Pakistan J. Zool., vol. 49(1), pp 221-228, 2017. DOI: http://dx.doi.org/10.17582/journal.pjz/2017.49.1.221.228

A Novel Mutation Ser34Phe in GNRHR causes Hypogonadotropic Hypogonadism during Pubertal Development in Boys

Misbah Riaz[1], Qaiser Mansoor[2], Maleeha Akram[1], Muhammad Ismail[2], Parveen Akhtar[3], Shakeel Mirza[4], Mazhar Qayyum[1], Afzaal Ahmed Naseem[1], Faheem Tahir[5] and Syed Shakeel Raza Rizvi[1]*

[1]Department of Zoology, Pir Mehr Ali Shah Arid Agriculture University, Shamsabad, Murree Road, Rawalpindi, Pakistan
[2]Institute of Biomedical and Genetic Engineering, Islamabad, Pakistan
[3]Department of Pediatrics, Fauji Foundation Hospital, Rawalpindi, Pakistan
[4]Department of Medicine, Military Hospital, Rawalpindi, Pakistan
[5]Reproductive Physiology, Public Health Laboratories Division, National Institute of Health, Islamabad, Pakistan

ABSTRACT

The signaling of G protein-coupled receptor 54 (GPR54) is a key regulator of secretion of gonadotropin-releasing hormone (GnRH), whereas GnRH is a crucial neurohormone regulating the secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) at puberty. The deficiency in release or action of GnRH leads to hypogonadotropic hypogonadism (HH) characterized by low FSH, LH and testosterone (T) and absent or impaired sexual development at puberty. Amongst others, mutations in *GPR54* and GnRH receptor (*GNRHR*) are possible causes of HH. This study aimed at identification of mutations in *GPR54* and *GNRHR* genes and their correlation with HH in Pakistani boys. Thirty one boys with delayed puberty and thirty one normal age matched controls were examined. Genomic DNA was extracted and amplified by PCR using specific primers for *GPR54* and *GNRHR* splice site exons. Mutations were analyzed by single-stranded conformation polymorphism (SSCP) and/or sequencing. No mutation was identified in *GPR54* 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 G at nucleotide position 123, which did not replace valine with any other amino acid. The other mutation determined at nucleotide position 101, was a missense mutation, which substituted serine with phenylalanine at 34th position of extracellular domain of *GNRHR*. The Ser34Phe mutation was manifested in phenotypic traits such as low concentrations of FSH, LH and T and delayed puberty. In conclusion, mutations in *GNRHR* may cause delay in male puberty in Pakistani population.

Article Information
Received 20 July 2016
Revised 31 August 2016
Accepted 07 September
Available online 25 December 2016

Authors' Contributions
SSRZ and MQ designed the experiment, supervised the research work and helped MR to write the article. MR conducted the experimental work. AAN and FT helped in hormonal analysis. SM and PA helped in providing patients. MA helped in sample collection. MI and QM performed genetic analysis of samples.

Key words
Male puberty, Hypogonadotropic hypogonadism, Gonadotropin releasing hormone receptor mutation, Delayed puberty, Luteinizing hormone, Follicle stimulating hormone, Testosterone

INTRODUCTION

The onset of male puberty takes place when the hypothalamus begins to secrete gonadotropin releasing hormone (GnRH), which causes the pituitary to release luteinizing hormone (LH) and follicle stimulating hormone (FSH) that stimulate the growth of the testes. The testes secrete testosterone (T), which causes the development of gonads, genitalia and secondary sex characteristics (Kalantaridou and Chrousos, 2002). The puberty is considered as delayed, if pubertal signs do not appear by the age of 15 years (Mushtaq and Wales, 2007). Some of the major causes of delayed puberty are constitutional delay in growth and development, hypergonadotropic hypogonadism and hypogonadotropic hypogonadism (HH) (Geffner, 2002). The pubertal and reproductive deficiencies in humans are caused by a number of genetic mutations in hypothalamic-pituitary-gonadal (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 in deficiency of either some or all of pituitary hormones. Some genetic mutations are also specific for gonads (Layman, 2002).

G protein-coupled receptor 54 (GPR54), a receptor of 54-amino acids peptide derived from KISS1 protein and expressed in the hypothalamus (Muir *et al*., 2001), is a regulator of GnRH secretion, essentially required for normal GnRH physiology (Beate and de Roux, 2005). GPR54 stimulates FSH and LH secretion via the release of

* Corresponding author: shakeel_raza@hotmail.com
0030-9923/2017/0001-0221 $ 9.00/0

GnRH (de Roux *et al*., 2003; Rakover *et al*., 2007) and plays a major role in the regulation of gonadotropic secretion at puberty (Seminara *et al*., 2003; Nimri *et al*., 2011). The mutations in GPR54 gene that have been reported to impair puberty, cause HH and reproductive disability (de Roux *et al*., 2003), indicating an association of inactivating mutations in GPR54 with HH (Papaoiconomou *et al*., 2011). Fifteen loss of function mutations have so far been described in GPR54 (Demirbilek *et al*., 2014). The presence of gain-of-function mutations in the GPR54 gene is very rare and only 2 mutations have yet been reported. The identification of naturally occurring genetic mutations has provided wide insight into the current knowledge of the human HPG axis (Latronico, 2009).

