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IDENTIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISMS IN GROWTH HORMONE SECRETAGOGUE RECEPTOR (*GHSR*) GENE IN NATIVE EGYPTIAN CHICKENS

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ABSTRACT

Growth hormone secretagogue receptor (GHSR) or Ghrelin receptor gene is legend for a peptide hormone produced by chicken proventriculus which stimulates growth hormone release and food intake. The purpose of this study was to determine single nucleotide polymorphisms (SNPs) in GHSR gene in six native Egyptian chicken breeds (Fayomi, Dandarawy, El-Salam, Dokki-4, Inshas, and Mandara) compared to Rhode Island Red (RIR). Blood samples were collected from these chickens at eight weeks of age. A PCR product of 598 bp, GHSR was amplified using polymerase chain reaction (PCR) and subsequently, subjected to sequence analysis to identify its different allelic patterns. Two different SSCP banding patterns (TT and TC) were detected. Nucleotide sequencing revealed presence of a novel T40C SNP in the first intron of GHSR at nucleotide number 59 before the second exon. Results from SSCP and sequencing denoted absence of CC genotype in all examined chicken breed. Although, we did not perform association analysis for these genotypes due to small population size, the present results put the basis for further studies to associate this SNP and others in GHSR with growth traits in local chicken breeds.

Keywords: Native chickens, Gherlin receptor gene, Sequencing, SSCP, SNPs.

INTRODUCTION

Egyptian chicken breeds vary in feather color, size and egg production as well as carcass characteristics and flavor preferred by Egyptian consumers. These breeds adapted to adverse Egyptian environmental conditions ***(Tixier-Boichard 2009)***. However, their meat production is low compared to broilers. Rearing of local chickens is insignificant in commercial farms, so that their meat production is insufficient for our needs. Several attempts have been done in Egypt to increase meat production of chickens by selection of high producing parents and crossing them for several generations which are time and money consuming. Also, these procedures lead to a slight improvement in growth and egg production traits. Selection methods depends mainly on detection of polymorphisms in genes affecting growth (growth axis genes) such as growth hormone secretagogue, or ghrelin, receptor (*GHSR*) are more efficient for improvement of growth traits.

GHSR is involved in many physiological functions, including pituitary growth hormone secretion, food intake and energy expenditure ***(Shuto et al., 2002; Liu et al., 2007)***. The chicken *GHSR* expression level was the highest in the pituitary, brain and moderately high in the liver, intestine and spleen ***(Tanaka et al., 2003)***. Ghrelin and its receptor have been identified in the pancreas ***(Kageyama et al., 2005)*** where ghrelin physiological control mechanism (calcium signaling via GHS-R) regulating cell development and insulin secretion ***(Date et al., 2002)***. The *GHSR* gene composed of two exons and one intron in both mammals and chicken, and its alternative splicing led to two isoforms of *GHSR-1a* and *GHSR-1b*. The *GHSR-1a* was produced by the splicing of exon 1 and exon 2 and had normal function. On the other hand, the *GHSR-1b* was a

truncated transcript due to lack of TM6 and TM7 domain ***(Howard et al., 1996; Tanaka et al., 2003)***. The higher *GHSR-1a* mRNA level than that of *GHSR-1b* was detected in many tissues ***(Geelissen et al., 2003; Tanaka et al., 2003)***.

In chicken, a total of 37 single nucleotide polymorphisms (SNPs) and one 6 bp indel were detected in the full length of chicken gene by using the denaturing high performance liquid chromatography (DHPLC) technology ***(Nie et al., 2005)***. Some of these SNPs were significantly associated with chicken fatness and muscle fiber traits ***(Lei et al., 2007)***. As *GHSR* modulate pituitary growth hormone secretion by binding to its ligand of ghrelin, it was a candidate gene for growth performance. However, genetic effect of *GHSR* gene polymorphism on chicken growth traits has not been reported until now.

Selection of local Egyptian chicken breeds depending on genetic bases was poorly studied. Therefore, the aim of this study is to determine the polymorphisms in *GHSR* gene and to calculate their allelic and genotypic frequencies in local Egyptian breeds as compared to RIR breed.

MATERIAL AND METHODS

Sampling and DNA extraction:

The samples were collected from six Egyptian local chicken strains (Fayoumi, Dandarawi, Dokki-4, Inshas, Mandara and El-Salam) and one foreign strain (Rhode Island Red) located at Al-Azzab Station of the Poultry Integrated Project at Fayoum governorate, Egypt. One hundred and twenty chickens (26 chickens from each strain) were used in this study.

