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Original Research

Polymorphic noncoding region of GNRH1 gene in male patients with impaired fertility

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Abstract

Impaired male fertility is a multifactorial pathological condition and genetic contribution is up to 20%. Gonadotropin Releasing Hormone 1 (GNRH1) is associated with maturation of spermatozoa. We aimed to study genetic sequence of GNRH1 gene in azoospermia males. We collected 50 clinically diagnosed infertile males after the informed consent. DNA was extracted and primers were designed to amplify the exonic and exon/intron boundaries of exon 1 and exon 2 region of GNRH1 gene. The amplicons were sequenced and analyzed using bioinformatic tools. We have identified sequence variations at c.1325-131del.A, c.1325+40 T>G, c.1325+34T>G, c.1325+124 Ins. A, c.1325+126 Ins. A, c.1325+181T>G, c.1325+264 Ins.G and c.1325+269 Ins. A position. Human Splicing Finder predicted that c.1325-131del.A, c.1325+34T>G, c.1325+124 Ins. A, c.1325+264 Ins.G and c.1325+269 Ins. A have impact on the splicing while c.1325+40 T>G, c.1325+126 Ins. A and c.1325+181T>G have relation with the splicing. The c.1325+40 reported in literature as rs2709608 but has no association with the male infertility. The c.1325+181T>G We could not find other variations in the literature therefore suggested to be novel. Our study suggests that intronic region of GNRH1 gene a highly variable in Pakistani infertile male patients and suggests to explore its correlation with the disease on a large scale.

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Introduction: Gonadotropin Releasing Hormone (GnRH) has a central role in controlling reproductive functions. It is manufactured in hypothalamic neurons and released in palpitate fashion into hypophyseal portal circulation to extend to anterior pituitary, where it binds to particular receptors (GnRHRs) and controls gonadal steroidogenesis in both males and females thus triggering synthesis and liberation of two gonadotropins i.e., follicle stimulating hormone (FSH) and luteinizing hormone (LH) [1]. The insufficient discharge or action of GnRH leads to lacking or diminished sexual growth at adolescence (Aslam et al. 2015). The principal management of reproductive proficiency in mammals is facilitated by the pulsatile discharge of GnRH-1 from their relative hypothalamic neurons [2] into the portal circulation of the pituitary gland, where its function is to control the discharge of gonadotropins and prolactin. If there is any impairment in this scheme, consequently infertile reproductive organs because of hypogonadism and hyperprolactinemia and leading to infertility [3]. Almost about 7% of all men face fertility problems. The etiology of damaged sperms production and their role can be associated with factors which act at testicular level directly or at pre or post testicular levels [4]. GnRH1 neurons play pivotal role in puberty and fertility[5]. *GNRH1* gene is uniquely exhibited in distinct population of neurons in hypothalamus. *GNRH1* gene positioned 8p21.2 encodes a 92 amino acids preproprotein (pre-pro-GnRH) [6]. *GNRH1* gene germ line mutations may result in idiopathic hypogonadotropic hypogonadism (IHH) in men and women [7]. IHH is a condition in which an individual fail to undergo puberty. It is due to irregular secretion of GnRH. *GNRH1* gene is also a contributing role[8]. The p.R31C mutation in *GNRH1* where arginine is replaced by cysteine[9]. A large deletion was revealed after investigation of hpg mouse *gnrh1* gene which left the promoter region only. One of the hypothesis stated that the inefficacy of GNRH neurons to manufacture and discharge GNRH was due to the gene shortening lead to non-functional mRNA precursor molecule of *GNRH1* gene[5].

The *GNRH1* gene was assumed to be the nominee contributory gene for HH for the presence of the hypogonadal (hpg) mouse model and the *gnrh1* homozygous gene loss in mice. Though human *GNRH1* gene alteration has not been recognized for many years, two self-determining sets have freshly defined homozygous frameshift alterations in patients with normosmic HH. A heterozygous mutation was recognized in the patients without indications or symptoms; hence, heterozygous transfer of the mutation is not considered to be a cause of HH. The main objective or our study was to explore the intronic sequence of *GNRH1* gene in infertile Pakistani male patients.