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 GNRHR, initiating a cell signaling cascade involving G proteins, typically Gαq, activates phospholipase Cβ resulting in the production of second messengers inositol triphosphate (IP3), diacylglycerol and calcium with subsequent secretion of both FSH and LH (Kim *et al*., 2010). GNRHR plays a pivotal role in normal gonadotropins secretion during puberty (Beate *et al*., 2011), whereas defects in GNRHR impair it's binding with GnRH and cause lower secretion of FSH, LH and T (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, brain, testes, prostrate, kidney and liver. In 1997, GNRHR inactivating mutations were first recognized as 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*., 2011). The current literature indicates the presence of thirty three different mutations (including 3 deletions) in exon 1, exon 2 and exon 3 of GNRHR gene (Ullo-Aguirre *et al*., 2014; Beneduzzi *et al*., 2014). Of these confirmed mutant alleles, Gln106Arg and Arg262Gln constitute ~50%, Gln106Arg represents 27.1% and Arg262Gln account for 17.4% of the total mutant alleles (Kim *et al*., 2010). These are mostly compound heterozygous missense mutations that affect the receptor binding or signal transduction (Chevrier *et al*., 2011).

The present study examined mutations in GPR54 and GNRHR genes in thirty one sporadic cases of hypogonadotropic hypogonadism with complaints of delayed puberty visiting our public sector hospitals.

MATERIALS AND METHODS

Subjects

Thirty one male patients, who failed to attain growth and signs of sexual maturation as late as 15 years of age visiting Pakistan Institute of Medical Sciences (PIMS), Islamabad, Military Hospital (MH) and Fauji Foundation Hospital (FFH), Rawalpindi were screened. The research protocol was approved by Research Ethical Review Committees of Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, PIMS Islamabad, FFH Rawalpindi and Institute of Biomedical and Genetic Engineering (IBGE), Islamabad. HH was defined as inappropriately low concentrations of T below 12 nmol/l in the setting of inadequately low, below or around the lowest limit of normal ranges of LH and FSH. The other diagnostic criteria included late or absent spontaneous pubertal maturation. The control group consisted of 31 age-matched boys with normal hormone levels and pubertal hallmarks as described by Tanner and Whitehouse (1976). All of the participants were briefed about the study and written consent was taken for their inclusion in the study.

Hormone measurements

Plasma concentrations of LH, FSH and GH were assayed by ELISA (Amgenix International Inc. 3444 Pinotin Ct. San Jose, CA 95148, USA). The minimum detectable concentration of LH and FSH was 2.5 IU/L and that of GH was 0.5 ng/ml. The plasma T concentrations were determined by Electrochemiluminescence Immunosay testosterone kit using automatic equipment (Roche Diagnostic Elecsys 2010 instruments). The detection range for T was 0.087-52.0 nmol/l.

Table I.- Primers for GPR54.

Primers	Sequences	T_m
GPR54- exon 1-F	5'GGGCGGCCGGGAGGAGGA3'	65°C
GPR54-exon 1-R	5'CCGGGACGGCAGCAGGTG3'	
GPR54-exon 2-F	5'GCCCAGCGCCCGCGCATC3'	65°C
GPR54-exon 2-R	5'GTCCCCAAGTGCGCCCTCTC3'	
GPR54-exon 3-F	5'CAGGCTCCCAACCGCGCAG3'	64°C
GPR54-exon 3-R	5'CGTGTCCGCCTTCTCCCGTG3'	
GPR54-exon 4-F	5'CTTCATCCTGGCTTGTGGCAC3'	58°C
GPR54-exon 4-R	5'CTTGCTGTCCTCCCACCCAC3'	
GPR54-exon 5-F	5'GCCTTTCGTCTAACCACCTTC3'	58°C
GPR54-exon 5-R	5'GGAGCCGCTCGGATTCCCAC3'	

Table II.- Primers for GNRHR.

