

*Full Length Research Paper*

# Association of single nucleotide polymorphisms in IGF-I, IGF-II and IGFBP-II with production traits in breeder hens of Mazandaran native fowls breeding station

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The purpose of this study was to detect the polymorphism in IGF-I, IGF-II and IGFBP-II marker loci and their association with body weight at 8 weeks, average egg weight and total number of eggs laid during first 12 weeks after flocks maturity in breeder hens of native fowls. Blood samples were collected randomly from 160 individuals and genomic DNA was extracted using modified salting out method. A set of specific primer pairs were used for amplification of target genomic DNA at each marker loci and polymorphisms were detected using PCR-RFLP method. For IGF-I and IGFBP-II marker loci, allele B was the most frequent allele and ranged from 0.61 to 0.63 while, allele A was identified as a dominant allele in IGF-I marker site due to the highest frequency (0.57). The frequency of AA homozygous genotype was the lowest among all marker loci (0.08), whereas, AB genotype showed the highest frequency (0.61). Analysis of phenotypic data showed that the average egg weight and total number of eggs laid during first 12 weeks after flocks maturity were significantly affected by IGF-II and IGF-I marker loci respectively. No significant associations were observed between IGFBP-II genotypes and production traits. Comparison between detected alleles in the present study with reported allele by other research groups revealed a new allelic pattern for the analyzed marker loci in breeder hens of Mazandaran native fowls breeding station.

**Key words:** Native fowls, IGF-I, IGF-II, IGFBP-II, polymorphism.

## INTRODUCTION

Insulin-like growth factors (IGFs) system is a complex system of peptide hormones (IGF-I and IGF-II), cell surface receptors and circulating binding proteins. IGF-I and IGF-II bind to the insulin-like growth factor 1 receptor, insulin receptor and activate their intrinsic tyrosine kinase domain activities (Adam et al., 2005). Several studies have shown that circulating IGF-I affects growth rate, body composition and lipid metabolism in poultry (Ballard et al., 1990; Beccavin et al., 2001; Zhou et al., 2005). It has been shown that plasma chicken IGF-I concentration was greater in genetic lines selected for high growth rate compared with that in slower growing lines (Scanes et al., 1989). The chicken IGF-I gene maps to 165.95 cM on

chromosome 1 and a QTL at 150 cM on chromosome 1 affecting abdominal fat weight has been detected in chicken (Ikeobi et al., 2002). The chicken IGF-I gene has been cloned and sequenced and is composed of four exons and three introns (Kajimoto and Rotwein 1991). It has been shown that an SNP of IGF-I gene (*Pst*I-RFLP) was associated with body weight at 2 and 4 months of age in the Wanzhai Yellow breed chickens (Wang et al., 2004). Bennett et al. (2006) was developed an experiment with PCR-RFLP method to genotype a chicken F2 population and to evaluate associations between each SNP genotype of IGF-I and multiple phenotypes. They have found significant associations between chicken IGF-I polymorphism and 5 week body weight. The chicken IGF-II gene maps to chromosome 5 and contains three exons and displays a biallelic pattern of expression (Yokomine et al., 2001; Nolan et al., 2001). An association between phenotypic variation at several

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**Table 1.** Primer sequences and the cyclic conditions used to amplify fragments of IGF-I, IGF-II and IGFBP-II genes in breeder hens of Mazandaran native fowls.

Locus	Primer sequences (5'-----3')	Denaturation	Annealing	Extension
IGF-I	F:CATTGCGCAGGCTCTATCTG R:TCAAGAGAAGCCCTTCAAGC	94 °C, 60 s	56 °C, 45 s	72 °C, 60 s
IGF-II	F:CCAGTGGGACGAAATAACAGGAGGA R:TTCCTGGGGGCCGGTTCGCTTCA	94 °C, 60 s	67 °C, 90 s	72 °C, 90 s
IGFBP-II	F:ACCGGTCTGAGAGCATCCTTG R:GGGAAAAAGGGTGTGCAAAAG	94 °C, 30 s	61 °C, 30 s	72 °C, 30 s

growth and carcass traits with one polymorphism at exon 2 of the chicken IGF-II gene has been reported (Amills et al., 2003). In serum of different species, over 99% of IGF molecules circulate as complexes to at least 7 specific and high affinity-binding proteins, which regulate bio-activity of IGF (Lei et al., 2005).