Methodology: We registered the infertile male (based on the semen profile data) from various diagnostic centers of Lahore region after the informed consent. After their willingness, 50 males participated in this study and we collected blood sample for DNA extraction. Each blood sample was shifted to the tube containing 500 µl of 0.5 M EDTA (ethylene diamine Tetra acetate), then stored at -20^{0}C till DNA extraction.

Genomic DNA was isolated from WBCs using standard organic method with some modifications. DNA samples were quantified and analyzed on 1.5% agarose gel for 30 minutes at 90-100V. Primers were designed to explore the intron-exon intersections of *GNRH1* using primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) as mentioned in the table 1.

PCR conditions were optimized, and all the DNA samples were amplified under the same conditions as mentioned below. Primer optimizing conditions were denaturation at 94^{0}C for 3 minutes, annealing at 94^{0}C for 1 minute, 55-65^{0}C for 1 minute and 72^{0}C for 1 minute. 10 cycles were repeated at these annealing conditions with the difference of -1^{0}C. Elongation was carried out at 94^{0}C for 1 minute, 55^{0}C for 1 minute and 72^{0}C for another 1 minute. This was repeated 25 times. In the last step temperature was set at 72^{0}C for 7 minutes to infinite hold at 4^{0}C. After PCR run, products were run on agarose gel and observed under gel doc for the confirmation of procedure. Size of each band was examined by comparing with ladder of size 1kb, according to size of primer sequence. The reagents required for PCR were; dNTPs =2.5 ul, $MgCl_2$ =2.5 ul, Buffer =2 ul,Taq=0.5 ul ,FP=0.5 ul ,RP= 0.5 ul,Water =14.5 ul, DNA sample=2 ul.

PCR products were analyzed on agarose gel 1.2% stained with ethidium bromide dye and bands were observed under U.V trans-illuminator.

Sanger sequencing was performed on the amplicons and data was analyzed using bioinformatics tools i.e., bioedit and exon splicing finder. Alignment showed single base changes in 15 samples as shown in the result section.

Results: After sequencing, results were analyzed using bioedit and alignment was carried out using BLAST tool and we observed 08 variations in the intronic regions.

Sequence Variations: We found one deletion A nucleotide in 10 patient samples at c. 1325-131 position (Figure 1). Similarly, T>G change at c.1325+40 observed in 9 patients. Addition of A nucleotide at c.1325+124 and c.1325+126 found in 3 and 2 male patients respectively.

Splicing effect prediction: Splicing effect was predicted using Human Splicing Finder a reliable an online tool for splicing site prediction [10]. This online tool predicted that c.1325-131del.A, c.1325+34T>G, c.1325+124 Ins. A, c.1325+264 Ins.G and c.1325+269 Ins. A are in the potential splice site regions. Therefore, suggested to have some role in the splicing of *GNRH1* transcript. As shown in the figure 3, the potential splice site indicating significance of the intronic region changes.

Discussion: We identified 08 variations (Table 2) out of 50 samples. Each position was analyzed db SNP and ENSEMBLE where only two rs numbers were found to have been reported. The variant position c.1325+40 where T was replaced by G had rs2709608. These variant overlaps 2 transcripts, has 3281 sample genotypes and is mentioned in 1 citation. Second type of reported mutation was at position c.1325+181 with T>G change had same variant in gene as c.1325+40. This variant in gene *GNRH1* has been cited by Zerno et al. (2016) in which homozygous change at this position observed in mother the offspring. Suggested that this change in *GNRH1* gene possibly associated with reproductive phenotype in males [11]. This polymorphism has also been investigated with rheumatoid arthritis but found no notable interrelation between *GNRH1* polymorphism and rheumatoid arthritis [12].

Certainly, the phenotype of mice homozygous for *Gnrh1* deletion was accordant with HH [13]. Nonethless, it was in 2009 when two groups working independently found homozygous *GNRH1* mutations in a male patient and in one sib-pair with normosic HH [3,14] additionally, infrequent variants were noticed and published [3]. Afterwards, patients have not been reported with *GNRH1* mutations, which lead to the interpretation that *GNRH1* mutations seem to be an exceedingly infrequent reason of nHH [15].