Primers	Sequences	T_m
GNRHR-exon 1.1-F	5'CAGGGACAAAATTTGACATACG3'	53°C
GNRHR-exon 1.1-R	5'ATGTTCCACATCCCATCCAG3'	
GNRHR-exon 1.2-F	5'TTCTGCTCTCTGCGACCTTT3'	56°C
GNRHR-exon 1.2-R	5'CTGACTTCCAGAACCCAAGC3'	
GNRHR-exon 2-F	5'GGCTAGCAGAGTACCAAAGAGAA3'	55°C
GNRHR-exon 2-R	5'TGCCACTCTGTTTTGAGCAT3'	
GNRHR-exon 3-F	5'TCCTTTTTGTCCACTTTGGTTT3'	52°C
GNRHR-exon 3-R	5'TCCCAGATGGAGAGATTCA3'	

Genetic screening

Genomic DNA was extracted from peripheral blood sample using the organic DNA extraction protocol described by Sambrook *et al*. (1989). The probable genetic mutations in the GPR54 and GNRHR were determined by Single Strand Conformation Polymorphism (SSCP) gel electrophoresis. The genomic DNA was amplified by PCR (Fermantas Lithuania PCR kit) to obtain amplicons. Primers designed for the exons of GPR54 and GNRHR genes are shown in Tables I and II. The amplicons were subjected to SSCP for further analysis. Furthermore, fragment showing an abnormal migration pattern and about one-third of the amplicons randomly chosen among those with a normal migration pattern were directly sequenced (3130 Genetic Analyzer (ABI)) for GPR54 and GNRHR genes.

RESULTS

Thirty one sporadic cases of HH boys between the ages of 15 and 21 years with late or absent sexual maturation were examined. The mean plasma concentrations of T (1.69-3.31 nmol/l), LH (0.31-0.92 IU/L) and FSH (1.27-2.27 IU/L) were significantly lower in HH patients than controls.

Probable gene mutation in GNRHR and GPR54

No mutation was detected in GPR54 gene in any of the 31 sporadic cases of HH.

Two mutations in one sporadic HH case were found in screening of GNRHR gene in thirty one HH patients. One novel C to T missense mutation was identified in exon 1.1 at nucleotide position 101. This change caused the serine substituted with phenylalanine, Ser34Phe, in the GNRHR (Figs. 1, 2, 3 and 4).

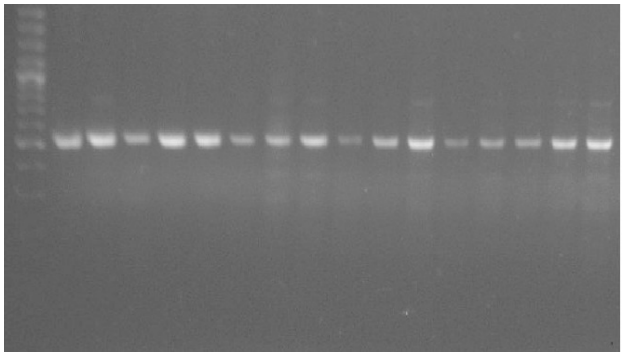

Fig. 1. GNRHR exon 1.1 bands on 2 percent agarose gel. All bands are clear and of proper size of 450 base pairs.

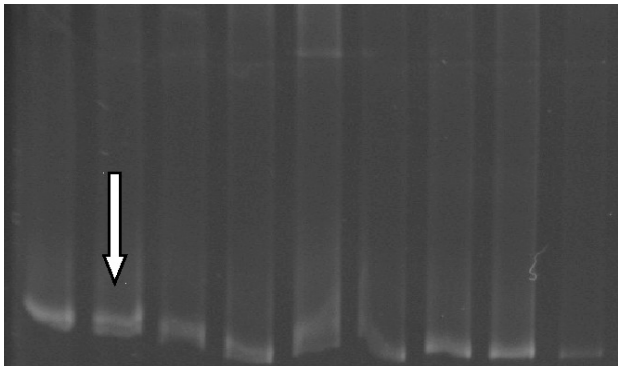

Fig. 2. GNRHR 1.1 bands on polyacrylamide gel: Arrow shows the sample with double bands, further sequenced for detection of mutation. All other bands are well defined and single.

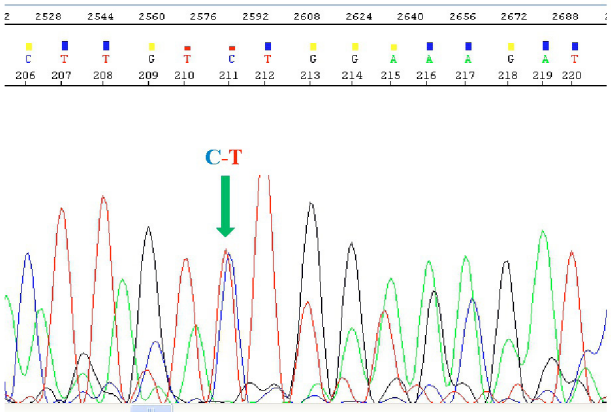

Fig. 3. Mutated sequence of GNRHR exon 1.1 C-T change at nucleotide position 101 causing Ser34Phe change. Mutation is indicated by arrow.

