Full Length Research Paper

Genomic growth hormone, growth hormone receptor and transforming growth factor β-3 gene polymorphism in breeder hens of Mazandaran native fowls

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Growth hormone axis and the transforming growth factor-β subfamily are the most important groups of genes that are involved in a wide variety of physiological functions such as growth and reproduction. As no information about the allelic characterization of growth hormone (GH), growth hormone receptor (GHR) and transforming growth factor β3 (TGF-β3) loci is available in breeder hens for Mazandaran native fowls breeding station, we studied their distribution in this population. A total of 156 blood samples were collected and a specific primer sets were used to amplify a fragment of GH, GHR and TGF-β3 loci using polymerase chain reaction (PCR). The PCR products from GH, GHR and TGF-β3 loci were digested with *Sac*I, *Hind*III and *Bsl*I restriction endonuclease, respectively. In GH and GHR loci, allele A was the most frequent and ranged from 0.99 to 0.79 while, allele B was identified as a dominant allele at TGF-β3 locus due to the highest frequency (0.81). The frequency of BB homozygous genotype was the lowest (average = 0.03) whereas, AA genotype showed the highest frequency among all loci. The amplified fragment in GH locus was characterized by a deletion of approximately 118 bp. Deletion of 118 bp fragment not only reduced the expected size of the PCR product, but also, introduced a new restriction site for *Sac*I enzyme at GH marker site. Further association analysis is required to clarify the effects of these marker genotypes on production traits in this breeder flock.

Key words: GH, GHR, TGF-β3, breeder hen.

INTRODUCTION

Growth hormone (GH) axis and the transforming growth factor-β subfamily are the most important groups of genes that are involved in a wide variety of physiological functions such as growth, and reproduction. The chicken growth hormone (cGH) gene is considered as one of the most important candidate genes that can influence chicken performance traits because of its crucial function in growth and metabolism (Vasilatos-Younken et al., 2000). The cGH gene contains 4 exons and 5 introns with an overall length of 4.1 kb and 5.2 kb in the chicken and duck respectively (Kansaku et al., 2008). Polymorphisms

in the cGH gene were widely studied by restriction fragment length polymorphisms or DNA sequencing (Yan et al., 2003).

Genomic DNA from four divergent chicken breeds was screened for single nucleotide polymorphisms (SNPs) in the cGH gene using denaturing high-performance liquid chromatography and sequencing (Nie et al., 2005). They have found a total of 46 SNPs of which 4 were in the 5' untranslated region, 1 in the 3' untranslated region, 5 in exons and with the remaining 36 in introns. They have found that, among other correlations, G + 1705A was significantly associated with body weight at all ages measured (Nie et al., 2005). Association analysis also shows that *Ava*I genotypes in the third intron of cGH are related to abdominal fat pad weight and abdominal fat percentage (Zhang et al., 2007).

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Table 1. Primer sequences and the cyclic conditions used to amplify fragments of cGH, cGHR and TGF-β3 genes in breeder hens of Mazandaran native fowls breeding station.

Locus	Primer sequences		Denaturation	Annealing	Extension
GH	F:5′-CTAAAGGACCTGGAAGAAGGG-3′ AACTTGTCGTAGGTGGGTCTG-3′	R:5′-	94°C, 30s	60°C, 60s	72°C, 60s
GHR	F:5′-GGCTCTCCATGGGTATTAGGA-3′ GCTGGTGAACCAATCTCGGTT-3′	R:5′-	94°C, 120s	59°C, 70s	72°C, 90s
TGF-β3	F:5′-TCAGGGCAGGTAGAGGGTGT-3′ GCCACTGGCAGGATTCTCAC-3′	R:5′-	94°C, 60s	58°C, 60s	72°C, 60s

It is widely accepted that hormones, growth factors and other agents exerts their biological effects on target tissues by binding to specific receptors on the plasma membrane. Certain hormones (steroid and thyroid hormones) bind to receptors in the cytoplasm or nucleus of a target cell but GH binds with high affinity and specificity to glycoproteins in the membrane and cytosol fractions of tissue and serum. The chicken growth hormone receptor (cGHR) resembled the mammalian GHR with the exception of two overall sequence homology and the absence of exon 3 homologue of the human GHR. Sex-linked dwarfism in chickens is an inherited disorder characterized by reduced body weight and longitudinal bone growth, despite normal levels of circulating plasma GH. A missense mutation resulting in the substitution of serine for the conserved phenylalanine was identified in the region of the cGHR cDNA encoding the extracellular domain.

