Genetic Analysis of *GnRH1* variants in patients with Idiopathic Hypogonadotrpic Hypogonadism in Pakistani Population

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Abstract
Idiopathic hypogonadotropic hypogonadism (IHH) is the reduction or absence of Gonadotropic releasing hormone (GnRH) secretion which stimulates FSH and LH (gonadotropins) from the anterior pituitary. This study was carried out to find the link of GnRH coding exon variants affecting final GnRH structure which lead to the disease. The clinical data and blood samples of 35 IHH patients were obtained with their informed consent. The exon 2 of GnRH1 gene, being the coding sequence of GnRH among all 4 exons, was amplified by Polymerase chain reaction (PCR). After PCR amplification, SSCP analysis was done to detect the existence of variations on the basis of difference in migration in Polyacrylamide gel. From SSCP, five samples were selected and sent for sequencing. A known missense variant NM_001083111.2: c.47G>C in exon 2 of GnRH1 gene was observed in two patients without any effect on final structure of protein. In conclusion, this study shows that the GnRH1 exon 2 variants affecting GnRH structure are either not common or in non-coding region for variations in promotor regions, transcriptional region.

Keywords: GnRH1, Idiopathic hypogonadotropic hypogonadism, infertility, sequence variance.

1 Introduction

Idiopathic hypogonadotropic hypogonadism is an autosomal recessive disease caused by the low secretions of gonadotropic releasing hormone resulting in the reduction of gonadotropins (LH and FSH) secretions from hypothalamic pituitary gonadal axis. This leads to reduced secretions of testosterone from Leydig's cells in testicles as a result of low LH secretions causing absence or incomplete sexual development at puberty (9 to 14 age)[1],[2],[3],[4]. The occurrence of IHH is 1 in 8000 in males and 1 in 4000 in females or 1 in 10,000 in males [5],[6],[7],[8]. IHH patients are mostly diagnosed late in adolescence due to difficulties in differentiation from cases of delayed puberty [9],[10]. Clinical examinations and laboratory tests are required to confirm the diagnosis of IHH [11]. For its treatment, combinational hormonal therapies are being used [12] to increase the LH, FSH, Testosterone and sperm count [13]. GnRH pump is another possible treatment in which GnRH is pumped for around every two hour into the subcutaneous tissue [14]. There are many genes associated with IHH from which 60 genes have been identified up to date for half of the IHH cases [1]. Most important in them are GnRHR, GnRH1, TAC3, KISS1, TACR3 and KISS1R [15]. GnRH gene was first discovered in relation to sexual development and it has 23 isoforms and most authentic one proved is GnRH related to the stimulation of FSH and LH [16]. GnRH is located on 8p21.2 and precursor protein which is processed to form gonadotropic releasing hormone. GnRH consists of a decapeptide having altered pyroglutamate (pGlu) at the N-terminal and an amine group at C-terminal with the sequence "pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2" [17]. There are four exons in which exon 2 contains a translation site encoding GnRH decapeptide, a signal peptide and a portion of GnRH associated peptide (GAP). The remaining exons encode other GnRH associated peptides and 3' UTR (untranslated region) and exon splicing enhancers [18]. The present study emphasizes on the variations present in GnRH coding exon responsible for IHH as it is directly related to GnRH structure and function.

2 Materials and Methods

2.1 Study Approval and sample collection

Approval of the study carried out on patients diagnosed with Idiopathic Hypogonadotropic Hypogonadism was acquired from Institutional review board (IRB) of Fatima Memorial Hospital College of Medicine & Dentistry

(FMHCMD). The approval letter along with informed consent of all the patients whose blood samples were taken was obtained for the study of 2 to 3 years duration. The approximate sample size was calculated statistically via using Solvin's Formula based on the occurrence of IHH in Pakistani population which came out to be 35. Blood samples were collected in EDTA vials. The conformation of IHH was done from FMHCMD under the observation of co-author / Ando Urologist. Written informed consent was obtained from patients before taking samples. All male normosmic patients with smaller size testes and volume, azoospermia, low semen volume with low LH, FSH & testosterone were included in the research. All patients below pubertal age and other hormonal or medical disorders were excluded from the study.

