagttcatgcc	60°C	453
GNRHR Ex_2-R	agaaacgtcagaagtataacactaaggagc		
GNRHR Ex_3-F	accccatatttcaaatccagattactttggc	60°C	581
GNRHR Ex_3-R	tccaacatttgtgttaatcattcccagatgg		

In silico *analysis*

Mutationtaster (http://www.mutationtaster.org/) software was used to predict the putative impact of amino acid substitution on the protein structure and function.

Ethical approval

The research protocol was approved by the Institutional Review Boards of Government College University, Lahore and Children Hospital, Lahore (Pakistan). All of the participants were briefed about the study and written consent was taken for their inclusion in the current research.

RESULTS

Total fifty two sporadic cases of HH girls, between the ages of 17 and 27 years, with late or absent sexual maturation were included in the genetic analysis. No mutation was detected in *GNRH1* gene for any of the 52 sporadic cases of HH. Two mutations in one sporadic HH case were found in screening of *GNRHR* gene in fifty two HH patients. One C to T missense mutation was identified in exon 1 at nucleotide position 101. This change caused the serine substituted with phenylalanine, Ser34Phe in the *GNRHR* (Figs. 1, 3). Another T-to-C mutation was identified at 221 nucleotide position in the same patient in exon 2 of *GNRHR*. This was a synonymous point change, which did not substitute histidine with any other amino acid (221His=) (Figs. 2, 3). No other mutation was found in *GNRHR* in any patient. The novel variants (Ser34Phe, 221His=) were absent in the control population of 52 healthy subjects.

The novel mutation causing substitution of Serine with phenylalanine, Ser34Phe was identified in a 20 years old girl presented with delayed puberty. The subject had infantile uterus and streak ovaries. Her plasma FSH, LH and E_2 concentrations were very low. Her breast was at stage 1 and pubic hair was at stage 2. The substitution of serine with phenylalanine, Ser34Phe might have disrupted the *GNRHR* function and caused low gonadotropins secretion and resulted in HH.

The novel mutation Ser34Phe was considered to be disease-causing by online bioinformatics *in silico* prediction program Mutationtaster (http://www.mutationtaster.org/). The sequence analysis demonstrated that protein serine 34 in *GNRHR* is highly conserved among different species and any alteration may result in altered structure and protein function (Table IV).

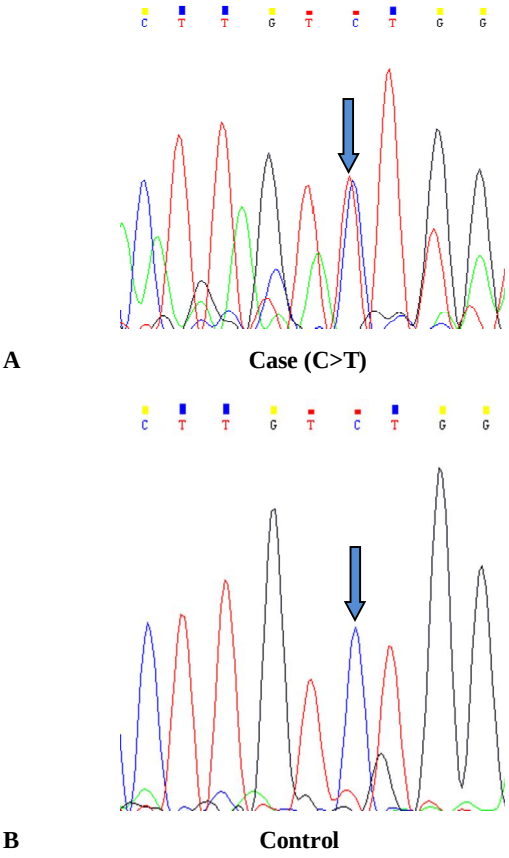

Fig. 1. Chromatogram representing the genetic polymorphism in Ser34Phe. A, CT; B, CC.

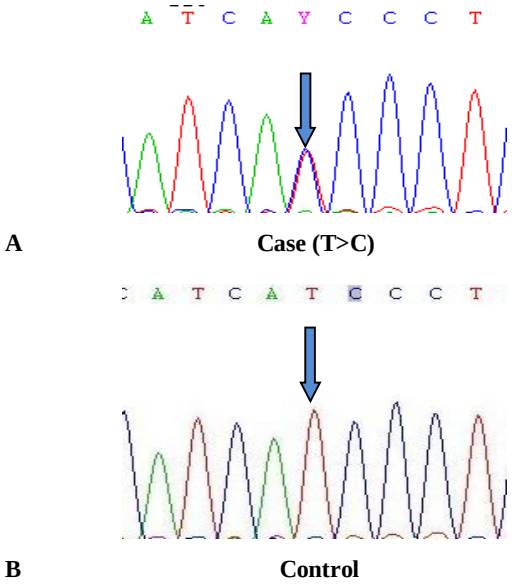

Fig. 2. Chromatogram representing the genetic polymorphism in 221His=. A, CT; B, CC.

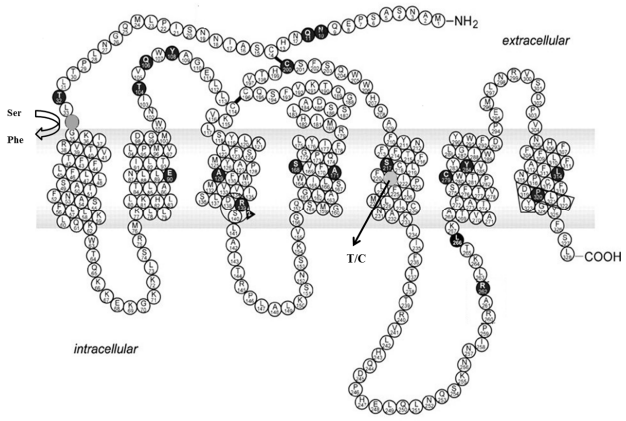

Fig. 3. Topology diagram of the amino acid sequence of the human *GNRHR* showing substitution Ser34Phe (grey) and 'T' to 'C' substitution (221His=) (grey). Black color indicates already reported mutations associated with HH.

