Pakistan J. Zool., vol. 51(3), pp 995-1001, 2019. **DOI**: http://dx.doi.org/10.17582/journal.pjz/2019.51.3.995.1001

Polymorphism and Allele Frequency Variation in Proximal End of Promoter and Exon 1 of Human Growth Hormone Gene in Pakistan- A Pilot Study

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Article Information
Received 26 May 2018
Revised 23 July 2018
Accepted 10 August 2018
Available online 28 March 2019

Authors' Contribution
AJS designed the experiments, analyzed the results and reviewed the manuscript. MK performed the experimental work and drafted the manuscript.

Key words
Polymorphism, Growth hormone gene family, Minor allele frequency, Vitamin D response elements.

ABSTRACT

Growth hormone gene belongs to a highly polymorphic gene family whose members contain regions of very similar conserved sequences making this gene a challenging candidate to analyze. Almost 90% of the sequence of growth hormone gene family members is similar and prone to single nucleotide variations. The sequence variation occurring in the non-coding region of the GH-1 gene leads to altered transcriptional regulation causing various developmental defects in humans. In this report, we have analyzed a short sequence of ~200-240 bp spanning the proximal promoter and exon 1 (proximal portion) of the GH-1 gene to analyze the SNPs and allele frequencies of respective SNPs. Genomic DNA from blood was extracted, and the selected region was amplified using PCR. Sequencing of the amplified product was performed using Sanger sequencing, and the resulting sequence was analyzed. We have recorded 11 SNPs in total that occur in the VDRE region, 5' UTR and untranslated region of exon 1. A greater number of the SNPs were observed to coincide with the reported data from various sources. Two SNPs at position -62 and -56 (relative to transcription start site) are novel and not reported elsewhere up to the best of our knowledge. The minor allele frequencies of SNPs at position -57, -50, -48, -40, -34, -1 and +58, respectively are higher than other populations. All the observed SNPs are located in the region involved in transcription factor binding. This study is the first report regarding the allele frequency data and SNPs of the GH promoter region and exon 1 in the Local population.

INTRODUCTION

The human growth hormone (GH) gene family is a family of homologous gene members. The gene family arose as result of gene duplication of a single ancestral gene followed by further duplication events. The GH gene family is organized into five highly similar genes namely growth hormone-1 (GH-1), chorionic somatomammotropin hormone-like-1 (CSHP-1), chorionic somatomammotropin hormone-1 (CSH-1); growth hormone-2 (GH-2) and chorionic somatomammotropin hormone-2 (CSH-2), spanning a region of 66-67kb on chromosome 17q23. The 5' end of the GH gene cluster is occupied by the GH-1 gene which is the most comprehensively studied member of the gene family (Chen *et al*., 1989). GH-1 is expressed in somatotroph cells of pituitary whereas other paralogues are all placentally expressed.

GH-1 is the primary gene responsible for the secretion of human growth hormone for regulating all postnatal growth. Growth hormone controls the overall growth pattern, stature *etc*. in human beings. GH mainly acts by binding to its receptor and stimulating the production of insulin-like growth factor (IGF). GH along with IGF are known to operate in the pathways that regulate cell proliferation. GH-1 gene is strictly regulated to achieve the desired gene expression under various physiological states and during the different stages of life in humans. A locus control region is present at ~14.5 kb upstream of the gene that interacts with the promoter of the GH-1 to control transcription (Jones *et al*., 1995). Various studies have identified SNPs in the LCR region that act in conjunction with the promoter SNPs and affect the activity of the promoter (Horan *et al*., 2003) and thus makes it a difficult candidate to study (Kopchick, 2004).

The promoter of the GH-1 is highly polymorphic, harboring a cluster of SNPs in different haplotypic combinations (Krawczak *et al*., 1999). Several studies have linked the promoter single nucleotide variations with increased and decreased risk of neoplasia. In Polish and Chinese cohorts the promoter SNPs were linked to breast cancer susceptibility (Millar *et al*., 2003; Le Marchand *et al*., 2002; Wagner *et al*., 2005; Ren *et al*., 2004; Canzian *et*

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0030-9923/2019/0003-0995 $ 9.00/0

al., 2005). Association between one SNP in the promoter and Intron 4 was found to cause variation in the secretion of GH and IGF profiles and linked with accelerated bone loss and short stature in children (Hasegawa *et al*., 2000). Various other SNPs in the promoter region including SNPs in pituitary specific transcription factor (Pit-1), Vitamin D response elements (VDRE), nuclear factor (NF) *etc*. are known to cause idiopathic short stature, Isolated growth hormone deficiency, dwarfism and other growth-related changes (Madeira *et al*., 2016; Giordano *et al*., 2006; Bona *et al*., 2004).