2.2 Clinical Analysis of patients

The patients were clinically examined at FMHCMD. Their testicular volume, pubertal delay, anosmia, testicular size and volume and erectile dysfunction were observed. Other diagnostic laboratory tests such as semen analysis and hormone assays ((LH, FSH, testosterone level, prolactin, TSH), and pituitary MRI were also performed in Fatima Memorial Hospital. Detailed History of patients was taken. Medical history and blood reports of patients was taken from hospital and genetic study was performed in School of Biochemistry and Biotechnology.

2.3 Genomic DNA extraction and PCR

Fresh blood samples were collected in EDTA vials. DNA extraction was done by standard procedure and confirmed by 1% agarose gel. DNA was stored at -20oC for further use [19]. The quantification of DNA was done via Nano Drop Spectrophotometer.

PCR amplification of GnRH1 exon 2 was performed with the help of forward and reverse primers. Primer sequences of exon 2 were obtained from literature and confirmed through NCBI data base, GRCh38.p12 with accession number: NG_016457.1 from position 6598-6981. No self-complementarity, GC content and absence of formation of hairpins and dimers was confirmed with Oligo Analyzer (version 3.1). The forward and reverse primer sequences of GnRH1 exon 2 are "5′-CCATCTTCTGCAGGGTTAGTG-3′" and "5′-GCCTTATCTCACCTGGAGCA-3′" respectively [20]. PCR products were confirmed on agarose gel electrophoresis (2%).

2.4 Single Stranded Conformational Polymorphism (SSCP) Analysis

SSCP analysis was performed with amplicons of affected and healthy samples in order to determine the presence of any nucleotide variation via difference in band migration patterns. The denaturation was done by adding PCR product formamide dye and incubating at 95oC for 15 minutes followed by immediate cooling at -20oC. Native Polyacrylamide gel electrophoresis was run with 8% polyacrylamide gel at 40 volts for 2-3 hours at room temperature. The difference in migration pattern of bands was observed with the help of ethidium bromide staining and visualized in UV light [21],[22].

2.5 Sequencing and Bioinformatics Analysis

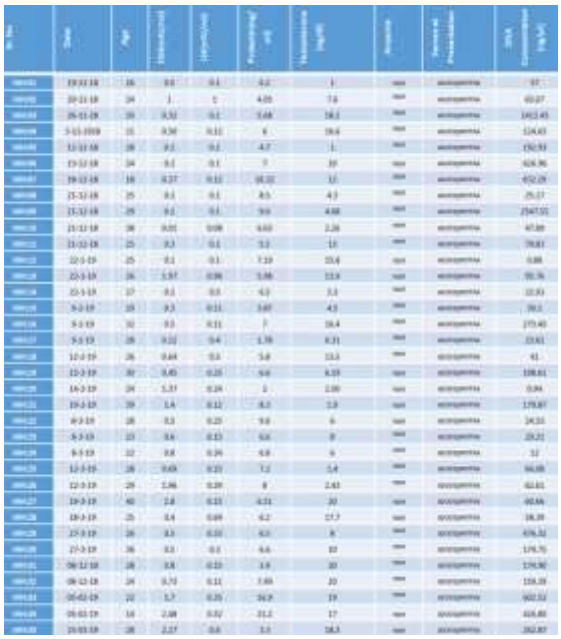
The selected samples from SSCP analysis were sent for sequencing after gel extraction of PCR products. The eluted PCR products were labelled and sent for sequencing. The results were analyzed with BioEdit software and NCBI. The results were confirmed by using Exome Aggregation Consortium browser and 1000 genomes database. Swiss Model was used to analyze the final protein structure of GnRH obtained from sequence of GnRH1 exon 2 of affected samples.

3 Results

3.1 Clinical data

In this study the average age of patients was 26.63±6.41. Following table 1 consists of hormonal values of patients and DNA concentrations.

Table 1: Patients Hormonal analysis and DNA concentrations.



The median (M) and interquartile range (IQR) of age is 26 and 5 respectively. The M and IQR of FSH is 0.5 and 0.73, LH is 0.15 and 0.25, prolactin is 6.6 and 1.81 and testosterone is 8 and 12.3 respectively.