Translation of this mutant transcript was indicated by the presence of GHR/GH binding protein in liver of normal and sex-linked dwarfism birds. Radiolabelled GH did bind to the hepatic membranes of most sex-linked dwarfism chicken (Hull et al. 1999). Serum GH binding activity, in contrast, was readily detectable, although at significantly lower levels than in normal bird. The missense mutation in the sex-linked dwarfism cGHR gene may thus affect targeting of cGHRs to hepatic plasma membranes (Hull et al., 1999).

The transforming growth factor β (TGF-β) subfamily is one of the most important groups of genes that are involved in development of growth and fitness traits. The growth factor β3 (TGF-β3) subfamily molecules are cytokines in the TGF-β superfamily. Chicken TGF-β3 maps to chromosome 5 and the biological activities of chicken TGF-β isomers appear to be similar to those of mammals (Groenen et al., 2000). The TGF-β superfamily members are multifunctional cell-cell signaling proteins that play pivotal roles in tissue homeostasis and development (Li et al., 2003). The objective of the present study was to identify polymorphism in GH, GHR and TGF-β3 loci in breeder hens of Mazandaran native fowls breeding station.

MATERIALS AND METHODS

Experimental population

Native fowls breeding station of Mazandaran located in the North of Iran have been established in 1988 with the objective of conserving the endangered population of native fowls in rural areas. The station has two main activities, namely extension and genetic improvement. Genetic improvement is done by selecting the best 100 cocks and 800 hens as parents of the next generations. Parents of each generation are selected among 6000 pedigreed and performance recorded birds produced each generation. The extension part is continuously producing and distributing 8 weeks old chicks among rural communities with the aim of increasing the population of native fowls in Northern provinces of Iran.

Sample collection and DNA isolation

A total of 156 blood samples were collected in EDTA treated tubes as an anticoagulant from randomly chosen of individuals. Samples were transferred to the laboratory and stored at 20°C for further analysis. DNA was isolated by standard salt procedure described by Miller et al. (1988). The quality and quantity of the extracted DNA was checked by spectrophotometer and agarose gel electrophoreses. DNA samples were adjusted to a concentration of 100 ng/µl and exactly 1 µl of the DNA samples were used as template for polymerase chain reaction.

GH, GHR and TGF-β3 gene amplification

The primers used to amplify GH, GHR and TGF-β3 loci are given in Table 1. For amplification of all the fragments PCR reactions of 25 µl were prepared separately as follows: 2.5 µl PCR buffer, 10 µM of each primer, 1.5 mM MgCl$_2$, 200 µM of each dNTPs, 1.0 U *Taq* DNA polymerase and 100 ng of genomic DNA. The 35 amplification cycles were carried out using a pre-programmed thermal cycler. The initial denaturation was done at 94°C for 4 min. and final extension at 72°C for 10 min. for each amplification reaction. Primer sequences and the cyclic conditions used to amplify fragment of cGH (Kansaku et al., 2003), cGHR (Feng et al., 1998) and TGF-β3 (Li et al., 2003) genes are given in Table 1.