In the current study, we have investigated the SNPs of the proximal promoter and exon-1 of the human growth hormone gene (GH-1) in a preliminary study in the local population to analyze the allelic variations of the SNP of GH-1 gene in Pakistan in comparison to reported data in other populations.

MATERIALS AND METHODS

Human subjects

One hundred human subjects that fit the criteria of exclusion and inclusion were recruited for the study. The study group consisted of normal statured local Pakistani population (male and female). Height and weight of the subjects were recorded. Venous blood was collected from the subjects. Informed consent according to Helsinki's guidelines was taken from the subjects. 3 ml of blood was collected from the subjects and added to K_2EDTA vacutainers (BD®). The vacutainers containing blood were stored at 4ºC and used for isolation of genomic DNA.

The criteria for inclusion of subjects was normal stature, no genetic abnormality, no chromosomal non-disjunction cases, no cases of dwarfism or gigantism, no history of breast cancer and no history of isolated growth hormone deficiency up to two previous generations in the family respectively. The criteria of exclusion was growth abnormalities, stunted growth, abnormally low or high BMI and genetic susceptibility to cancer.

Isolation of genomic DNA and restriction digestion

Genomic DNA was isolated from the blood collected from all subjects. DNA extraction was performed as described previously (Farooq and Sami, 2013). Restriction of DNA was performed with EcoR1 to obtain a 2.6 kb fragment specific to GH-1 gene. Restriction was performed in a 50 µl reaction containing 2 µg genomic DNA, 4 µl of EcoR1 enzyme (Thermo Scientific), 4 µl of 10X EcoR1 Buffer and nuclease-free water. The reaction mixture was incubated at 37°C for 16 h. The restriction pattern was observed on a 1.2% agarose gel by electrophoresis.

Primer designing

Primers were designed against the gene sequence of human growth hormone-1 gene (Accession number NG_011676.1). Primers were designed to amplify exon 1 and the 5' proximal promoter region of the gene for polymorphism analysis. The primers were validated by primer blast software.

Amplification and sequencing

DNA was amplified in a 25µl reaction using 50 ng of genomic DNA, 0.4 µM of each primer (Forward and Reverse), 12.5 µl GoTaq Green Master mix (Promega) and nuclease-free water, using PCR amplification conditions as described previously (Farooq and Sami, 2013). The resulting PCR products (240bp) were visualized on 1.2% agarose gel by electrophoresis. The amplified product was excised from the gel and cleaned using GeneJET PCR purification kit (ThermoFischer Scientific). The amplified product was subjected to DNA sequencing in a 10µl reaction by Sanger sequencing method using Applied Biosystems BigDye® Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, CA, USA) per the manufacturer's instructions. Sense and Antisense sequencing were performed for confirmation of single nucleotide changes. The sequences were assembled by ABI PRISM sequence analysis software. The final interpretation of sequence was carried out using Chromas software visually by reading the electropherograms.

Bioinformatics and statistical analysis

Individual sequences were aligned with reference sequence NG_011676.1 using multiple sequence alignment by Clustal Omega. Individual SNPs were analyzed, and genome coordinates were found using Ensemble Genome browser version 92 (https://asia.ensembl.org/index.html) from Human genome (Build GRCh38.p12). Existing Minor allele frequency data (1000 Genomes Project) of individual SNPs was downloaded and compared with the SNP data of this study.