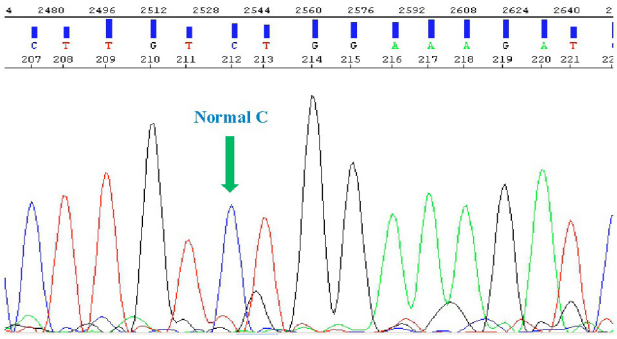

Fig. 4. Normal sequence of GNRHR exon 1.1. Arrow indicates the normal C position.

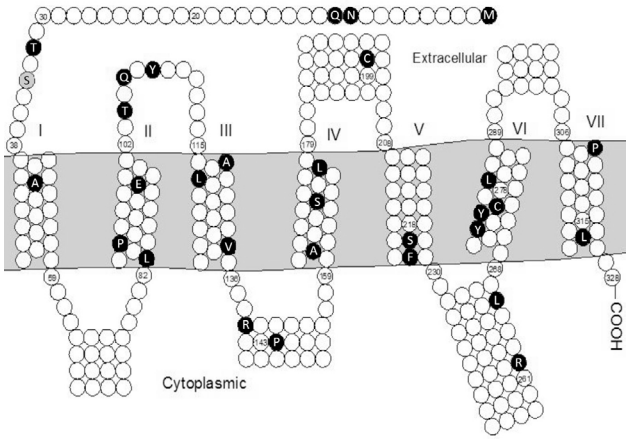

Fig. 5. Topology diagram of the amino acid sequence of the human GNRHR protein, showing all mutations depicted so far (black circles with white letters) including Ser34Phe (grey circle with black letter) (deletions are not mentioned).

This is the 34th mutation and is depicted in Figure 5 along with all other mutations reported so far. Another T-to-G mutation was identified at 123 nucleotide position in the same patient in exon 1.1 of GNRHR. This was a synonymous point change, which did not substitute valine with any other amino acid (Figs. 6 and 7).

The novel mutation causing substitution of serine with phenylalanine, Ser34Phe was identified in a 16 years old boy, who weighed 68 kg and had a height of 166 cm. The subject was presented with delayed puberty, retractile testes and genitalia, urinary retention, pain in testes when pulled down, fatigue, back pain and dysuria but his penis was of normal size. His plasma FSH, LH and T concentrations were very low. His facial hair was at stage 1 and pubic hair was at stage 3. His penile length was 7 cm and testicular volume was 3 mL. The boy exhibited normal FSH and LH response to GnRH administration at 13 and 16 years of age. Nevertheless, the response of testes in terms of the release of T following hCG stimulation was inadequate when tested at 14 years of age. He was being treated with testovirone (testosterone enanthate) injections at an interval of 2 weeks.

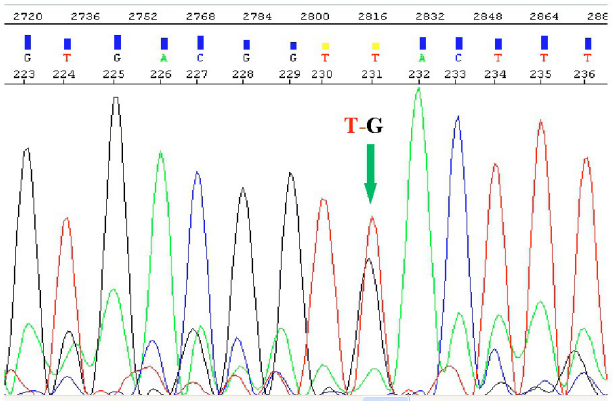

Fig. 6. T-G synonymous mutation in GNRHR exon 1.1 at nucleotide position 123. Arrow indicates the mutated nucleotide. Lines for T (red) and G (black) are superimposed at the mutation.

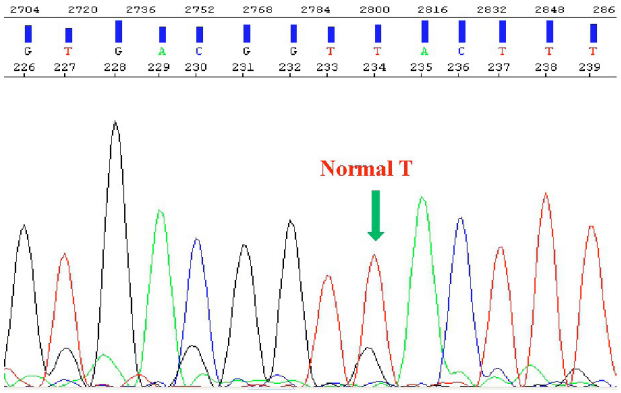

Fig. 7. Normal sequence of GNRHR exon 1.1. Arrow indicates the normal T position.