Restriction endonuclease digestion

For the PCR-RFLP assays, 10 µl of each PCR products from GH, GHR and TGF-β3 were digested with 5 units of *Sac*I, *Hind*III and
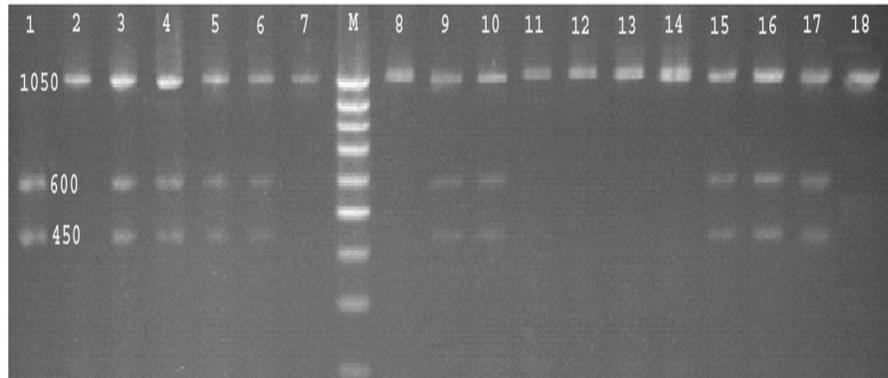
Figure 1. *Sac*I PCR-RFLP of chicken GH gene. Lane 1: genotype AA; Lane 2, 7 - 8, 11 - 14: genotype BB; Lane 3 - 6, 9 - 10, 15 - 17: genotype AB; Lane M: Molecular weight marker.
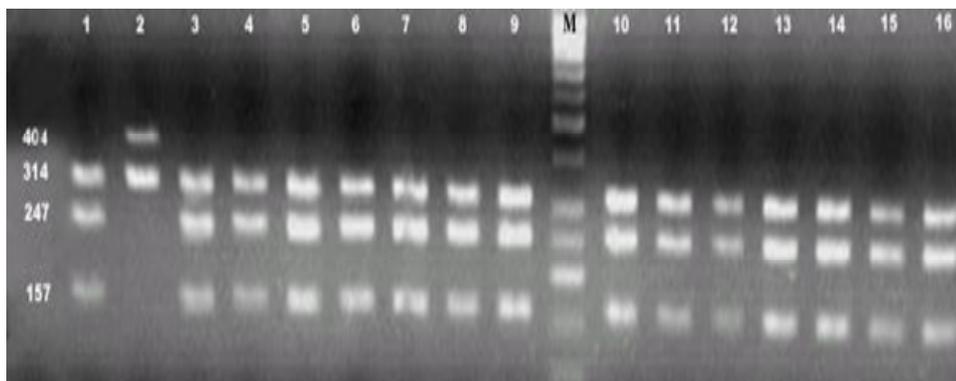
Figure 2. *Hind*II PCR-RFLP of chicken GHR gene. Lane 1, 3 - 16: genotype AA; Lane 2: genotype BB; Lane M: Molecular weight marker.

*Bsl*I restriction endonuclease at 37°C overnight, respectively. Digested products were separated by electrophoresis on 2.5% agarose gel in 1×TBE (Tris-Boric Acid-EDTA) buffer at 80 V for 4 h. The 50 bp DNA ladder (Fermentas SM1133) was used in each gel as molecular size standard. The gels were stained with ethidium bromide and the fragments were visualized using UV transilluminator.

Allele and genotype detection

The PCR products in each locus were determined by further digestion with suitable restriction enzyme. The polymorphism of GH was detected by digestion of PCR products using *Sac*I restriction enzyme with one restriction site at the amplified fragment as described by Kansaku et al. (2003). The A allele contained a 1050 bp band and the B allele included bands of sizes 450 and 600 bp (Figure 1). The polymorphism of GHR was detected by digestion of PCR products using *Hind*III with two restriction site at the amplified fragment (Figure 2). The A allele contained two bands of 404 and 314 bp and three bands of 314, 247 and 157 bp for B allele according to Feng et al. (1998). The polymorphism of TGF-β3 was detected by digestion, the PCR products using *Bsl*I restriction endonuclease. This enzyme had two or three restriction sites at the amplified fragment of TGF-β3 locus.

The A allele contained three bands, one band of 145 and two band of 75 bp and the B allele included one band of 125, two bands of 75 and one band of 20 bp as described by Li et al. (2003). The 20 bp DNA fragment was not appeared on the agarose gel (Figure 3). The size of the bands on the gel was estimated by 50 bp molecular size marker. A χ^2 test for goodness-of-fit was performed to verify if genotype frequencies agreed with HWE expectations. The gene frequencies were calculated by counting method as:

$$P = \frac{2(AA)+(AB)}{2N} \qquad q = \frac{2(BB)+(AB)}{2N}$$

Where $P = $ the gene frequency of allele (A), $q = $ the gene frequency of allele (B) and $N = $ the total number of birds tested.