All patients are managed by endocrinologist and andrologist and were recommended injection hCG and hMG. Those patients who were treated with hCG, hMG or S. testosterone injection showed comparatively improved hormonal values.

3.2 Genomic DNA extraction and PCR amplification

Genomic DNA of IHH patients were extracted and visualized on 1% agarose gel electrophoresis. GnRH1 exon 2 primers were optimized with healthy control sample. PCR genotyping of all affected samples with GnRH1 exon 2 was performed and results were visualized on 2% agarose gel.

3.2 Single-Stranded Conformational Polymorphism (SSCP)

SSCP analysis was performed with PCR amplified products of healthy and control and the difference in migration pattern of bands was observed. Figure 1(a) shows the SSCP results of PCR products selected for sequencing in random patients.

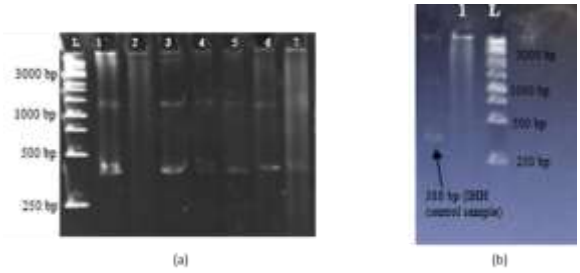


Fig1. Denatured GnRH1 on 8% Native PAGE (a) from left 1 kb DNA ladder Thermo Scientific GeneRuler in Lane L; Lane 1,3-6: PCR product with sample 16, 30, 17,19, 9 (b) from right 1 kb DNA ladder Thermo ScientificGeneRuler in Lane L; SSCP of GnRH1 exon 2 PCR product of control sample in Lane1.

3.3 Bioinformatics analysis of sequencing results

NCBI data base was used for sequence comparison of GnRH1 exon 2 through BLAST. Bioedit was used for sequence alignment and interpretation of all consensus DNA sequences. The single nucleotide variation found in the target DNA sequences were confirmed with Exac (Exone aggregation consortium) browser and 1000 genomes database. The prediction of protein was done through Swissmodel "http;//www.swissmodel.expasy.org". The sequenced samples included 5 patients selected after SSCP analysis to the coding sequence of GnRH1 being studied in this work. The figure 2 shows a single nucleotide variation in the affected samples as compared to the reference sequence and figure 3 shows the same variation in DNA chromatograms.

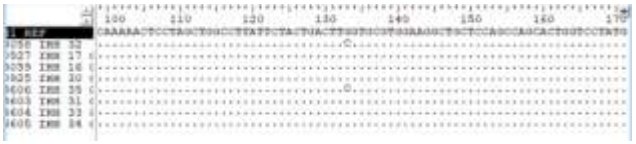


Fig2. Sequencing results of IHH affected samples amplified with GnRH1 exon 2 variation from G to C in 2 affected samples i.e. IHH: 32 and IHH: 35 in comparison with reference sequence obtained from NCBI.

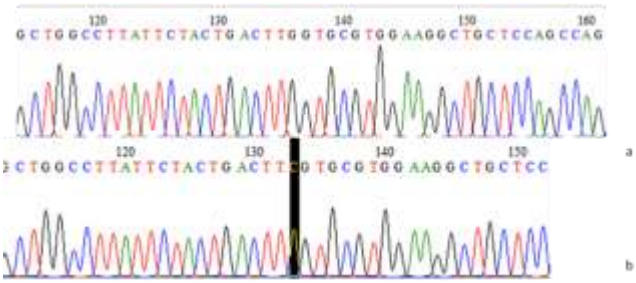


Fig3. DNA sequencing Chromatogram of IHH:32 amplified with GnRH1 exon 2 (a) wild type sequence (b) query sequence of IHH:32 amplified with GnRH1 exon 2 showing SNP from G to C.

The variation leads to change of amino acid from tryptophan to serine at position 16 (p.W16S) in the signal peptide of 92 amino acid precursor protein studied by NCBI. For the prediction of GnRH structure affected by this variant, SwissModel was used. There were no structural modifications found in the protein structure of GnRH as showed in figure 4.