DISCUSSION

In the current study, GPR54 mutation was not detected in any of the 31 sporadic cases of HH, although mutations in GPR54 gene have previously been found to be involved in the pathophysiology of HH (de Roux *et al*., 2003). Thus, two novel synonymous mutations were observed in one HH patient of Turkish-Cypriot and Afro-Caribbean mixed ancestry with delayed puberty. One was cysteine to arginine at 233 position (Cys223Arg) in 5th transmembrane helix and other was arginine to leucine at 297 position (Arg297Leu) in 3rd extracellular loop. These

novel mutations provided evidence that HH in humans may be caused by loss of function mutations in GPR54 (Semple *et al*., 2005).

Similarly, in a highly consanguineous family, a novel homozygous GPR54 mutation with T to C change resulting in substitution of phenylalanine with serine at position 272 (Phe272Ser) leading to HH was identified in six highly related patients belonging to two families of Israeli Muslim Arab. The males were presented with same clinical phenotypes of cryptorchidism, short penis and no spontaneous puberty (Nimri *et al*., 2011). Furthermore, a homozygous point mutation of GPR54 Leu102Pro observed in two Arab Muslim unrelated families with HH patients completely inhibited GPR54 signaling and resulted in a more quantitative than qualitative defect of activation of gonadotropic axis (Rakover *et al*., 2007). In addition, a GPR54 mutation L148S found in a Saudi Arabian family with 6 affected members from first cousin marriages impaired signaling of GPR54 receptor resulting in IHH (Seminara *et al*., 2003). In the same study, a non-related male with IHH was also found to have GPR54 mutations, R331X and X339R, causing elongation of the receptor sequence due to a disrupted stop codon (Seminara *et al*., 2003).

In the current investigation, two mutations in GNRHR were found in one sporadic case. One missense mutation (C to T) was identified in exon 1.1 at the position of 101 nucleotide, which translated in substitution of serine with phenylalanine at 34th position (Ser34Phe) in the N-terminal extracellular domain (ECD) of GnRHR. In a recent study, a missense mutation serine168arginine in fourth transmembrane domain of GNRHR gene was identified in a homozygous state in one male with complete HH (Fathi *et al*., 2013), which resulted in complete loss of receptor function because of full impairment of hormone binding to the receptor. In contrast, a compound mutation (Gln106Arg and Arg262Gln) found in another male patient caused partial HH. It was observed that Gln106Arg mutation located in the first extracellular loop of GNRHR decreased but did not eliminate GnRH binding, whereas Arg262Gln mutation located in the third extracellular loop of GNRHR negatively affected signal transduction. The authors found a plausible correlation between genotype and phenotype in their study. The patient, who was homozygous for completely inactivating S168R mutation exhibited complete IHH, whereas the affected patient, who was compound heterozygous for Gln106Arg-Arg262Gln mutations, showed partial HH (Fathi *et al*., 2013). In a similar study, three different heterozygous GNRHR mutations among 146 subjects with delayed puberty were reported. One female carried missense mutation c.317A>G (p.Q106R), and two females and three males carried 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.

In the present study, the GNRHR mutation Ser34Phe was identified in a 16 years old boy, who had low FSH, LH and T concentrations and retractile testes and genitalia. In a previous study, a 22 year old male diagnosed with delayed puberty at 18 years was found to have compound heterozygous GNRHR mutations Gln106Arg and Arg262Gln. Gln106Arg mutation in ECL1 markedly impaired GnRH agonist binding to the receptor. Both the Gln106Arg and Arg262Gln in ICL3 lowered IP3 production by 50% and the IP3 efficiency 50 fold (Kim *et al*., 2010). These compound heterozygous mutations were manifested by low FSH, LH and T concentrations and decreased libido, small testes, and a small penis. Nevertheless, the sperm count was normal but the motility was low (Kim *et al*., 2010). Another GNRHR gene mutation L102P in 1st ECL that resulted in substitution of leucine with phenylalanine caused HH by birth and bilateral cryptorchidism (Rakover *et al*., 2007).

The present study also provides some clues as to the molecular mechanism(s) that may be playing a prominent role in the hormonal regulation. For example, the mutation Ser34Phe, in itself is probably a very intriguing manifestation of the architecture of the cellular signaling in such hormonal control. Serine residue is well known to play a key role in the cellular signal transduction pathways due to its ability to be phosphorylated by up-stream kinase molecules or alternatively by its dephosphorylation by specific phosphatases. Therefore, this aspect of the regulation of GNRHR remains to be determined by conducting phosphorylation studies and its possible effect on the cellular signaling.