In 2009 a homozygous frame shift mutation (c.18-19insA) in GNRH1 was reported in the terminal region containing amino group in GNRH's precursor of protein. This contained a single peptide obtained from a teenager sister and a brother where both of them had normosmic Induced Hypogonadotropic Hypogonadism. This study was of particular importance as efforts made by certain other teams had never concluded recognition of modification in *GNRH1* gene in patients with IHH. Certain variation in *GNRH1* has been reported to be a genetic cause of normosmic IHH [14]. In case of *GNRH1*, pre-mRNA maturation also correlated with neuron specific splicing factors [16]. The possible mechanism explained by Son et. al (2005) suggested that the deletion of genome of last two exons containing exonic splicing enhancers was followed by the assembly of first transcripts retaining intron which can be transferred to cytoplasm and hinder the translation of conserved first-intron transcripts into GnRH peptides, stated the overpowering effect intron 1 hold on the translational action of downstream open reading frame which ultimately results in insufficiency of functional GnRH and hypogonadism in mouse [17].

GNRH1 has been reported in Pakistani patients but the reported variations are in the coding region and were associated with HH [18]. There is a huge number of citations in the published literature manifesting the significance of intronic regions. We have found intronic region a polymorphic but may impact on the splicing. Splicing is very crucial in maturation of pre-mRNA of *GNRH1* gene. Promoter sequence of GNRH1 gene has regulatory role in GNRH expression [19]. In present study, all the variations were found in the intronic regions which might have effect in male fertility. There might be some other factors or genes involved for causing infertility in patients whose samples were taken. It suggested to explore these variations based on case-control study on a large cohort.

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Table. 1. Primer Sequences used for amplification

S. No.	Primer ID	5'----------3'	GC%	Tm(°C)	Product Size
1	GnRH1_Ex1_Hum_F	AACTAAAGGCAAGCCAGCAA	55.00	60.36	449bp
2	GnRH1_Ex1_Hum_R	GCCTTATCTCACCTGGAGCA	45.00	60.02	
3	GnRH1_Ex2_Hum_F	TTATCTCCAGATCCCCATGC	50.00	60.00	452bp
4	GnRH1_Ex2_Hum_R	TGAAACACGGTCCTACCACA	50.00	59.85	

Table. 2. Summary of 08 variations identified in *GNRH1* gene

Sr. no.	Frequency	Position	Intron/Exon	Splicing effect	Base change
1	9	c.1325+40	Intron	No effect	T>G
2	1	c.1325+34	Intron	Affected	T>G
3	10	c.1325-131	Intron	Affected	A (deletion)
4	1	c.1325+264	Intron	Affected	G (addition)
5	1	c.1325+269	Intron	Affected	A (addition)
6	3	c.1325+124	Intron	Affected	A (addition)
7	2	c.1325+126	Intron	No effect	A (addition)
8	1	c.1325+181	Intron	No effect	T>G

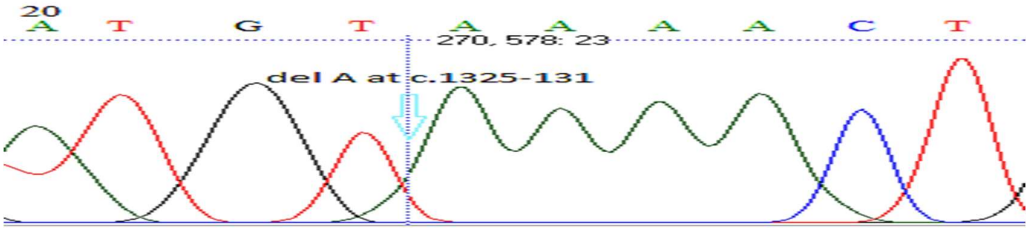

Fig. 1. Chromatogram showing deletion of A at c. 1325-131 position.