The IGF-binding protein type II (IGFBP-II) is sensitive to dietary protein level and may play an important role in the modulate the growth promoting effect of circulating IGF-I by making the IGFBP complex in ruminant and chicken (Kita et al., 2002; Lee et al., 2005). The chicken IGFBP-II gene spans approximately 38 kb and is located on chromosome 7 and consists of 4 short exons and 3 long introns (Schoen et al., 1995). Association between different SNP of IGFBP-II and some growth traits were reported in several chicken populations (Nie et al., 2005; Lei et al., 2005). The objective of the current study was to identify SNPs of chicken IGF-I, IGF-II and IGFBP-II using PCR-RFLP and evaluate associations between the variation at these marker loci and that of some production traits in breeder hens of native fowls.

## 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. In 1986 about 5000 cocks and hens were purchased from rural communities across the Mazandaran province and were transferred to a quarantine farm. In 1987 and after practicing quarantine procedures about 2500 birds of two sexes were kept to produce hatching eggs and chicks produced from these eggs were transferred to the station in 1988. Since then birds have been individually tagged and trap nest has been used for pedigree recording. A multiple trait animal model has been used for genetic evaluation of the birds for body weight at 8 weeks, age of the hens at first egg, average egg weight and total number of eggs laid during first 12 weeks after flocks maturity (when 5% of the flock are in egg production). Economic indexes are calculated for these traits and birds of two sexes are selected based on their aggregate genotypes for these traits. The station has two main activities, namely extension and genetic improvement. 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. 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 from among about 6000 pedigreed and performance recorded birds produced each generation.

### Sample collection and DNA extraction

DNA was isolated from 160 blood samples of breeder hens from Mazandaran native fowls breeding station. Five microliter blood samples were collected in EDTA treated tubes as an anticoagulant. The collected blood samples kept in ice, shifted to the laboratory and stored at - 20 °C until used in assay. Genomic DNA was extracted using modified salting-out procedure as described by Miller et al. (1988). At the final step of DNA extraction procedure, the collected DNA pellet was washed in 70% ethanol, dried and dissolved in TE buffer. The quality and quantity of the extracted DNA was checked by spectrophotometer and agarose gel electrophoreses. The extracted DNA samples were adjusted to a concentration of 50 ng  $\mu\text{l}^{-1}$  and exactly 1  $\mu\text{l}$  of the DNA samples were used as a template in polymerase chain reaction.

### Amplification of IGF-I, IGF-II and IGFBP-II genes

The sequences of three primer pairs used to amplify a target fragment in IGF-I, IGF-II and IGFBP-II loci in chicken are shown in Table 1. PCR reactions of 25  $\mu\text{l}$  were prepared separately for amplification of each selected marker loci as follows: 2.5  $\mu\text{l}$  PCR buffer, 20  $\mu\text{M}$  of each primer, 2.5 mM  $\text{MgCl}_2$ , 0.2 mM of each deoxynucleotide triphosphate, 1.0 U Taq DNA polymerase and 50 ng of genomic DNA. The 40 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. The cyclic conditions used to amplify fragment of chicken IGF-I (Moody et al., 2003), IGF-II (Amills et al., 2003) and TGF- $\beta$ 3 (Lei et al., 2005) marker loci are presented in Table 1.

### Genotyping of IGF-I, IGF-II and IGFBP-II genes

A specific primer pairs was used to amplify a fragment of 900 bp corresponding to the 5' end of promoter region of chicken IGF-I gene sequence. The polymorphism in IGF-I marker site was detected by digestion of PCR products using *Hinf* I restriction enzyme which contains one restriction site at the amplified fragment. The PCR products were digested at 37 °C overnight with 7 unit of *Hinf* I enzyme. Digestion of the PCR products yielded two restriction fragments of 700 and 200 bp. The A and B alleles obtained at IGF-I marker site contained one band of 900 and two bands of 200 and 700 bp, respectively. The typing of IGF-II gene

**Table 2.** Allele and genotype frequency of IGF-I, IGF-II and IGFBP-II genes in breeder hens of Mazandaran native fowls (N=160).