Blood samples (3 ml/chicken) were collected from the wing vein in a vacuum plastic tube containing EDTA and kept on ice. The samples were stored at -25°C until used for genomic DNA extraction using phenol /chloroform precipitation protocol **(John et al., 1991).**

Polymerase Chain Reaction (PCR):

The locus of *GHSR* gene was amplified by PCR using specific primer (Table1) designed by primer 3.0 software based on the published sequences of *Gallus gallus* (Gen Bank accession number, AB095994).

Table (1): Primer sequences, annealing temperature (Ta) and PCR product size of *GHSR* gene

Gene	Forward primer (5´-3´)	Reverse primer (3´-5´)	Ta (°C)	size
GHSR	TGGTTGAAAAGAGAGAATGCT	CCACACGTCTCCTTTTATATTC	59	598

The PCR reactions were performed with a total volume 25 **µl** containing 2.0 µL DNA template (approximately 50 ng), 0.5 µL (0.20 mM) dNTP, 2.5 µL buffer, 1.5 µL (2.5 mM) MgCl2, 1.0 µL 10µmol/L forward primer, 1.0 µL 10µmol/L reverse primer, 0.5 µL 10× Taq DNA polymerase (5 U/µL, Fermentas, #K1071) and 16.0 µL nuclease free water. Thermal cycling parameters were as follows: initial denaturation at 94 °C for 3 min, 35 cycles of amplification (94 °C for 30 s for DNA denaturation, annealing temperatures 59 °C for 40 s, extension at 72 °C for 1 min) and final extension at 72 °C for 5 min. The samples were held at 4 °C. PCR products were electrophoresed on 1% agarose gels using

1X TAE buffer containing 200 ng/ml ethidium bromide. After running of the stained gel visualized with UV light of gel documentation system (Biometra Biomedizinische Analytik. GmbH).

DNA sequencing:

PCR products with expected size were purified using PCR purification kit following the manufacturer protocol (Jena Bioscience # pp-201×s) to remove primer dimmers, primers, nucleotides, proteins, salt, agarose, ethidium bromide and other impurities (Jena Bioscience # pp-201×s/Germany). The purified PCR products were sequenced in automated sequencer (Applied Biosystem, USA). The Sequences were analyzed using the Chromas Lite 2.1 program (http://technelysium.com.au/?page_id=13) and the identity of the sequenced PCR product was examined using Blast search against GenBank database of *Gallus gallus* (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The alignments, annotations and assembly of the sequences were performed using Geneious 4.8.4 software http://www.geneious.com/web/ geneious/home.

Statistical analysis:

Genotypic and allelic frequencies of T40C SNP at nucleotide number 59 before exon 2 of *GHSR* of selected chicken breeds were directly calculated ***(Snedecor and Cochran 1976).*** Chi squared used to examine the significance of genotype frequencies

RESULTS

Gel electrophoresis revealed the presence of intact bands near wells which means that they are genomic DNA and they are good enough to be used in the subsequent procedures (Fig. 1).

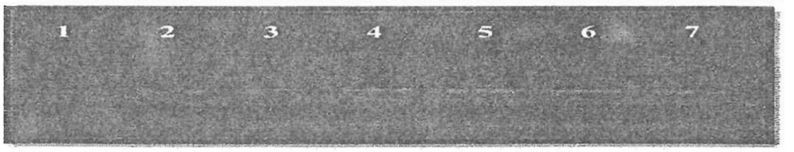

Fig. (1): Ethidium bromide stained agarose gel showing extracted genomic DNA. Lane 1: Fayomi, lane 2 Dandarawy, lane 3: RIR, lane 4: Dokki-4, lane 5: Inshas, lane 6: Mandara, lane 7: El-Salam.

A fragment of 598 bp of *GHSR* gene was amplified using PCR (Fig. 2).