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 *et al*. (1997) studied a 22 year old male with delayed puberty at 18 years, who had small testes and small penis. His T and gonadotropins were low. He was found to exhibit compound heterozygous GNRHR mutations, Gln106Arg and Arg262Gln. The Gln106Arg mutation impaired GnRH binding to GnRHR. His sister exhibited compound heterozygosity for these two mutations. The unaffected parents were heterozygous for both mutations and an unaffected daughter was heterozygous, indicating that it is an autosomal recessive inheritance (Kim *et al*., 2010). Another 19 years old HH boy was identified with an A to T point mutation at codon 168 of GNRHR gene, resulting in a change from

Serine to Arginine. This mutation was homozygous in patients, whereas it was heterozygous in his both parents, who were phenotypically normal (Pralong *et al*., 1999). Similarly, Layman *et al*. (1998) reported a family with compound heterozygous GNRHR mutations Arg262Gln and Tyr284Cys, affecting one male and three females. They all had IHH with low gonadotropins levels and delayed puberty. Furthermore, Costa *et al*. (2001) found novel heterozygous mutations in GNRHR in four siblings (two males and two females) causing HH with low plasma LH concentration that was responsive to stimulation by GnRH.

GNRHR mutations can be classified into partial or complete loss of function mutations. The partially inactivating substitutions of GNRHR frequently found in familial HH are Q106R and R262Q (Fathi *et al*., 2013). The homozygous mutations in GNRHR may show variable phenotypes, including apparent delayed puberty (Lin *et al*., 2006). Thus, homozygous R262Q mutation in GNRHR identified in two brothers having delayed puberty at 15 years of age caused one boy to progress through puberty after a short treatment with T but his brother showed little progress after treatment (Lin *et al*., 2006). de Roux *et al*. (1997) also identified an A129D mutation in three siblings with variable phenotypic outcome. A comparison of compound heterozygous and homozygous patients suggests that their phenotype and the response to GnRH is determined by the GNRHR variant with less severe loss of function causing less changes in the phenotype (Fathi *et al*., 2013).

The mechanism whereby the mutation Ser34Phe caused HH in a boy is not properly understood. Nevertheless, it may be suggested that the mutation Ser34Phe observed in extracellular domain of GNRHR might affect proper binding of GnRH to it. The ECDs and/or transmembrane domains (TMDs) are involved in the formation of the ligand-binding pocket. Furthermore, ECD is involved in the binding of GnRH with GnRHR and GNRHR mutations Asn10Lys and Gln11Lys in ECD decrease binding of GnRH with GnRHR and IP3 signaling (Kim *et al*., 2010). In view of foregoing observations, it may be suggested that GNRHR mutation Ser34Phe might have reduced binding of GnRH to mutant GnRHR, resulting in reduction of signal transduction and decreased secretion of FSH, LH and T. Since GNRHR is also expressed in testes, it may also be suggested that GNRHR expressed in the testes that may be responsive to peripherally produced GnRH may be directly involved in their proper descend and development, and a mutation in GNRHR may lead to small retractile testes.

The other T-to-G mutation, identified at 123 nucleotide position in exon 1.1, was a synonymous point change, which did not replace valine 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.

CONCLUSION

In conclusion, we report here the identification of one novel missense mutation in GNRHR gene in one sporadic case. The missense mutation Ser34Phe resulted in lower secretion of FSH, LH and T and delayed puberty. This research has undoubtedly a direct significance in the current genetic, clinical and basic research and would certainly aid in understanding the molecular mechanisms involved in the hormonal regulation, signal transduction and determination of the cause of delayed onset of puberty.

ACKNOWLEDGMENTS

The author is thankful to PMAS Arid Agriculture University, Rawalpindi and IBGE, Islamabad, Pakistan for providing funds for execution of the project.

Conflict of interest statement

We declare that we have no conflict of interest.

REFERENCES

Beate, K. and de Roux, N., 2005. Molecular genetics of isolated hypogonadotropic hypogonadism and Kallmann syndrome. *Endocrin. Devel*., **8**: 67-80. http://dx.doi.org/10.1159/000084094

Beate, K., Joseph, N., de Roux, N. and Wolfram, K., 2011. Genetics of isolated hypogonadotropic hypogonadism: Role of GnRH receptor and other genes. *Int. J. Endocrinol*., 1-9.

Beneduzzi, D., Trarbach, E.B., Le-Min, Jorge, A.A.L., Garmes, H.M., Renk, A.C., Fichna, M., Fichna, P., Arantes, K.A., Costa, E.M.F., Zhang, A., Adeola, O., Wen, J., Carroll, R.S., Mendonca, B.B., Kaiser, U.B., Latronico, A.C. and Silveira, L.F.G., 2014. Role of gonadotropin-releasing hormone receptor mutations in patients with a wide spectrum of pubertal delay. *Fertil. Steril*., **102**: 838-846. http://dx.doi.org/10.1016/j.fertnstert.2014.05.044