RESULTS AND DISCUSSION

Allele and genotype frequencies observed in the analyzed samples are reported in Table 2. The AA homozygous genotype frequency in three loci was dominantly high. In GH and GHR loci, allele A was the most frequent
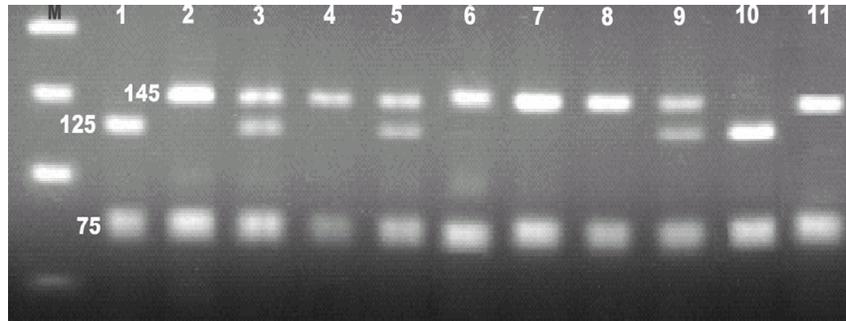
Figure 3. *Bs*I PCR-RFLP of chicken TGF-β3 gene. Lane 1, 10: genotype *AA*; Lane 2, 4, 6, 7, 8, 11: genotype *BB*; Lane 3, 5, 9: genotype *AB*; Lane M: Molecular weight marker.

Table 2. Frequencies of alleles and genotypes on GH, GHR and TGF-β3 genes in breeder hens of Mazandaran native fowls breeding station (N=156).

Loci	Genotype frequencies			Allele frequencies	
	AA	**AB**	**BB**	**A**	**B**
GH	0.62 (96)	0.36 (57)	0.02 (3)	0.798	0.202
GHR	0.99 (155)	0.00 (0)	0.01 (1)	0.990	0.010
TGF-β3	0.66 (103)	0.28 (44)	0.06 (9)	0.190	0.810

and ranged from 0.99 to 0.79 while, allele B was identified as the dominant allele in TGF-β3 locus due to the highest frequency (0.81). The frequency of BB homozygous genotype was the lowest among all loci (average = 0.03) whereas, AA genotype had the highest frequency (average = 0.76). The probability of random mating in the population was estimated by Chi-square (χ^2) test to examine Hardy-Weinberg equilibrium (HWE) at each locus. The χ^2 test showed that all three loci deviated from HWE.

The PCR product with the expected size of 1168 bp was not amplified in the present study in GH locus. This expected fragment (1168 bp) after digestion with *Sac*I restriction enzyme produces two fragments with 144 and 1024 bp (Kansaku et al., 2003). In the present study a fragment size of 1050 bp was amplified in GH locus. A deletion with the length of 118 bp has occurred at GH locus in this population. The resulting fragment was digested with *Sac*I restriction enzyme and produced two fragments with 450 and 600 bp (Figure 1). The A allele was cleaved into two fragments 600 and 450 bp, while the B allele remained uncut at 1050 bp because of the absence of a *Sac*I restriction site. A *Sac*I polymorphism in the fourth intron of the GH gene was reported to be associated with the number of tissues with tumors in Marek's disease virus-infected white leghorn chicken (Liu et al., 2001).

Selection for abdominal fat appears to affect allele frequencies, and some alleles of these RFLPs were associated with juvenile body weight, egg weight, and egg specific gravity (Feng et al., 1997; Kuhnlein et al., 1997). A 50 bp deletion in intron 4 of the cGH gene was found in Chinese native Taihe Silkies chickens (Nie et al., 2002). The cGH gene in another native breed, Yellow Wai Chow, was found to have one silent substitution, 31 insertions, and other substitutions spread among the introns (Stephen et al., 2001). PCR-RFLPs have been characterized in the introns of cGH gene of White Leghorn and it has been suggested that the alleles identified were linked to egg production traits and avian leukosis (Kuhnlein et al., 1997).

It has been reported an SNP with G to A substitution of GH gene was significantly associated with abdominal fat pad weight, abdominal fat pad ratio, and crude fatty content of the breast muscle (Lei et al., 2007). Recently, the effects of bovine growth hormone gene polymorphism at codon 127 and 172 were determined on carcass traits and fatty acid compositions in Japanese Black cattle using allele specific-multiplex PCR (Astrid et al., 2009). They found that GH gene polymorphism affected carcass traits and fatty acid compositions.

