Polymorphisms of prolactin gene in a native chicken population and its association with egg production

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Summary

The induction and regulation of broodiness is of the most important role of prolactin in avian species. The promoter region of the prolactin gene is an appropriate model for studying tissue-specific and hormonally-regulated activation of gene transcription. In this study, the association between prolactin promoter region alleles and egg production in Fars native chickens was investigated. In total, 100 laying hens, randomly selected from the flock of the Breeding Center for Fars Native Chicken, were genotyped for two single nucleotide polymorphisms (C-2161G and C-2402T), one 24-bp insertion-deletion at the site -358. The observed genotypic frequencies in the C-2161G site were CC (0.437), CG (0.435) and GG (0.101). Two alleles were found for the SNP of C-2402T with frequency of 0.34 for T and 0.66 for C. The 24-bp insertion-deletion at the site -358 was shown to be polymorph in the studied sample, with the observed genotyping frequency of 0.417 in II, 0.457 in ID and 0.126 in DD. Significant relationship was found between the SNPs and insertion-deletion genotypes with egg production (P<0.01).

Introduction

There is considerable interest in the genetic control of broodiness in the chicken. Broodiness is an economically important trait which is controlled at least by two dominant autosomal genes that induce and inhibit the behavior, respectively, with equal influences (Romanov et al., 2002). Prolactin (PRL), produced in the lactotropes of the pituitary gland, has varied functions in invertebrate species (Freeman et al., 2000), including the initiation of incubation in the avian species, resulting in regression of the ovary and cessation of egg production (Sharp, 1997). Injection of PRL induced incubation behavior (Ishida et al., 1991; Shimida et al., 1991), whereas active immunization against PRL inhibited the development of broodiness (Cristomo et al., 1997; Sharp, 1997). Similarly, active immunization against vasoactive intestinal peptide (VIP) disrupted the physiological pauses that occur during the ovulatory sequences which modulates PRL levels that interfere with the follicular development and oviposition (El-Halawani et al., 2000; Reddy et al., 2002). The expression of PRL mRNA was significantly higher in the low egg production strains, indicating that egg production was related to PRL mRNA expression in chickens (Shimida et al., 1991). The complete sequence of the chicken PRL gene that has been determined (Ohkubo et al., 2000; Au and Leung, 2002). Cui et al. (2006) recently reported an association between PRL gene and egg production in chickens. The chicken *PRL* gene was cloned and recombined-derived PRL was produced to actively immunize bantam hens, however, this did not influence the photo-induced onset or subsequent rate of egg production (March et al., 1994).

The pituitary specific transcription factor (PIT-1) activates transcription of PRL gene (Bradford et al., 2000). Prolactin gene

transcription is also regulated by factors such as activin (Lacerte *et al*., 2004) and CCAAT/enhancer binding protein (Enwright *et al*., 2003). In turkey and Xinghua chicken (a Chinese native breed), potential Pit-1 binding sites in the 5′ flanking region of the PRL gene have been identified (Kurima *et al*., 1995; Ohkubo *et al*., 2000; Au and Leung, 2002), but the precise molecular mechanism of Pit-1 mediated *PRL* gene activation is not clear.

Promoter region of *PRL* gene is an appropriate model for studying both tissue-specific and hormonally-regulated activation of gene transcription (Seyfred and Gorski, 1990; Elsholtz *et al*., 1991; Ebrahimi, 2011). Compared to the extensive studies on the promoter region of mammalian *PRL* gene the information on *PRL* gene in birds is rather limited (Kurima *et al*., 1995; Weatherly *et al*., 2001). The length of promoter region shows differences between mammals and birds. A super upstream promoter is determined in the 5′ flanking region of human *PRL* gene and it can trigger the human *PRL* expression even in absence of Pit-1 (Gellersen *et al*., 1995), which infers that the structural variations in 5′ promoter could explain the differences in the regulatory mechanism (Gellersen *et al*., 1995; Kurima *et al*., 1995; Weatherly *et al*., 2001). To elucidate the genetics of PRL, genomic structures of *PRL* in avian were studied by Kansaku *et al*. (2005). An abundance of SNP was reported in the 5′-flanking region of chicken PRL, and the 24-bp insert-deletion was significantly associated with broody behavior and egg production (Jiang *et al*., 2005; Cui *et al*., 2006), indicating its usefulness as a molecular marker for egg production.

Fars native chickens show a high frequency of broodiness, producing 20-70 eggs during each laying period (8 months). The objective of this study was to investigate the potential association between the PRL promoter region polymorphisms and egg production in Fars native chickens.