Beranova, M., Oliveira, L.M., Bedecarrats, G.Y., Schipani, E., Vallejo, M., Ammini, A.C., Quintos, J.B., Hall, J.E., Martin, K.A., Hayes, F.J., Pitteloud, N., Crowley, U.B. and Saminara, S.B. JR., 2001. Prevalence, phenotypic spectrum, and modes of inheritance of gonadotropin-releasing hormone receptor mutations in idiopathic hypogonadotropic

hypogonadism. *J. clin. Endocrinol. Metab*., **86**: 1580-1588. http://dx.doi.org/10.1210/jcem.86.4.7395

Chevrier, L., Guimiot, F. and de Roux, N., 2011. GnRH receptor mutations in isolated gonadotropic deficiency. *Mol. Cel. Endocrinol*., **346**: 8-21. http://dx.doi.org/10.1016/j.mce.2011.04.018

Costa, E.M.F., Bedecarrats, G.Y., Mendonca, B.B., Arnhold, I.J.P., Kaiser, V.B. and Latronico, A.C., 2001. Two novel mutations in the gonadotropin releasing hormone reception gene in Brazilian patients with hypogonadotropic hypogonadism and normal olfaction. *J. clin. Endocrinol. Metab*., **86**: 2680-2686. http://dx.doi.org/10.1210/jcem.86.6.7551

de-Roux, N., Young, J., Misrahi, M., Genet, R., Chanson, P., Schaison, G. and Milgrom, E., 1997. A family with hypogonadotropic hypogonadism and mutations in the GnRHR. *New Engl. J. Med*., **337**: 1597-1602. http://dx.doi.org/10.1056/NEJM199711273372205

de-Roux, N., Genin, E., Carel, J.C., Matsuda, F., Chaussain, J.L. and Milgrom, E., 2003. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc. Natl. acad. Sci. USA*., **100**: 10972-10976. http://dx.doi.org/10.1073/pnas.1834399100

Demirbilek, H., Ozbek, M.N., Demir, K., Kotan, L.D., Cesur, Y., Dogan, M., Temiz, F., Mengen, E., Gurbuz, F., Yuksel, B. and Topaloglu, A.K., 2014. Normosmic idiopathic hypogonadotropic hypogonadism due to novel homozygous nonsense c.C969A (p.Y323X) mutation in the KISS1R gene in three unrelated families. *Clin. Endocrinol*., **82**: 429-438.

Fathi, A.K., Hu, S., Fu, X., Huang, S., Liand, Y. and Luo, X., 2013. Molecular defects of the GnRH receptor gene in Chinese patients with idiopathic hypogonadotropic hypogonadism and the severity of hypogonadism. *Int. J. Pediatr. Endocrinol.,* **25**: 659-668. http://dx.doi.org/10.1186/1687-9856-2013-s1-o25

Geffner, M.E., 2002. Hypopituitarism in childhood. *Cancer Cont*., **9**: 212-222.

Kalantaridous, S.N. and Chrousos, G.P., 2002. Monogenetic disorders of puberty. *J. clin. Endocrinol. Metab*., **87**: 2481-2494. http://dx.doi.org/10.1210/jcem.87.6.8668

Kim, H.G., White, J.F., Bhagavath, B. and Layman, L.C., 2010. Genotype and phenotype of patients with gonadotropin-releasing hormone receptor mutations. *Front. Horm. Res.,* **39**: 94-110. http://dx.doi.org/10.1159/000312696

Lanfranco, F., Gromoll, J., von Eckardstein, S., Herding, E.M., Nieschlag, E. and Simoni, M., 2005. Role of sequence variations of the GnRH receptor and G protein-coupled receptor 54 gene in male idiopathic hypogonadotropic hypogonadism. *Eur. J. Endocrinol*., **153**: 845-852. http://dx.doi.org/10.1530/eje.1.02031

Latronico, A., 2009. The GPR54 gene mutations as a cause for hypogonadotropic hypogonadism. *Eur. Soc. Endocrinol*., **20**: 1-3.

Layman, L.C., Cohen, D.P., Xie, M., Li, Z., Reindollar, R.H., Bolbolan, S., Bick, D.P., Shernis, R.R., Duck, L.W., Sellers, L.C. and Neill, J.D., 1998. Mutations in gonadotropins-releasing hormone receptor gene cause hypogonadotropic hypogonadism. *Nat. Genet*., **18**: 14-15. http://dx.doi.org/10.1038/ng0198-14

Layman, L.C., 2002. Human gene mutations causing infertility. *J. med. Genet*., **39**: 153-161. http://dx.doi.org/10.1136/jmg.39.3.153

Lin, L., Conway, G.S., Hill, N.R., dattani, M.T., Hinmarsh, P.C. and Archermann, J.C.A., 2006. Homozygous R262Q mutation in the gonadotropins-releasing hormone receptor and presenting as constitutional delay of growth and puberty with subsequent borderline oligospermia. *J. clin. Endocrinol. Metabol*., **91**: 5117-5121. http://dx.doi.org/10.1210/jc.2006-0807