Loci	Allele frequencies		Genotype frequencies		
	A	B	AA	AB	BB
IGF-I	0.39	0.61	0.18	0.42	0.40
IGF-II	0.57	0.43	0.39	0.36	0.25
IGFBP-II	0.37	0.63	0.08	0.61	0.31

involved the analysis of one polymorphism site on a 1300 bp fragment, corresponding to the exon 2, intron 2 and most of exon 3 of the chicken IGF-II gene. The IGF-II polymorphism was detected by digesting of PCR products with the *Hsp92II* enzyme at 37°C overnight. The A allele as a result of enzyme digestion showed two bands of 350 and 950 bp while the B allele showed two bands of 550 and 750 bp in IGF-II loci. The IGFBP-II was typed for *Bsh1236 I* RFLP in exon 2 of type II IGF binding protein gene. The digested of 950 bp PCR products with *Bsh1236* restriction enzyme showed an uncut 950 bp (A) allele and a digested B alleles with two bands of 100 and 850 bp. Restriction digests were electrophoresed for 1 h at 80 V on a 2% agarose gel with ethidium bromide. Individual PCR-RFLP fragment sizes in each sample were determined, based on standard DNA molecular weight markers for each gene, by viewing the banding pattern under ultraviolet light.

#### Statistical analysis

Genotypes of individual birds at the different marker loci were recorded by direct counting of the bands. The gene frequencies were calculated by counting method as:

$$p = \frac{2(AA) + (AB)}{2N} \quad q = \frac{2(BB) + (AB)}{2N}$$

where  $p$  = the gene frequency of allele A,  $q$  = the gene frequency of allele B and  $N$  = the total number of birds tested.

A chi-squared test for goodness-of-fit was performed to verify if genotype frequencies agreed with Hardy-Weinberg equilibrium (HWE) expectations using POPGENE software. Marker-trait analysis was performed by SAS GLM procedure (SAS Institute, 1999) and the genetic effects on body weight at 8 weeks, average egg weight and total number of eggs laid during first 12 weeks after flocks maturity (when 5% of the flock are in egg production) were analyzed by mixed procedure according to the following model:

$$Y_{ijk} = \mu + G_i + H_j + e_{ijk}$$

where  $Y_{ijk}$  was an observation on the trait,  $\mu$  was the overall population mean,  $G_i$  was the fixed effect associated with genotype  $i$ ,  $H_j$  was the fixed effect associated with hatch  $j$  and  $e_{ijk}$  was the random error. The fixed effect of hatch ( $H_j$ ) was not significant for any trait and therefore was not included in the model. Statistical significance threshold was determined as  $P < 0.05$ . The average egg weight and total number of eggs were measured for up to twelve weeks after sexual maturity. Sexual maturity is the age when the first egg was recorded from each hen. The average of egg weight is the sum of egg weight laid divided by the number of eggs collected for up to twelve weeks after sexual maturity from each hen.

## RESULTS AND DISCUSSION

Breeder hens (160) from Mazandaran native fowls breeding station were examined for the IGF-I, IGF-II and IGFBP-II gene polymorphisms in the current study. Allele and genotype frequencies observed in the analyzed samples are given in Table 2. For IGF-I and IGFBP-II loci, allele B was the most frequent allele and ranged from 0.61 to 0.63 while, allele A was identified as a dominant allele in IGF-I locus due to the highest frequency (0.57). The frequency of AA homozygous genotype was the lowest among all loci (average = 0.08) whereas, AB genotype had the highest frequency (average = 0.61). The probability of random mating in the population was estimated by Chi-square ( $\chi^2$ ) test to examine Hardy-Weinberg equilibrium (HWE) at each locus. The  $\chi^2$  test showed that both IGF-I and IGF-II loci deviated from HWE, while IGFBP-II marker site did not deviate from HWE. For IGF-I marker site, digestion of the PCR product yielded two restriction fragments of 700 and 200 bp. In the other study, the restriction enzyme *Hinf I* produced fragment sizes of 622 and 191 bp for the 2 inbred lines of chicken, whereas the broiler line had fragment sizes of 378, 244 and 191 bp (Zhou et al., 2005). The *Hinf I* RFLP was located in the 5' untranslated region of the IGF-I gene, near a putative TATA box and consisted of one A to C substitution. The fragment size and also the size of alleles resulted by restriction enzyme in the present study, was different from other reports (Amills et al., 2003; Zhou et al., 2005). The IGF-II polymorphism was detected by digesting of PCR products with the *Hsp92II* enzyme in breeder hens of Mazandaran native fowls breeding station. The A allele as a result of enzyme digestion showed two bands of 350 and 950 bp, while, the B allele showed two bands of 550 and 750 bp in IGF-II loci. The size of the amplified PCR fragment and alleles obtained in the present study was different from other reports (Amills et al., 2003; Zhou et al., 2005). Three genotypes of AA, AB and BB were detected in two chicken strains of the Black Penedesenca breed in IGF-II marker site. This SNP which could be detected by *Hsp92 II* restriction enzyme is a neutral substitution of nucleotide C to T at exon 3 of IGF-II gene (Amills et al., 2003). In the present study, The IGFBP-II was typed for *Bsh1236 I* RFLP in exon 2 of IGFBP-II