DISCUSSION

In the current study, *GNRH1* mutation was not detected in any of the fifty two sporadic cases of HH. The available data demonstrates that *GNRH1* mutations are very rarely associated with HH. Two independent studies have reported homozygous and only mutation so far in *GNRH1* in one sib-pair (Bouligand *et al.*, 2009) and in a male patient (Chan *et al.*, 2009) with HH. Thereafter, no patients have been reported with *GNRH1* mutations.

In the current study, two novel mutations in *GNRHR* were found in one sporadic case. One missense mutation was identified in exon 1 at the position of 101 nucleotide. The said mutation was translated in the form of substitution of serine with phenylalanine at 34th position in the N-terminal extracellular domain (ECD) of *GNRHR* and is designated as Ser34Phe (C to T). In a recent study, a missense mutation serine168arginine (S168R) in the fourth transmembrane domain of the *GNRHR* gene was identified in a homozygous state in one male with complete HH (Fathi *et al.*, 2013), which had previously been shown to cause complete loss of receptor function because hormone binding to the

Table IV.- Amino acid sequence alignment of GNRHR among species: Ser34Phe is indicated in Bold.

Species	AA	Alignment
Human	34	LMQGNLPTLTLSGKIRVTVTFFL
Mutated	34	LMQGNLPTLTL**P**GKIRVTVTFFL
P. troglodytes	34	LMQGNLPTLTLSGKIRVTVTFFL
M. mulatta	34	LMQGNLPTLTLSGKIRVTVTFFL

receptor was completely impaired. In another patient, a compound mutation (Gln106Arg and Arg262Gln) was found in a male with partial HH. The Gln106Arg mutation located in the first extracellular loop of *GNRHR* decreased but did not eliminate GnRH binding, while Arg262Gln mutation located in the third extracellular loop of GnRH negatively affected the signal transduction (Fathi *et al*., 2013). In a similar study, three different heterozygous *GNRHR* mutations among 146 subjects with delayed puberty have been reported. One female carried the missense mutation c.317A>G (p.Q106R), and two females and three males carried the missense mutation c.785G>A (p.R262Q). Both mutations were shown to partially inactivate the *GNRHR* (Vaaralahti *et al.,* 2011). One heterozygous R262Q mutation of *GNRHR* was also identified in 45 patients (Lanfranco *et al.,* 2005), rendering them infertile.

A large-scale screening has shown that *GNRHR* mutations account for about 3.5% to 16% of the sporadic cases of HH and up to 40% of familial cases of HH (Beranova *et al.,* 2001). De Roux and his colleagues (1997) found two compound heterozygous *GNRHR* mutations Gln106Arg and Arg262Gln in a female patient and her brother. The Gln106Arg mutation impaired the GnRH binding to the *GNRHR*. Similarly, Layman *et al*. (1998) reported a family with compound heterozygous *GNRHR* mutations in which one male and three females were affected. They all had low gonadotropins levels and delayed puberty. The compound heterozygous *GNRHR* mutations Arg262Gln and Tyr284Cys were also identified by DNA sequencing. These mutations were observed in all four affected HH patients of that family. Furthermore, Costa *et al.* (2001) found novel heterozygous mutations in *GNRHR* in four siblings (two males and two females). Affected patients had HH with low plasma LH concentration that was responsive to stimulation by GnRH.

The mechanism whereby the mutation Ser34Phe observed in the present investigation caused HH in a girl is not properly understood. Nevertheless, it may be suggested that the mutation Ser34Phe observed in extracellular domain of *GNRHR* in the present study might affect proper binding of GnRH to it. It has been demonstrated that the ECDs and/or TMDs are involved in the formation of the ligand-binding pocket (Kim *et al.*, 2010). It has earlier been shown that ECD is involved in the binding of GnRH with *GNRHR* (Kim *et al.,* 2010). The *GNRHR* mutation Asn10Lys in ECD has been shown to reduce ligand binding and IP_3 signaling (Kim *et al.,* 2010). Similarly, *GNRHR* mutation Gln11Lys also decreases the binding of GnRH with *GNRHR* and IP_3 signaling (Kim *et al.,* 2010). It has also been shown that a *GNRHR* gene mutation L148S in 2nd intracellular loop (ICL) that leads to substitution of Leucine with Serine causes low levels of GnRH and gonadotropins and results in delayed puberty (Seminara *et al*., 2003; Wacker *et al*., 2008). In view of the foregoing observations, it may be suggested that *GNRHR* mutation Ser34Phe observed in the present study might have reduced receptor binding of the ligand due to the down regulation of the signal transduction, resulting in reduced secretion of FSH, LH and E_2 in the said patient of HH.

The other T-to-C mutation was identified at 221 nucleotide position in the same patient in exon 2 of *GNRHR* was a synonymous point change, which did not replace histidine with any other amino acid residue. As this mutation did not cause any change in the structure of the translated protein, it does not appear to have any additive effect on the severity of HH in said patient.

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Conflict of interest statement

The authors have no conflict of interest to declare

CONCLUSION

We report the identification of two novel mutations in *GNRHR* gene in one sporadic case of HH. Of the two novel mutations identified in *GNRHR* gene, one was missense mutation and the other was synonymous mutation. However, further multicentric studies at larger cohort of HH patients are needed to fully elucidate the genetics of HH in our local population.

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