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Amino Acid Substitutions in Growth Hormone and Growth Hormone Receptor Genes Mutants in *Camelus dromedarius*

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ABSTRACT

The identification of genetic polymorphisms in genes enable us to estimate biological similarities and obtain a better perspective of quantitative traits that play key roles in development and regulating growth. The present study was conducted to characterize genetic variability in the growth hormone (GH) and growth hormone receptor (GHR) of *Camelus dromedarius*. GH is secreted and synthesized from somatotroph cells of the anterior pituitary lobe and plays significant role in growth, development, metabolism, lactation, and reproduction. Growth hormone interact with GHRs and influence on metabolism and growth. Changes in GHR concentration affect signalling pathways, binding capacity and alters activity of GH. Therefore, the GH and GHR genes are considered important candidate genes. DNA samples of Marecha camel were collected from the Camel Breeding and Research Station at Rakhmani Bhakar, Pakistan. Two significant polymorphic sites were identified in GH gene and three in GHR gene. Out of these, T1720A polymorphism in GH and A211927G polymorphism in GHR genes changed the amino acid from leucine to histidine and methionine to valine, respectively. These polymorphic results suggest that Marecha camel, a major breed in Pakistan, has genetic variability in the GH and GHR genes. These results suggest that Marecha camel has genetic variability in growth-related genes and identified polymorphisms may be helpful for association studies and future selection programs.

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Authors' Contribution
AN designed the study. SS carried out the genomic work. SH collected the samples. SS, AN, MJ and MYZ, analyzed the data and wrote the manuscript. AN and ASH revised the manuscript.

Key words
Polymorphism, GH gene, GHR gene, growth, Marecha, *Camelus dromedarius*

INTRODUCTION

Pakistan ranks eighth among the main camel-raising countries of the world (Faye, 2015). The camel population is approximately 1.2 million in Pakistan (Economic Survey, 2016-17) representing 22% of total livestock production. Nowadays, research interest on genetic polymorphisms of candidate genes has increased due to their utilization in genetic selection, as well as being helpful to differentiate evolutionary relationships among different livestock breeds (Sodhi *et al*., 2007). Association of single nucleotide polymorphisms (SNPs) with economically important traits has been commonly used as a selection tool in various other livestock species such as cattle (Ge *et al*., 2003), sheep (Bastos *et al*., 2001) and goats (Gupta *et al*., 2007). Various candidate genes associated with different growth traits are mainly present on the growth hormone axis. Growth hormone (GH) is a polypeptide hormone and has various biological actions including somatogenic, lactogenic, diabetogenic and insulin-like effects (Ishag *et al*., 2010). GH is considered the primary regulator in metabolism and postnatal growth that has effects on health, growth rate, milk production, body composition and aging by controlling expression of many genes (Lincoln *et al*., 1995; Sumantran *et al*., 1992). The growth hormone receptor (GHR) facilitates the biological function of GH by stimulating myogenic signals and influences transcription of many genes (Argetsinger *et al*., 1996). GHR mediates its effect by signal transduction of the target cells. Allelic variations in GHR affects its signalling pathways and binding capacity. Mutation in the GHR gene causes short stature and Laron syndrome in human. GHR gene has nine untranslated exons and encoded by BTA20 in cattle. The binding capacity of GH associated with polymorphisms of GHR is considered as the candidate gene which influences development, growth and carcass traits in livestock animals (Blair *et al*., 2002). The camel GH gene is about 1900 bp in length and is comprised of five exons and four introns (Maniou *et al*., 2001). It is a 22 kDa single chain polypeptide and secreted in a circadian and pulsatile manner by somatotrophs of the anterior pituitary gland (Dybus, 2002). GH and GHR genes

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are considered important candidate genes for growth, milk and carcass traits in livestock. This study attempted to identify polymorphisms in GH and GHR genes which may be used for association of polymorphisms with growth traits in the Marecha camel population, leading to marker assisted selection for future camel breeding plans.

MATERIAL AND METHODS

Selection of animals and blood samples

The blood samples (n =105) of Marecha camels were collected from the Camel Breeding and Research Station (CBRS) at Rakhmahni Bhakkar, Pakistan. Blood samples (10 mL) were collected aseptically from the jugular vein of animals into falcon tubes containing 0.5 M ethylenediamine tetra-acetic acid (EDTA) as an anticoagulant.