Materials and Methods

In total, 100 laying hens, randomly selected from the flock of the Breeding Center for Fars Native Chicken, were studied. In this flock, the replacement hens and roosters are selected yearly based on the age at puberty, 12-week body weight, 3-month egg production, and the mean egg weight at 28, 30 and 32 weeks of age.

Genomic DNA was used to evaluate two single nucleotide polymorphisms (C-2161G and C-2402T), one 24-bp insertion-deletion at the site -358 in the promoter of prolactin gene. The fragment containing SNP of C-2402T and C-2161G of PRL gene was amplified using primers in Table 1.

The following primer was used to amplify the PRL gene fragment containing one 24-bp insertion-deletion at the site -358.

The PCRs reactions were performed in final volume of 20 μL containing 100 ng of genomic DNA, 0.2 μM primer, 200 μM dNTPs, 1.75 mM MgCl$_2$, 2 U of *Taq* DNA polymerase (Cinagen Co., Tehran) and 1 x reaction buffer on a thermal cycler with the following cycles: initial denaturation of 5 min at 94°C; 35 cycles of 94°C for 30 s, 62°C (primer pair 5 F) or 54°C (primer pair 24 BP) for 30 s, and 72°C for 30 s with a final extension of 5 min at 72°C.

The fragment amplified by primer pair 5 F was digested with *Alu*I (Metabion, Germany) and *Csp6*I (Metabion, Germany) endonucleases (followed by electrophoresis on 3% agarose gel) for genotyping the polymorphisms of C-2402T and C-2161G, respectively. The fragment amplified by primer pair p2 was electrophoresed directly on 3% agarose.

Statistical analysis

The association between prolactin gene alleles and egg production was analysed using the GLM procedure of SAS (2004). The used models included the genotype of each site, hatch number, age at puberty, and weight at initiation of lay and duration period as fixed effects. Values were considered significant at P<0.01 and presented as least squares means ± standard errors. The gene substitution effect was also determined using the following formulae:

$$\alpha_1 = q(a + d(q - p)) = q\alpha$$

$$\alpha_2 = -p(a + d(q - p)) = -p\alpha$$

α_1= Allelic replacement for allele 1
q= Allelic frequency for allele 1

p= Allelic frequency for allele 2
a= Additive effect of gene
d= Dominance deviation

Results

Three genotypes were found at the site of -2402 (CC, CT, TT) and three genotypes at the site of -2161 (CC, CG, GG) after digestion with *Alu*I and *Csp6*I (Figs. 1 and 2). The 24-bp insertion-deletion at the site -358 was shown to be polymorph in the examined sample with two alleles, I and D. The observed genotypic frequencies in the C-2161G site were CC (0.464), CG (0.435) and GG (0.101). Two alleles were found for the SNP of C-2402T with frequency of 0.34 for T and 0.66 for C. The 24-bp insertion-deletion at the site -358 was shown to be polymorph with the observed genotypic frequency of 0.417 in II, 0.457 in ID and 0.126 in DD (Table 2) (Fig. 3).