**Table 3.** Phenotypic traits of body weight at 8 weeks, average egg weight and total number of eggs laid during first 12 weeks after sexual maturity in the samples used for SNP analysis in breeder hens of native fowls.

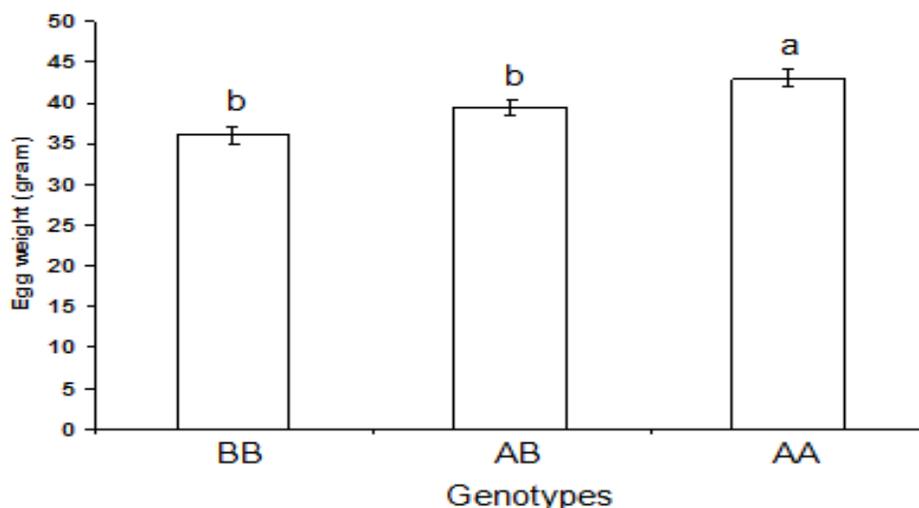
Traits	Mean	SD	CV (%)	Min.	Max.
Body weight	511	103.25	20.21	270	850
Number of egg	29	13.41	46.24	6	68
Egg weight	43.8	5.80	13.24	34.6	55.7

**Table 4.** Effects of polymorphism of IGF-I, IGF-II and IGFBP-II on body weight at 8 weeks, average egg weight and total number of eggs laid during first 12 weeks after sexual maturity in the samples used for SNP analysis in breeder hens of native fowls.

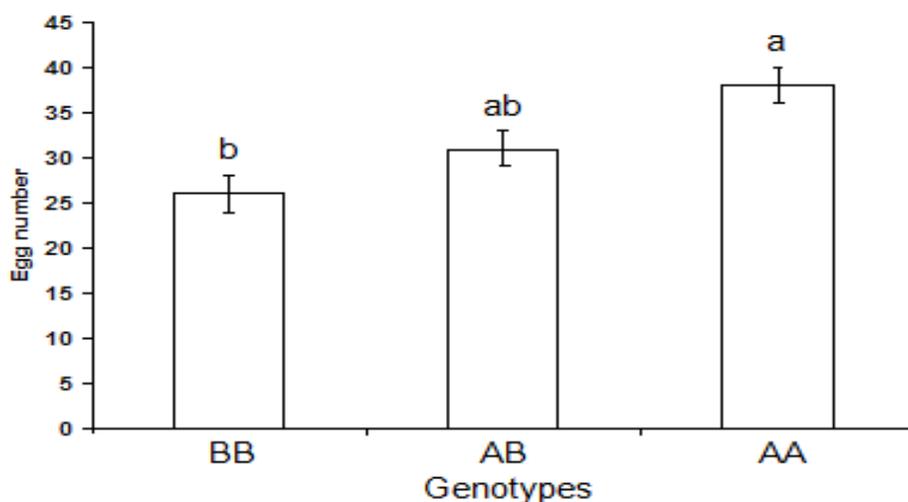
Loci	Traits		
	Body weight	Egg number	Egg weight
IGF-I	NS	NS	P < 0.05
IGF-II	NS	P < 0.05	NS
IGFBP-II	NS	NS	NS