DNA extraction and quantification

DNA was isolated from white blood cells (WBCs) suspended in the preserved samples using a standard phenol-chloroform-isoamyl alcohol DNA extraction protocol (Sambrook *et al*., 2001). The quantity and quality of every DNA sample was evaluated using a gel electrophoresis (0.8 % agarose gel) and NanoDrop ND-1000 spectrophotometer (Nano DropTechnologies). All samples were brought to the same concentration level, namely 50 ng/µL.

Designing of primers

Three primers for GH genes and nine for GHR were designed by using Primer3 software (http://frodo.wi.mit.edu/) from NCBI database (GenBank accession number GH: AJ575419 and GHR: 105097468) to amplify 15 exon regions. Primer sequences were optimized using OligoTM primer design software (Table 1).

Table I. List of primers for amplification of growth hormone (GH) and growth hormone receptor (GHR) genes.

Primer Name	Sequence	Length	GC content	Tm	Product Length
GHF1	AAAATAAGTGGGGGCAGAGG	20	50	60.31	187
GHR1	TCACCCTTCCGTACACATCA	20	50	59.96	----
GHF2	CTTCTGAATGTGAGCGTGGA	20	50	59.98	839
GHR2	TTGGAAAAGAGCAAGGAGGA	20	45	59.93	----
GHF3	GTGGAGGGGAAGAGAAGGAG	20	60	60.19	716
GHR3	CTGGGGAGGGGTAACAGATT	20	55	60.18	----
GHRF1	ATTTGCCATGAGGTGGTTCT	20	45	59.41	493bp
GHRR1	TCGGCTGCTACTTGGAACTT	20	50	60.02	----
GHRF2	CCAGAAGGTTTCCATTTGCT	20	45	59.17	396bp
GHRR2	CAATTTCGGTCAGTCATCCA	20	45	59.50	----
GHRF3	GAAGCTGTGACCCAGGAAAA	20	50	60.23	439bp
GHRR3	GCCAGATCTCCAGTGCCTAA	20	55	60.36	----
GHRF4	TAGCCTCCTATTGGTCTGC	19	52	55.48	500bp
GHRR4	ACCTCCATCCACCTACAGA	19	52	55.30	----
GHRF5	CAGAAGAAACCACTCCGTCAG	21	52	59.89	439bp
GHRR5	CCATGTAAGCACCTGCTGTCT	21	52	60.33	----
GHRF6	TATGTCCGTGTCTCCACCAG	20	55	59.26	581bp
GHRR6	GCCACCTGCCCATTAATATC	20	50	59.94	----
GHRF7	AGCCTTTAACGGCACACACT	20	50	59.80	483bp
GHRR7	TGTCCTATCCAACTTCCCAGA	21	47	59.54	----
GHRF8	TTTTGCCTGGTCATTTACTGC	21	42	60.12	695bp
GHRR8	CTGTCTGTGTCTGAGCCTTCA	21	52	59.19	----
GHRF9	TGGGTGGAATTTATCGAGCTA	21	42	59.56	945bp
GHRR9	CAGCCAACTCTTTGCCATTA	20	45	58.92	----

Amplification of GH gene

Amplification of the GH and GHR genes were performed by using a touchdown PCR protocol with annealing temperature range (GH: 62°C-52°C and GHR: 64°C-54°C) on a Bio-Rad thermo cycler. Polymerase chain reaction was carried out in a volume of 25 µL using 50 ng/µL genomic DNA, 10x PCR buffer, 2.5 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 U *Taq* Polymerase, and 0.5 µL of each primer. Twelve primers were used for the analysis. The thermal cycler was programmed for 5 min initial denaturation at 95°C, followed by 30 sec denaturation at 94°C, 30 sec primer annealing at 37°C, 45 sec extension at 72°C for 35 cycles and then final extension at 72°C for 10 min. PCR products were examined on a gel documentation system after running on the gel. The gel results were analysed against a ladder of 50 bp. Sequencing of PCR products was performed using an ABI Prism 3100 Genetic Analyzer (Applied Biosystem Inc. Foster City, CA).

BLAST

All the sequences were aligned using NCBI online software blast (http://www.ncbi.nlm.nih.gov). SNPs were identified from the aligned sequences. BioEdit translate tool was used to analyse SNPs to look for changes in codons and hence amino acids and protein sequence.