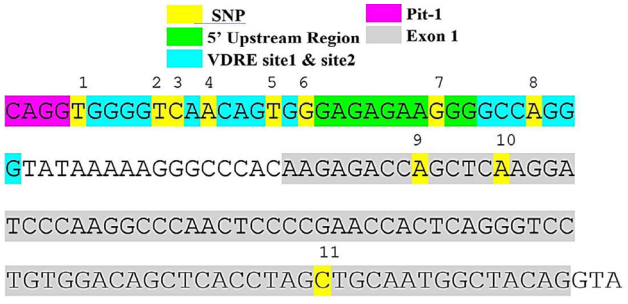

Fig. 1. Map of GH-1 gene with SNP locations.

Table I.- Primers for amplification of Exon 1 and promoter of GH-1.

	Sequence of primer (5'-3')	Location*	T_m (°C)	GC (%)
Forward primer	AGCACAAGCCCGTCAGTGGC	-112 to -93	68.22	65
Reverse primer	GGTCGCTGCCTCTCCCCTCA	+111 to +130	69.50	70

*Relative to transcription start site.

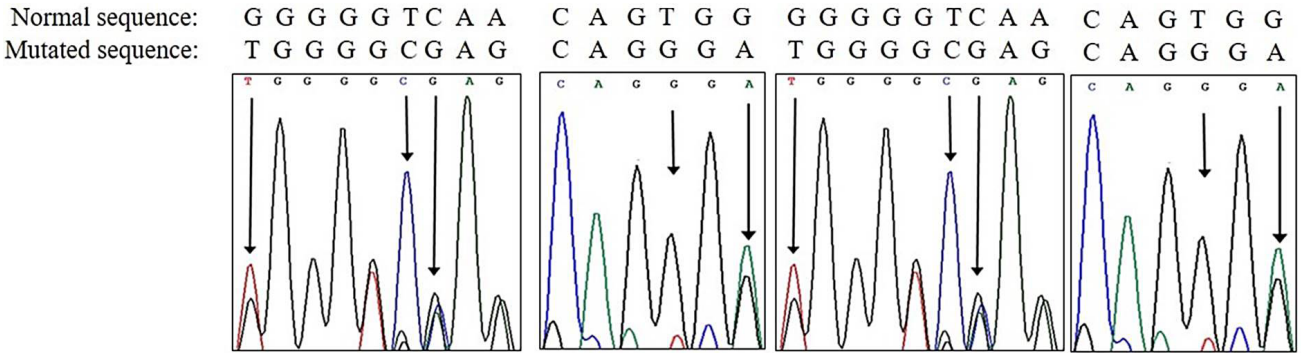

Fig. 2. Electropherograms indicating SNPs in proximal promoter of the GH1.

The age and height data of the subjects included in the study were statistically analyzed including standard error and standard deviation. Frequencies of SNPs were calculated, and each SNP was tested for Hardy Weinberg Equilibrium.

RESULTS AND DISCUSSION

GH-1 along with other members of the paralogue is a set of highly polymorphic genes (Chen *et al*., 1989). The non-coding (especially the proximal promoter) and coding regions of the GH gene has a high frequency of occurrence of allelic variations and SNPs. The GH promoter contains various closely spaced cis elements for different trans-acting transcriptional regulators and are prone to sequence variations. In this study, we analyzed the proximal region of the promoter including the VDRE, 5'UTR, TATA box and exon 1 of the GH-1 gene.

PCR product of the GH-1 promoter and exon 1 was validated by electrophoresis. A 240 bp fragment was observed on the agarose gel (Farooq and Sami, 2013). The fragment was cleaned and sequenced using the same primers (forward and reverse).

The growth hormone-1 gene region under study is reported to contain over 20 single nucleotide polymorphisms in the ensemble genome viewer and the single nucleotide polymorphism database (bdSNP) of NCBI (Stenson *et al*., 2017; Zheng-Bradley *et al*., 2017; Day, 2010). 11 SNPs were recorded in the proximal end of the gene (region: vitamin D response element to intron 1). 9 of the observed SNPs are similar to the previously reported SNPs in the SNP database by different researchers. (Giordano *et al*., 1997; Wagner *et al*., 2005; Esteban *et al*., 2007; Adkins *et al*., 2005; Millar *et al*., 2003; Hasegawa *et al*., 2000; Horan *et al*., 2003). One novel SNP is still under investigation for a possible triallelic variation. All the allele frequencies were found to be in Hardy-Weinberg equilibrium (Table III). The SNPs tend to be clustered in the vitamin D response elements (VDRE) and near the transcription start site (Filion *et al*., 2006).