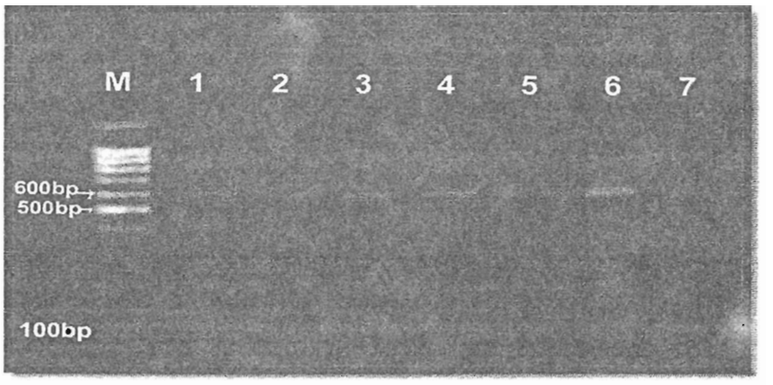

Fig. (2): Ethidium bromide stained agarose gel of PCR products representing amplification of *GHSR* gene in chicken breeds with size of 598 bp (lane 1: Fayomi, lane 2: Dandarawy, lane 3: El-salam, lane 4: Dokki-4, lane 5: Inshas, lane 6: Mandara, lane 7: RIR). M represents 100 bp ladder.

After purification of these fragments, sequencing was carried out in order to verify the identity of the PCR product and to detect any SNPs. The obtained sequences were examined compared to previously known sequences published in GenBank database using Blast search. The results of this step confirmed that the sequences, as expected, showed high similarity with *Gallus gallus GHSR*.

Two unique SSCP banding patterns (TT/TC) were detected in *GHSR* (Fig. 3). The sequences of the *GHSR* in different chicken breeds (submitted to GenBank with accession number KF957990, KF957991, KF957992, KF957993) of Dokki-4, El-Salam, Inshas, Mandara; respectively. Sequence analysis showed one novel T40C SNP in the first intron of *GHSR* gene at nucleotide number 59 before exon 2 of El-Salam, Inshas, Dokki-4 and Mandara was on the other hand this SNP was not found in the pure Egyptian breeds, Fayoumy and Dandarawy (Fig. 4).

Allelic and genotypic frequencies were calculated and differences were observed (Table 2). In T40C SNP, the allele T showed a significantly high frequency (0.86) and so genotype TT showed a high frequency (0.71). The two genotypes were in Hardy-Weinberg equilibrium ($p < 0.05$), as shown in Table (2). Genotype CC was not found and accordingly frequency of allele C was low (0.14). Genotype CT showed a low frequency (0.29).

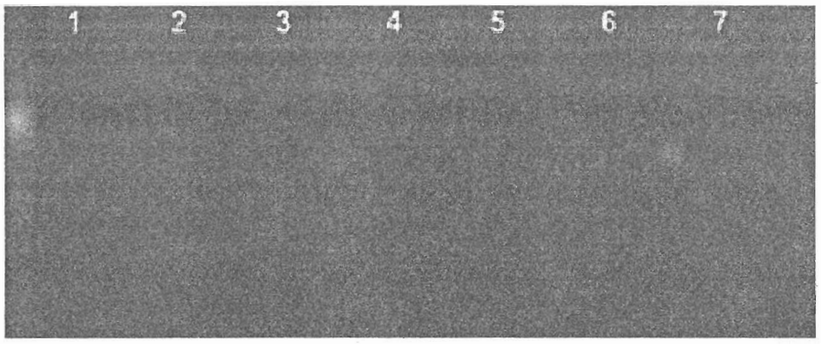

Fig. 3. PCR-SSCP patterns of *GHSR* in Egyptian chickens show the genotypes. Dimorphic SSCP patterns were determined; genotype TC (lane 2: mandara) which has two bands SSCP patterns and TT genotype show monomorphic bands patterns (Lane 1: El-Salam, lane 3: Dokki-4, lane 4: Inshas, lane 5: Dandarawy, Lane 6: Fayoumy, lane 7: Rhode Island Red).