Marker-trait association analysis

Amongst genotypes of the 24-bp insertion-deletion, laying performance (egg number and rate of lay) of II genotype was not significantly different from that of ID
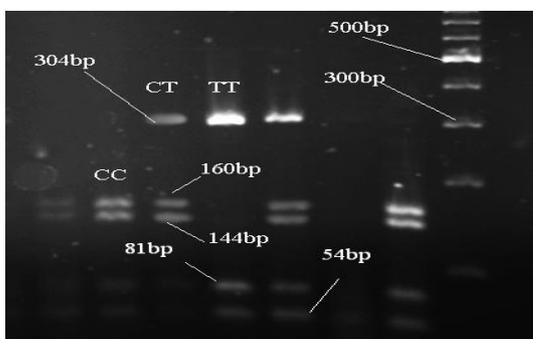
Fig. 1: Restriction analysis of PRL 439 bp PCR products digested with AluI by 3% agarose gel electrophoresis. CC genotypes: Restriction fragments of 160, 144, 81 and 54 bp, CT genotypes: Restriction fragments of 304, 160, 144, 81 and 54 bp, and TT genotypes: Restriction fragments of 304, 81 and 54 bp
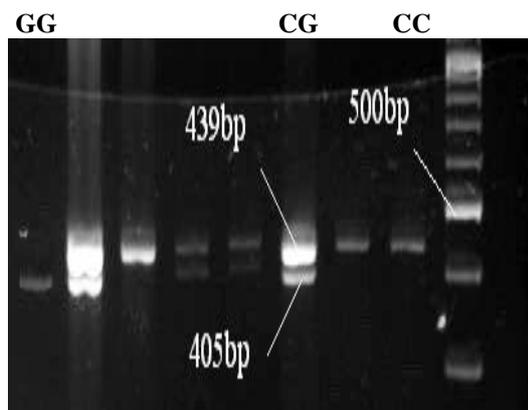
Fig. 2: Restriction analysis of PRL 439 bp PCR products digested with Csp6I by 3% agarose gel electrophoresis. GG genotypes: Restriction fragments of 405 bp, CG genotypes: Restriction fragments of 439 and 405 bp, and CC genotypes: Restriction fragments of 439 bp
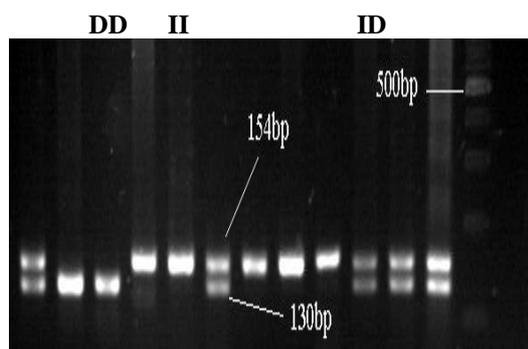
Fig. 3: Genotypes of the 24-bp insertion-deletion at the site of -358 by PCR with agarose gel electrophoresis analysis

Table 2: Allelic and genotypic frequencies of the 3 polymorphic sites

Polymorphism	Allelic frequency		Genotypic frequency		
Alu1-RFLP	C 0.66	T 0.34	CC 0.437	CT 0.448	TT 0.115
CSP6I-RFLP	C 0.68	G 0.32	CC 0.464	CG 0.435	GG 0.101
Insertion-Deletion	I 0.65	D 0.35	II 0.417	ID 0.457	DD 0.126

Table 1: Primer characteristics (Cui *et al*., 2006)

Primer name	Length	Primer	Sequence
p1 (C-2161G and C-2402T)	439	Sense	AGAGGCAGCCCAGGCATTTTAC
		Antisense	CCTGGGTCTGGTTTGGAAATTG
p2 (24-bp insertion-deletion)	130-154	Sense	TTTAATATTGGTGGGTGAAGAGACA
		Antisense	ATGCCACTGATCCTCGAAAACTC

genotype, but laying performance of DD genotype was significantly different from those of II and ID genotypes (Table 3). Amongst *Alu*I-RFLP genotypes, laying performances of CC and CT were not significantly different but laying per-formance of TT genotype was significantly different from those of CC and CT genotypes (Table 4). Amongst *CSP6I*-RFLP genotypes the laying performances of CC and CG genotypes were not significantly different from each other, but the laying performance of GG genotype was significantly different from that of the CC and CG genotypes (Table 4). Estimation of allelic replacement effect indicated a positive effect of I, C (*ALU*I-RFLP) and C (*CSP6*I-RFLP) on egg production (Table 5).

Table 3: Least squares means ± standard errors of egg number and laying rate according to 24-bp insertion-deletion genotypes

Genotype	Egg number	Laying rate
II	52.03 ± 1.0^a	0.80 ± 0.01^a
ID	48.78 ± 1.0^a	0.75 ± 0.01^a
DD	34.38 ± 1.7^b	0.54 ± 0.02^b

[a, b] Within each column, means with similar letter are not significantly different (P>0.01)

Table 4: Least squares means ± standard errors of egg number and laying rate according to PCR-RFLP genotypes

	Genotype	Egg number	Laying rate
Alu1-RFLP	CC	49.55 ± 1.1^a	0.79 ± 0.02^a
	CT	46.96 ± 1.3^a	0.76 ± 0.02^a
	TT	42.01 ± 1.9^b	0.67 ± 0.03^b
CSP6I-RFLP	CC	48.75 ± 1.3^a	0.79 ± 0.02^a
	CG	46.80 ± 1.4^a	0.77 ± 0.02^a
	GG	39.88 ± 2.9^b	0.66 ± 0.04^b

[a, b] Within each column and for each polymorphism, means with similar letter are not significantly different (P>0.01)

Table 5: Allelic replacement effect for three genotypes, CT, CG and ID

Allele	α_1	Allele	α_2
C	+1.06	T	-0.54
C	+1.73	G	-0.80
I	+2.61	D	-1.44

α_1: Allelic substitution effect for allele 1, and α_2: Allelic substitution effect for allele 2