gene and three genotypes of AA, AB and BB was observed in the analyzed samples. The size of amplified PCR fragment for IGFBP-II in the present study and also the size of the allele resulted by *Bsh1236* restriction enzyme was different from the report of Lei et al. (2005) in a chicken population which was made up of reciprocal crosses between White Recessive Rock and Xinghua breed. The variations in PCR fragment and allele size obtained in the present study and other reports may be because of different chicken populations. For the final conformation of the different allelic variants obtained in the present study for IGF-I, IGF-II and IGFBP-II marker sites, the amplified fragment at each marker loci should be sequenced. The phenotypic traits of body weight at 8 weeks, average egg weight and total number of eggs laid during first 12 weeks after flocks maturity measured in the samples used for SNP analysis (Table 3). The effects of polymorphism of IGF-I, IGF-II and IGFBP-II marker loci on these traits are presented in Table 4. There were no significant associations between these three loci and body weight at 8 weeks of age, whereas there were significant associations between IGF-I and IGF-II loci on average egg weight and total number of eggs, respectively. In contrast, study of the same mutation of IGF-I in 2 genetically diverse maternal and paternal Black Penedesenca chicken strains, significant association of the IGF-I polymorphism was found for average daily gain (Amills et al., 2003). In the previously study, It has been shown that chickens inheriting IGF-I broiler alleles had heavier body weight at all ages to market weight and average daily gain than birds with the Leghorn allele (Zhou et al., 2005). In the present study, the effect of homozygous AA genotype of IGF-I marker site was greater than the heterozygous AB and homozygous AA

genotypes for egg weight trait (Figure 1), but the frequency of AA genotype was the lowest among the other two homozygous AA or heterozygous AB genotypes. The obtained result may indicate that the individuals with AA genotype may be not selected as a breeder hen for the next generation because of a low record for egg number and also body weight at 8 weeks of age. In the present study, for IGF-II marker site, there was no significant differences between homozygous AA and heterozygous AB genotypes on total egg number, but a significant differences ( $P < 0.05$ ) was found between homozygous AA and BB genotypes (Figure 2). The individuals with AA genotype showed higher number of egg than birds with BB genotype. For IGF-II marker site, the frequency of AA genotype was higher than other two AB and BB genotypes. No association was found between IGFBP-II polymorphism and production parameters studied in this population. There was no significant association between the same single SNP at IGFBP-II marker site and growth and carcass traits, but there were significant associations of haplotype genotypes in IGFBP-II marker sites with growth and carcass traits with the mixed model analyses (Lei et al., 2005). It has been proposed that the traditional approach for studying both trait association (marker vs. trait) and linkage disequilibrium (marker vs. marker), single-marker analysis has created many problems, such as noisy, unsatisfied and obscured important localization information (Daly et al., 2001). Therefore, haplotype or haplotype block provided a practical solution to resolve these problems (Daly et al., 2001). However, the same haplotype construct model should be proposed for IGFs marker sites for the future research work in breeder flocks of Mazandaran native fowls breeding station.



**Figure 1.** The effect of IGF-I genotypes on average egg weight during first 12 weeks after sexual maturity in the samples used for SNP analysis in breeder hens of native fowls.



**Figure 2.** The effect of IGF-II genotypes on total number of eggs laid during first 12 weeks after sexual maturity in the samples used for SNP analysis in breeder hens of native fowls.

## Conclusion

In the current study, the new Hinf I, *Hsp92* II and for *Bsh1236* I polymorphism were found for IGF-I, IGF-II and IGFBP-II genes, respectively. For the final conformation of the observed new allelic pattern the amplified target fragments should be sequenced. Considering that the polymorphism in IGF-I and IGF-II marker loci had a beneficial effect on egg weight and number of eggs, it would be possible to make selection schemes favoring the desired genotypes for increasing the egg related parameters in chickens. To make the selection schemes applicable, it would be necessary to further analyze the

effects of IGFs polymorphisms by using populations from different generations and increasing the size of samples.

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