Muir, A.I., Chamberlain, L., Elshourbagy, N.A., Michalovich, D., Moore, D.J., Calamari, A., Szekeres, P.G., Sarau, H.M., Middleton, J.K., Darker, J.G., Larminie, C.G., Wilson, S., Bergsma, D.J., Emson, P., Faull, R., Philpott, K.L. and Harrison, D.C., 2001. AXOR12, a novel human G-protein-coupled receptor, activated by peptide KISS-1. *J. biol. Chem*., **276**: 28969-28975. http://dx.doi.org/10.1074/jbc.M102743200

Mushtaq, T. and Wales, J.K., 2007. Diagnosis, investigation and treatment of delayed puberty in children. *Pediatr. Child Hlth*., **17**: 349-355. http://dx.doi.org/10.1016/j.paed.2007.06.005

Nimri, R., Lebenthal, Y., Lazar, L., Chevrier, L., Phillip, M., Bar, M., Hernandez, M.E., de Roux, N. and Gat, Y.G., 2011. A novel loss-of-function mutation in GPR54/KISS1R leads to hypogonadotropic hypogonadism in a highly consanguineous family. *J. clin. Endocrinol. Metab*., **96**: 536-545. http://dx.doi.org/10.1210/jc.2010-1676

Noel, S.D. and Kaiser, U.B., 2011. G-protein coupled receptors involved in GnRH regulation: Molecular insights from human disease. *Mol. cell. Endocrinol*.,

346: 91-101. http://dx.doi.org/10.1016/j.mce.2011.06.022

Papaoiconomou, E., Msaouel, P., Makri, A., Diamanti, K.E. and Koutsilieris, M., 2011. The role of kisspeptin/GPR54 in the reproductive system. *In Vivo*, **25**: 343-354.

Pralong, F.P., Gomez, F., Costillo, E., Cotecchia, S., Abuin, L., Aubert, M.L., Partmann, L. and Gaillard, R.C., 1999. Complete hypogonadotropic hypogonadism associated with a novel inactivating mutation of gonadotropins-releasing hormone receptor. *J. clin. Endocrinol. Metab.,* **84**: 3811-3816. http://dx.doi.org/10.1210/jcem.84.10.6042

Rakover, Y.T., Ducos, M.C., Iovane, A., Aumas, C., Admoni, O. and de Roux, N., 2007. Neuroendocriniie phenotype analysis in five patients with isolated hypogonadotropic hypogonadism due to a L102P inactivating mutation of GPR54. *J. clin. Endocrinol. Metab.,* **92**: 1137-1144. http://dx.doi.org/10.1210/jc.2006-2147

Sambrook, J., Fritschi, E.F. and Maniatis, T., 1989. *Molecular cloning: a laboratory manual*. Cold Spring harbor Laboratory Press, New York.

Seminara, S.B., Messanger, S., Chatzidaki, E.E., Thresher, R.R., Acierno, J.S., Shagoury, J.K., Abbas, Y.B., Kuohung, W., Schwinof, K.M., Hendrick, A.G., Zahn, D., Dixon, J., Kaiser, U.B., Slaugenhaupt, S.A., Gusella, J.F., Rahilly, S.O., Carlton, M.B.L., Crowley, W.F., Aparicio, S.A. and Colledge, W.H., 2003. The GPR54 gene as a regulator of puberty. *New Engl. J. Med*., **349**: 1614-1627. http://dx.doi.org/10.1056/NEJMoa035322

Semple, R.K., Achermann, J.C., Ellery, J., Farooqi, I.S., Karet, F.E., Stanhope, R.G., Orahilly, S. and Aparicio, S.A., 2005. Two novel missense mutations in G protein-coupled receptor 54 in a patient with hypogonadotropic hypogonadism. *J. clin. Endocrinol. Metab*., **90**: 1849-1855. http://dx.doi.org/10.1210/jc.2004-1418

Tanner, J.M. and Whitehouse, R.H., 1976. Clinical longitudinal standards for height, weight, height velocity, weight velocity and stages of puberty. *Arch. Dis. Child*., **51**: 170-179. http://dx.doi.org/10.1136/adc.51.3.170

Ulloa-Aguirre, A., Zarinan, T., Dias, J.A. and Conn, P.M., 2014. Mutations in G protein coupled receptors that impact receptor trafficking and reproductive function. *Mol. cell. Endocrinol*., **382**: 411-423. http://dx.doi.org/10.1016/j.mce.2013.06.024

Vaaralahti, K., Wehkalampi, K., Tommiska, J., Laitinen, E.M., Dunkel, L. and Raivio, T., 2011. The role of gene defects underlying isolated hypogonadotropic hypogonadism in patients with constitutional delay of growth and puberty. *Fertil. Steril*., **95**: 2756-2758. http://dx.doi.org/10.1016/j.fertnstert.2010.12.059