Protein structures

Protein structure was retrieved from mRNA sequences of genes from the NCBI GenBank database and induced mutations were identified by using BioEdit ver. 7.0 software. Then sequences of mRNA were back-translated into protein through the Lasergene 7.1 software. For visualising the three-dimensional structure of GH and GHR, we used the SWISS-MODEL server available on the ExPASy website (https://www.expasy.org/) (Bahrami *et al*., 2012).

RESULTS

Polymorphisms in the GH gene

In this study we used three primers to flank the GH gene comprised sequence lengths of 287 bp from Exon 1, 161 bp from Exon 2, 165 bp from Exon 3, 156 bp from Exon 4, and 198 bp from Exon 5 of camel GH. Amplified fragments of 187 bp, 839 bp and 765 bp were obtained. This amplified GH sequence of Marecha breed was compared with GenBank reference sequence of AJ575419.1. Two polymorphic sites (SNPs) were identified by using BLAST, one in the non-coding region (intron 4) at nucleotide position 753 and the other in the coding region (Exon 5) at nucleotide position 1720 (Table II). The mutation in intron 4 was an insertion (A < G) and the mutation in Exon 5 was a transversion (T<A). The nucleotide substitution at G180 revealed a leucine (L) to histidine (H) polymorphism (L180H) (Fig. 1A).

Polymorphisms in the GHR gene

Nine primers were used for PCR amplification of the GHR gene's exons with product sizes of 493, 396, 439, 500, 439, 581, 483, 695 and 945 bp, the last one amplifying to two exon regions (Fig. 2A). This amplified sequence was then compared with GenBank reference sequence of 105097468. Three polymorphic sites (SNPs) were identified by using BLAST, two in the non-coding region and the other in the coding region as shown in Table III. By sequencing GHR, three SNPs were found in GHR, two from intron and one from Exon 4. SNP A211927 changed the amino acid from methionine to valine, resulting in a codon change from ATG to GTG (Fig. 1B).

Table II. Identified Polymorphisms in genomic region of the growth hormone (GH) and growth hormone receptor (GHR) genes.

Genes	Nucleo-tide position*	Muta-tion	Codon change	Nature of mutation	Amino acid substitution	Change type	Allele frequency		Major allele frequency
GH	753	A>G	-	Intronic	-	-	A: 0.6585	G: 0.3415	0.6585
	1720	T>A	CTT>-CAT	Exon	Leucine >Histidine	Non-syn-onymous	T: 0.6041	A: 0.3959	0.6041
GHR	211,834	C>A	-	Intronic	-	-	0.5099	0.4901	0.5099
	211,927	A>G	AT-G>GTG	Exon	Met>Val	Non-syn-onymous	0.8096	0.1904	0.8096
	108,709	C>A	-	Intronic	-	-	0.6056	0.3944	0.6056

Accession Number, GH, AJ575419.1; GHR, 105097468.

Fig. 1. Growth hormone (GH) gene (A) and growth hormone receptor (GHR) gene (B) of Merecha camel. A shows Leu/His polymorphism while B shows Mathionine/Valine polymorphism.

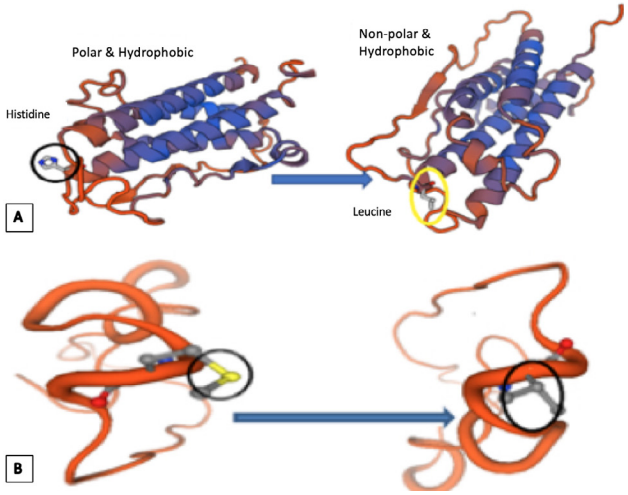

Fig. 2. The change in amino acid in GH protein (A) and GHR protein of Marecha camel. A shows change from Leu to His, while B shows from leucine to histidine.