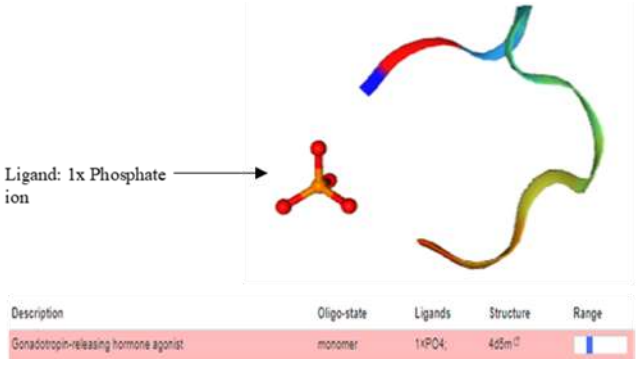


Fig4. Predicted 3D structure of GnRH1 decapeptide (QHWSYGLRPG) from SwissModel indicating no change in the final protein structure due to SNP in signal peptide.

The variant NM_001083111.2: c.47G>C was observed in 2 IHH patients. The parents from the IHH were also genotyped and amplified products of GnRH1 exon 2 were sequenced. The variation was not found in any of the parental samples showing that this variant was not inherited from any of the parents.

4 Discussion

The present study was conducted on genetic screening of GnRH1 in patients suffering from IHH as it is a candidate gene for controlling the reproduction and puberty. It encodes 92 amino acids which give GnRH after final processing. First 23 amino acids form signal protein or proganadoliberin-1 isoform 2 preprotein. Other amino acids form gonadotropic releasing hormone (GnRH) and a chain of GnRH associated peptide 1 or GAP-1. After PCR amplification, the presence of single nucleotide mutation was detected with the help of single stranded conformational polymorphism analysis which showed variation in migration of bands in the gel. The results of sequencing indicated the presence of a single nucleotide missense variant NM_001083111.2: c.47G>C observed in 2 IHH patients (IHH: 32 and IHH: 35) in exon 2 at position 6757 of GnRH1 gene. The variation leads to amino acid change at position 16 i.e., Tryptophan to Serine (p.W16S) in the precursor protein encoded by the GnRH1 gene. Mutation in a signal peptide also known as progonadoliberin-1 isoform 2 preproprotein. The filtering allelic frequency of variant is 0.5345 which is greater than its total population frequency 0.2332 according to Exome Aggregation Consortium database. This shows that this variant is too common in a population mainly Asia and Africa to be disease causative. Though the final protein structure of GnRH predicted with the help of SwissModel (http;//www.swissmodel.expasy.org) browser did not have any change in it mainly because of the signal peptide being cleaved before final processing of GnRH. This variant has been previously reported in familial patients of central precocious puberty [23], postmenopausal women and osteoporosis patients associated with bone density in Japan [24], nIHH patients in Russia [25], breast cancer in Netherlands [26] and associated with lower levels of testosterone and follicles in PCOS patients [27]. However, it has not been previously reported as main cause of Idiopathic Hypogonadotropic Hypogonadism further confirmed by this study. It has also not been reported in any IHH cases in Pakistan as per the results of a study conducted in Pakistan by Aslam and co-workers in which they found no variants in GnRH1 gene [28]. Tryptophan amino acid is incorporated in protein structure and function. It is stable with its intermittence in proteins and its codon [29]. Serine is without a bulky side chain and is close to most other amino acid residues. The inequalities have made apparent that amino acid tryptophan is highly conserved and serine being fast-evolving having high number of mutations [30]. Thus, it supports the fact that mutations in GnRH1 gene causing an aberration in final protein structure are either very rare or eliminated in generations. They might also be due to change in some other regulatory elements or co-exist with some other mutations. As a result they do not directly affect the GnRH structure and eventually its function. Despite not having a direct involvement in IHH, this variant can be related with other phenotypic characteristics and not only in IHH but also in other reproductive disorders as observed in previous studies [23],[25],[27] . Therefore, a complete analysis of whole GnRH1 and its relation to phenotype is required to find the key elements responsible for GnRH deficiency causing Idiopathic hypogonadotropic hypogonadism.

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