Table II.- Height and weight data of subjects.

Gender	Age (year)	Height (cm)	Weight (kg)
Male	37.5 ± 9.0	174.6 ± 7.0	73.3 ± 12.3
Female	32.5 ± 8.0	162.5 ± 5.0	55.5 ± 8.2

Values are Mean ± SD.

The sequence changes observed were compared with the already reported data, and the sequence variation is found to agree with the published data. However, the minor allele frequencies at various SNPs are observed to be higher in the group of the local population. SNP 1 (T→G) is present at the 5' end of the VDRE site 1 (position -62) and is not reported elsewhere to the best of our knowledge; it is detected first time in the local population with a MAF of G=0.17 (Table III). SNP 2 at position -57, a change of T→C was observed with a frequency of 0.7 (Fig. 2). SNP 3 at position -56 contains a possible novel triallelic

Table III.- Single neucletoide polymorphism data with chromosomal coordinates and minor allele frequency data.

S. No	SNP ID	HGVS Name	Chromosomal location	Allele	Occurrence (%)	Frequency	MAF global	Homozygous	Heterozygous	Location on gene	Functional Region
1	not reported	NG_011676.1g.4939T>G	17:63918900	T	83	0.83	No MAF data	GG 07	GT 20	-62	Pit-1
				G	17	0.17		TT 73			
2	rs2005172, CR031008	NG_011676.1g.4944T>G	17:63918895	G	22.7	0.227	T=0.4529/2268	CC 55	GC 45	-57	VDRE Site 1
		NG_011676.1g.4944T>C		C	77.3	0.773		GG 0			
3	not reported	NG_011676.1g.4945C>G	17:63918894		Possible Triallelic variation Under investigation		No MAF data			-56	VDRE Site 1
4	not reported	NG_011676.1g.4943A>G	17:63918892		Very low frequency		No MAF data			-54	VDRE Site 1
5	rs788661715	NG_011676.1g.4951T>G	17:63918888	G	85.2	0.852	G=0.005/25	GG 70	GC 30	-50	VDRE Site 1
		NG_011676.1g.4951T>C		C	14.8	0.148		CC 0			
6	rs61762493	NG_011676.1g.4953G>A	17:63918886	G	67	0.67	A=0.0178/89	GG 34	GA 66	-48	VDRE Site 1
				A	33	0.33		AA 0			
7	rs71651678	NG_011676.1g.4961G>C	17:63918878	G	50	0.50	C=0.0192/96		GC 100	-40	5' UTR
				C	50	0.50					
8	rs759606522	NG_011676.1g.4967A>C	17:63918872	A	88.6	0.866	MAF data not available	AA 77	AC 33	-34	VDRE Site 2
				C	11.3	0.113		CC 0			
9	rs6171, CR031006	NG_011676.1g.4995A>G	17:63918844	G	30.7	0.307	G=0.36/1820	AA 41	AG 57	-6	5' UTR
				A	69.3	0.609		GG 2			
10	rs695	NG_011676.1g.5000A>T	17:63918839	A	62.5	0.625	T=0.078/394	AA 30	AT 70	-1	5' UTR
				T	32.9	0.329		TT 0			
11	rs771309275	NG_011676.1g.5058C>T	17:63918781	C	72	0.77	T=0.0002/25	CC 61	CT 39	58	Exon 1
				T	23	0.23		TT 0			

Table IV.- Comparison of single polymorphism with GH paralouge genes.