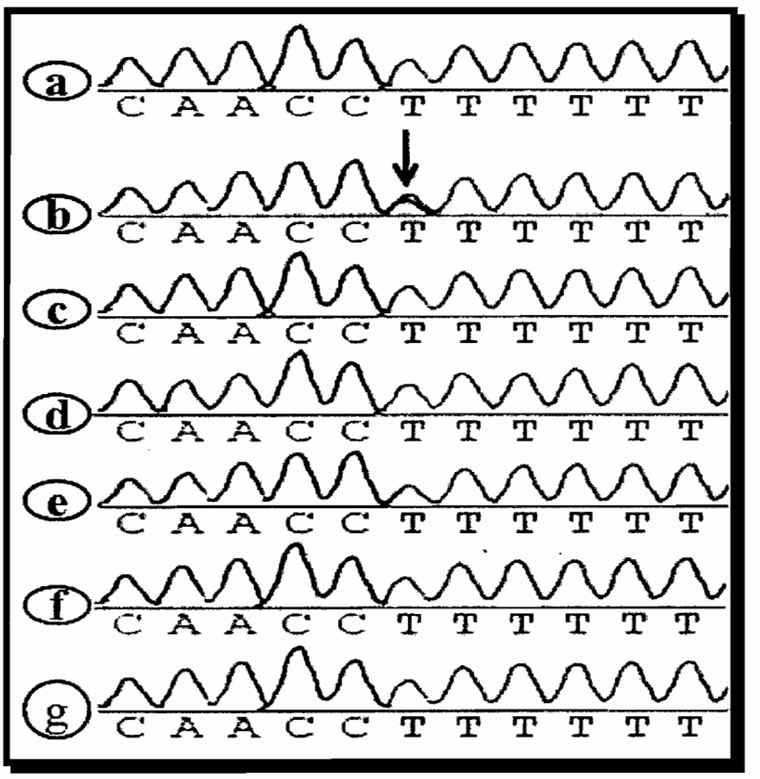

Fig. (4): The sequences of *GSHR* gene showed one novel SNP, T40C SNP was detected in intron 1at nucleotide number 59 before exon 2 (a: El-Salam, b: Mandara, c: Dokki-4, d: Inshas, e: Dandarawy, f: Fayoumy, g: Rhode Island Red). The arrows indicate the position of SNPs.

Table 2: Genotype distribution and allelic frequencies at T40C SNP of chicken breeds *GHSR*.

Gene Locus	Genotype frequencies		Allele frequencies		χ^2	*P* value
	TT	CT	T	C		
GHSR (T40C)	0.71	0.29	0.86	0 14	20 19	<0.05

DISCUSSION

In spite of local Egyptian chickens are wide spread breeds adapted to adverse environmental conditions and their meat flavor desired by Egyptian consumers, still suboptimal local chicken meat production are provided. Phenotypic selection up till now is the most used method for chicken breeding program. Since the main aim of breeding goals is genetic improvement, direction toward improving the quantitative traits of high economic values specially the growth trait. The genes controlling growth must be identified. This can be achieved by determination the marker genes affecting the growth and the growth related axis genes such as growth hormone secretagauge receptor (*GHSR*) gene which was considered as candidate gene for marker assisted selection (MAS).

Polymorphisms in *GHSR* gene were related to muscle cell proliferation are currently a new strategy for genetic improvement of growth traits and subsequently meat production ***(Goddard et al., 1996).*** In the present study a novel T40C SNP in intron 1 at nucleotide number 59 before exon 2 of *GHSR* gene of El-Salam, Inshas, Dokki-4 and Mandara was detected. This SNP was not found in the pure Egyptian breeds, Fayoumy and Dandarawy. It was not also present in the examined foreign breed, RIR. El-Salam, Inshas, Dokki-4 and Mandara breeds are not pure Egyptian breeds and they produced by crossing between the original (pure) breeds (Fayoumy and Dandarawy) and other foreign breeds characterized by their high growth rate and meat production. Because these cross breeding chickens have higher body weight, growth rate and meat production as compared to the two original (pure) Egyptian breeds, it is possible that T40C SNP might be associated with growth traits in these chickens.

In accordance, five polymorphisms (four SNPs and a 'GGTACA' indel) of chicken *GHSR* gene were significantly associated with body weight ***(Fang et al., 2010)***. In addition, two other SNPs were detected in poultry *GHSR* gene: C1459T SNP in chicken and A3427T SNP in duck and have been shown to be significantly associated with abdominal fat weight and subcutaneous fat thickness ***(Nie et al., 2009).*** C3286T and T640C were detected in sire and dam lines of a broiler chicken and were association with the growth and body composition traits ***(Darzi Niarami et al., 2014).***

In this study we have found only two genotypes (TT and TC), however no CC genotype was observed in all examined chicken breeds. Although, we did not perform association analysis for these genotypes due to small population size, the preliminary results of this study provides the researchers with raw data which could be used as a basis for further studies to associate this SNP and others in *GHSR* with growth traits in local chicken breeds.

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