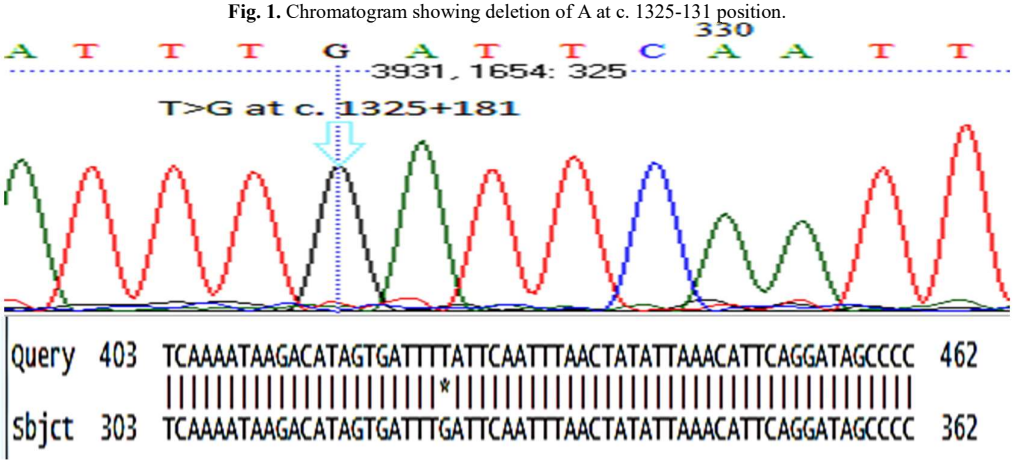

Fig. 2. Chromatogram showing transversion T>G at position c.1325+181

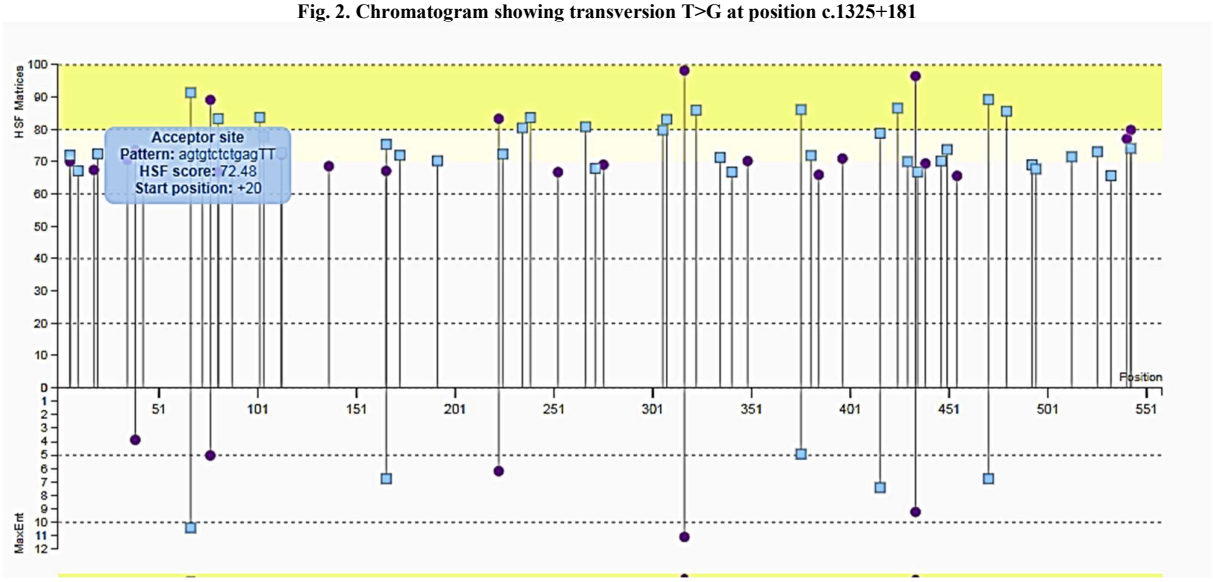

Fig. 3. The graphical presentaion of potential splice site through HSF tool.