In the present study, the amplified fragment at GHR locus was 718 bp, involving the intron 2 and partial of exon 3 on Z chromosome. The nucleotide substitution on amplified fragment of GHR gene creates a *Hind*III recognition site (Feng et al., 1998). Digestion of the 718 bp PCR product of GHR gene with *Hind*III restriction enzyme revealed two patterns of restriction fragments, one with 314 and 404 bp (allele A), and second with 314, 247 and 157 bp (allele B). No genotype with uncut frag-

ment (718 bp) was found as a function of no recognition site for *Hind*III enzyme on GHR gene in the present study (Figure 2). Several strains of meat type and egg laying chicken were found to segregate for a *Hind*III RFLP located in the intron preceding exon 4 of GHR locus. Analysis of two meat type strains derived from a common genetic base, but divergently selected for the size of the abdominal fat pad revealed that the frequency of the *Hind*III + allele was significantly higher in the lean line than the fat line (Feng et al., 1998).

It has been reported that selection for body weight, feed efficiency and egg production over nine generations have led to an increase of the incidence of the *Hind*III + allele (Feng et al., 1998). Three single nucleotide polymorphisms (SNP) have been genotyped in a F_2 designed full-sib resource population to analyse their associations with chicken growth and fat deposition traits. One out of three SNP (G6631778A) was associated with body weight, dressed weight, hatch weight and subcutaneous fat thickness (Ouyang et al., 2008). In the present study, the amplified fragment at TGF-β3 locus was 295 bp, involving the exon 3 of TGF-β3 on chromosome 5 in chicken. The nucleotide substitution on amplified fragment of TGF-β3 gene creates a *Bsl*I recognition site. Digestion of the 295 bp PCR product of TGF-β3 gene with *Bsl*I restriction enzyme revealed two patterns of restriction fragments, one with 145, 75 and 75 bp (allele A), and second with, 125, 75, 75 and 20 bp (allele B). The 20 bp DNA fragment was not appeared on the agarose gel (Figure 3).

The TGF-β3 polymorphism was associated with traits of growth and body composition, such as body weight, average daily gain in broiler and leghorn chickens (Li et al., 2003). It has been reported that birds with TGF-β3-BB genotype (two broiler alleles) had higher body weight at 6 and 8 week of age and greater average body weight gain between 4 to 6 and 6 to 8 week of age than birds with TGF-β3-LL genotype (two leghorn alleles) in the F_2 population (Li et al., 2003). The heterozygote genotype did not differ from the broiler homozygote, suggesting dominance of the broiler TGF-β3 on breast muscle weight as was also found for total body weight and average daily gain. There was no significant difference between F2 TGF-β3-BB and TGF-β3-LL birds in percentage breast muscle weight (Li et al., 2003).

Conclusion

Comparison of detected alleles with reported ones from other research groups in GH locus shows a new allelic pattern at GH site in breeder hens of Mazandarn native fowls breeding station. The amplified fragment in GH locus was characterized by a deletion of approximately 118 bp. Deletion of the 118 bp fragment not only reduces the size of the PCR fragment, but also, introduced a new restriction site for *Sac*I enzyme. However, further sequencing analysis will be needed to confirm the obtained new

allelic pattern at GH site.

The main objectives of the current strategy in commercial broiler breeding programs aim to increased growth rate, increased breast muscle yield, decreased abdominal fat pad content, increased feed efficiency and increased overall fitness. The relationship between these traits is very complex and some of the traits are very difficult to measure. Therefore, molecular marker-assisted selection (MAS) is needed. In a MAS program the relative frequency of the QTL alleles is of paramount importance. If the favorable allele(s) is (are) already at high frequency in the population under selection, then little can be gained from MAS while if the favorable allele(s) is (are) rare a larger impact can be obtained (Fontanesi et al., 2007).

The obtained results in the present study indicate that the A allele frequency in both GH and GHR loci was identified as the dominant allele whereas, it was at the lowest level in TGF-β3 site. It can be concluded that the incidence of higher A allele frequency in GH and GHR loci and B allele in TGF-β3 locus may be as a long term selection strategy sued in this population. Further association analysis will be required to clarify the effects of these marker genotypes on production traits in this breeder flock.

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