DISCUSSION

Genetic improvement has been considered to be an important tool for improving growth and meat production. Detection of genes and polymorphisms associated with quantitative and qualitative traits is an important component of genetic improvement for these economic traits. When the relationship between candidate genes and traits are well recognized, then the genetic potential of breeding animals may be improved. Information about polymorphisms can give vital information about the genomic regions that have been targeted by selection, leading to phenotypic variation.

In the present study, five polymorphic sites were identified in GH and GHR genes of *Camelus dromedarius*. This study was designed to identify the novel SNPs in these growth-related genes in camels. The finding of polymorphisms in these genes can be used to assess effect of these candidate genes on camel growth and then can be used for marker assisted selection in a genetic improvement program. GHR gene has not been studied before in camel and is novel to the current study. However, SNPs in GH gene have been documented in camel and other species. For example, more than 10 SNPs in the GH gene were detected in cattle (Musa, 2007), and 24 and 19 in Osmanabadi and Sangamneri goat breeds, respectively (Wickramaratne *et al*., 2009) and seven SNPs in sheep (Bastos *et al*., 2001) have been reported, while only one SNP was detected in the GH gene of Pakistani (Shah *et al*., 2006) and Sudanese (Ishag *et al*., 2010) dromedaries. Several studies found significant associations of GH polymorphisms with growth and production traits in numerous species. Moradian *et al*. (2013) observed a significant association of a GH polymorphism with growth traits such as birth weight, weaning weight and six- and nine-month weight in Makooei sheep.

In our study, we identified two SNPs in the GH gene, out of which one SNP was found in the exon and one in the intron region. SNP 1720A was found to be non-synonymous and showed an amino acid change from leucine to histidine. Hediger *et al*. (1990) studied GH gene polymorphism in the fifth exon at 127 amino acid position and observed that a substitution of cytosine (C) to guanine (G) showed a positive association with growth. Lucy *et al*. (1993) reported substitution of C for G nucleotide at position 2141. This changed the amino acid from leucine to valine with a corresponding increase in growth. Histidine is a key glycoprotein and plays an important role in biological functions such as coagulation, immune response system, fibrinolysis, apoptosis and angiogenesis (Poon *et al*., 2011). It interacts with various molecules such as plasmin, thrombospondin, vascular endothelial growth factor (VEGF), and the heparin fibroblast growth factor (FGF) family (Poon *et al*., 2011). Leucine is considered an essential amino acid for protein synthesis and metabolic function. It plays an important role in regulating blood sugar

level, growth, muscle recovery and stimulating production of GH. Leucine also plays an important role in weight loss as well as muscle building. T1720A substitutions causes a change in amino acids (leu>his) which has influence on the protein structure and biological function of GH.

In GHR, two transversion mutations were detected at positions 211,834 C>A and 108,708 C>A in Introns 4 and 2 respectively, while a transition mutation at position 211,927 A>G was found to be non-synonymous: this changes the amino acid from methionine to valine. Simmental bulls with Leu/Leu and Leu/Val genotypes had significantly higher breeding values for a meat trait as compared with the Val/Val genotype (Schlee *et al*., 1994). In the GH gene of Japanese cattle, animals with methionine showed a high beef marbling score. This association of methionine with high beef marbling scores was only found in Japanese cattle and is considered a characteristic of this cattle breed (Chikuni *et al*., 1994; Chikuni *et al*., 1997). Given its importance in other livestock species, this gene can also be used as an indicator for the genetic improvement of growth traits in *Camelus dromedarius*.

In Balinese cattle, a significant association of GHR with weaning weight and average daily weight gain was observed (Maskur *et al*., 2014). GHR regulate biological activities of GH by stimulating signal transduction in target cells. Hale *et al*. (2000) identified significant associations of GHR with growth, milk production, and carcass traits, and reported a candidate gene associated with growth in Angus cattle. Deepika *et al*. (2013) found a polymorphism in the exon and intron region of GHR and it has a significant association with milk and growth traits in an indigenous cattle breed. Overall these findings will be helpful to give more insight about potential of these genes in marker assisted selection for growth in a camel breeding program.

CONCLUSION

Following gene sequencing, five polymorphisms were identified in GH and GHR genes in Marecha camels. This is the first study conducted for molecular characterization of GH and GHR genes in the camel. These identified polymorphisms in marecha camel may be useful markers for selection in a camel breeding program for higher growth and meat production.

Statement of conflict of interest

The authors declare no conflict of interest.

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