S. No	SNP ID	Chromosomal location	Position on gene	GH1 Allele	Frequency	GH Paralogues			
						GH2	CSH1	CSH2	CSHP1
1	not reported	17:63918900	-62	T	0.83	T	T	T	T
2	rs2005172, CR031008	17:63918895	-57	G	0.227	A	T	T	G
				C	0.773				
3	not reported	17:63918894	-56			G	C	C	C
4	not reported	17:63918892	-54	No data		A	A	A	A
5	rs788661715	17:63918888	-50	G	0.852	C	C	C	C
				C	0.148				
6	rs61762493	17:63918886	-48	G	0.67	A	A	A	A
				A	0.33				
7	rs71651678	17:63918878	-40	G	0.5	G	C	C	G
				C	0.5				
8	rs759606522	17:63918872	-34	A	0.886	A	A	A	A
				C	0.113				
9	rs6171, CR031006	17:63918844	-6	G	0.307	A	G	G	A
				A	0.693				
10	Rs695	17:63918839	-1	A	0.625	A	T	T	C
				T	0.329				
11	Rs771309275	17:63918781	58	C	0.77	C	T	C	C
				T	0.23				

variation and could not be confirmed by conventional sequencing methods, so it is under further study and analysis. SNP 4 at position -54 is a transition SNP A→G with a frequency of ~1%. SNPs 2, 5-8, 10 and 11 are similar to previously reported SNPs, however, the frequencies of minor alleles are higher as compared to Polish, German, Japanese and Caucasian populations (Table III) (Wagner *et al*., 1997, 2005).

Another critical SNP at position -34 (A→C) lies in the VDRE site2 of the gene with an MAF of 0.11 for C allele. At all instances the C allele was present in the heterozygous form. The SNPs 1-8 all lie in the vitamin D response element and should be considered significant as they can cause a variation in the binding of trans-element (Vitamin D-Vitamin D receptor complex) of the VDRE thus causing a change in transcriptional regulation of GH-1 (Seoane *et al*., 2002). The Human GH VDRE is an imperfect direct repeat, and the Glu42 of the VDR interacts with the -57 T with higher affinity to exert a negative regulatory effect. The change at -57 from T to any other base affect the binding of cis-trans elements creating a change in the expression profile of the GH-1 gene (Shaffer and Gewrith, 2002).

The SNPs 10 and 11 at positions -6 and -1 respectively are present near the start of transcription start site. SNP 10 is at -1 position, A to T change was recorded with a frequency of 0.329 for T allele, and the rate of heterozygosity was 100%. SNP at position -6 that is present in the 5' Untranslated Region (UTR) and has a similar MAF as reported by 1000 genomes project (Sayers *et al*., 2011).

The portion of the GH-1 gene analyzed for SNPs was aligned with the paralogues of the GH gene family and most of the variations sites that exist in the GH1 sequence differ from that of the paralogue genes.

The alignment of the SNPs with the paralogues indicated that 4 out of 11 SNPs (~36%) in the GH-1 gene does not correspond to any of the other four genes (Table IV) other SNPs match with one of the four paralogues, respectively. The possible mechanism of the sequence recombination and high variation rate is due to possible gene conversion or transfer where the other four paralogue genes act as donors. Our results confirm the existing data of the GH gene polymorphism and highlight the variations in allele frequency data of the local population from other studied populations including German, Polish, Japanese, Chinese, and Caucasian *etc*. (Esteban *et al*., 2007; Farooq and Sami, 2013).

SNP 1 and 3 have not been reported in any population till date, all other SNPs are published by various researchers in different populations. Some of the SNPs of the GH-1 promoter and exon 1 region are observed and reported in short stature subjects in multiple studies furthermore the haplotype map of the patients with Idiopathic short stature,

and isolated growth hormone deficiency varies from that of the normal statured population (Hirschhorn *et al*., 2001; Dennison *et al*., 2004).

CONCLUSION

GH-1 promoter is susceptible to sequence variations and polymorphisms. There exists a high density of closely spaced and clustered single nucleotide polymorphisms in the proximal region of promoter including transcriptional regulator binding elements and the 5'UTR. Along with the promoter, the exon 1 (proximal portion) including the signal peptide also contains sequence changes and are frequently reported till date. The comparison of the observed SNP in the current study indicates same changes as given in the previous work. Some base changes are also comparable to the SNPs found in idiopathic short stature and isolated growth hormone deficiency patients. In future the results reported could be helpful to design mutant variants to perform functional studies and analyze the effect of SNPs on the expression and functionality of the gene.

ACKNOWLEDGEMENT

Authors are grateful to Higher Education Commission for providing indigenous PhD. Fellowship to the first author to carry out this research work.

Statement of conflict of interest

Authors declare no conflict of interest.

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