Discussion

Prolactin has an important role in the initiation of incubation in domestic birds (Sharp, 1997). Although PRL release from the mammalian pituitary gland is mainly controlled by the inhibitory action of hypothalamic dopamine, prolactin secretion in birds is said to be under the control of vasoactive intestinal peptide (VIP) which stimulates PRL release (Rozenboim *et al*., 1993). The inhibitory effect of dopamine on PRL release is mediated through dopamine receptor DRD2 (Youngren *et al*., 1998), while dopamine stimulates PRL secretion via activation of DRD1 receptor at the hypothalamic level by operating through VIP (Porter *et al*., 1994). Xu *et al*. (2010) recently demonstrated that polymorphisms of DRD1 receptor and their haplotypes were associated with the frequency of broodiness and some production traits in the chicken.

The avian *PRL* gene is highly conserved and most sequence polymorphisms in the chicken *PRL* gene occur in 5′ flanking region, 3′ flanking region, and the coding region of the signal peptide (Kansaku *et al*., 2008). In Chinese native chickens, Cui *et al*. (2006) detected 3 SNPs in PRL exons 2 and 5, and 1 SNP in *cPRL* intron 2. Liang *et al*. (2006) demonstrated that polymorphism existed in the 5′ flanking regions of *cPRL* gene from two Chinese breeds and the White Leghorn chickens. In the proximal part of *cPRL* gene promoter, a 24-bp insertion-deletion and a polyA length polymorphism were identified, and 4 SNPs were found in the conserved distal region of *cPRL* gene promoter. We found 3 polymorphisms in Fars native chicken *PRL* gene promoter. The frequency of I (insertion-deletion poly-morphism), C (*ALU1*-RFLP) and C (*CSP6I*-RFLP) were 0.65, 0.66, and 0.68, respectively. Therefore, these alleles seem to be the preponderant genes affecting egg production in the studied population.

In this study, chickens with II, CC (at C-2402T site) and CC (at C-2161G site) genotypes had significantly greater egg production (number) and laying rate than DD, TT and GG genotypes, respectively (P<0.01). Therefore, it may be assumed that the PRL gene affected egg production by

regulating the activity of reproduction in chickens. Fars native chickens show strong broodiness and produce some 50 eggs during each laying period (unpublished observations). However, Ohkubo *et al.* (1998) reported that differences in the expression of broodiness in White Leghorn and bantam hens could not be explained by differences in the amounts of *PRLR* mRNA in the hypothalamus or in the transcription or gross structure of the *PRLR* gene.

In avian species, changes in plasma PRL level are associated with the expression of PRL mRNA in the anterior pituitary (Wong *et al.*, 1991). The expression of PRL is regulated by the 5′-flanking region sequence by binding with specific transcription factors (Jiang *et al.*, 2005). Studies in mammals and birds have shown that Pit-1/GHF-1 (Frisch *et al.*, 2000), estrogen receptors (Maurer and Notides, 1987), the CCAAT-enhancer binding protein-α (Enwright *et al.*, 2003) and other proteins are essential in regulating the expression of PRL via specific promoter binding sites. The sequence variation in the 5′-proximal region of PRL may lead to changes of transcription factor binding sites and alter the expression of PRL. A possible ecotropic viral integration site-1 encoded factor (Evi-1) binding site (score 93) was presented in the 5′ flanking region of the chicken *PRL* gene because of the 24-bp insertion (Cui *et al.*, 2006). The Evi-1 has 2 zinc-finger domains and Evi-1 has been shown to be involved in transcription of many genes as a repressor (Hirai *et al.*, 2001; Palmer *et al.*, 2001; Vinatzer *et al.*, 2001). Thus, Evi-1suppresses transforming growth factor-*β* (TGF-*β*) signaling and antagonizes the growth-inhibitory effects of TGF-*β* (Kurokawa *et al.*, 1998; Izutso *et al.*, 2001). The presence of an Evi-1 binding site suggested the possible transcriptional regulation of the chicken *PRL* gene by Evi-1. It is possible that Evi-1 represses the expression of *PRL* gene in White Leghorn chickens by binding the Evi-1 binding site and further prevents broodiness (Jiang *et al.*, 2005), which can improve egg production to some extent.

In conclusion, this study showed the presence of an association between prolactin gene polymorphisms and egg production in Fars native chickens, but further studies are needed to confirm this association.

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