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Mutations in the exon 10 of prolactin receptor gene change the egg production performance in Wanjiang white goose

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Abstract To select the molecular genetic markers related to egg performance of Wanjiang white goose, prolactin receptor gene (PRLR) was adopted to be a candidate gene in our study. Five pairs of primers (P1–P5) were designed to detect the SNPs of PRLR gene by PCR-SSCP method. The results revealed that polymorphisms were discovered in the PCR products amplified with P4 primers in PRLR exon 10, three genotypes were found: AA, AB and AC. The sequence of AB genotype is the same as original sequence (DQ660982) in NCBI. There are five mutations in AA genotype: C → A at 840 bp, C → T at 862 bp, T → C at 875 bp, T → A at 963 bp, A → T at 989 bp, resulting in amino acid mutations: His → Asn, Thr → Ile, Asn → Lys, Thr → Ser, and synonymous mutation at 875 bp. Sequencing revealed five mutations in AC genotype: G → T at 816 bp, A → T at 861 bp, C → T at 862 bp, T → C at 875 bp, A → G at 948 bp, causing amino acid mutations of Val → Phe, Thr → Phe, synonymous mutations at 875 and 963 bp. Besides, there are an *N*-glycosylation site (NQSR), three casein kinase II phosphorylation sites including SIIE, SKTE, and SLMD in AA genotype; three casein kinase II phosphorylation sites including SIIE, SKTE, and TLMD in AB genotype; three casein kinase II phosphorylation sites including SIFE, SKTE, and TLMD in AC genotype. The annual egg yielding of AB genotype geese are significantly more than those of AA and AC genotype geese on the average ($P < 0.05$). It is suggested for the first time that PRLR is a promising candidate gene that can affect egg performance in Wanjiang white goose.

Keywords Goose · Prolactin receptor gene · PCR-SSCP · Egg production performance

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Introduction

Prolactin (PRL) is a protein peptide hormone secreted by oxyphil cells of anterior pituitary, which is vital in the process of poultry propagation. It is related to reproductive performance, maternal behavior, and broodiness in the poultry [1, 2]. PRL plays its role through specifically combining with prolactin receptor (PRLR) in the membrane of effector cells. PRLR belongs to cytokine receptor family; it is widely distributed in various tissues as well as its binding sites [3–6]. It is discovered that the alternative splicing types of PRLR transcripts can produce various types of membrane-binding PRLR. PRLRs can be divided into three types according to their different lengths: short, medium and long type [7, 8]. The diversities of PRLR structure are mainly caused by the mutations in intracellular domains. PRLR is located on Z chromosome in chicken, and has close correlation with animal reproduction performance. As a result, it is suggested that PRLR gene could be regarded as an excellent candidate gene for animal broodiness research.

Wanxi white goose and Sichuan white goose are national protected poultry in China. However, there are many disadvantages in the process of rearing. The annual

egg yield of the Wanxi white goose is only about 25 eggs. As for the Sichuan white goose, its body is relatively small (about 3–4 kg) [9]. Wanjiang white goose, the progeny of the two-line hybridization of the Wanxi white goose and Sichuan white goose are of superior quality from their parental generations (relatively high egg yield and large body size). However, its fertility is unstable (including fertilization rate of eggs, hatching rate, average egg weight, and annual egg yield), thus the productivity is limited. Consequently, molecular biology methods have been applied to investigate the relationship between PRLR gene and egg production of the Wanjiang white goose in order to improve the laying performance.

PRLR polymorphism researches are mainly focus on livestock [10–13]. Drogemuller et al. detected polymorphism in PRLR of different lines of German pigs via PCR-RFLP [14], Vincent et al. also found polymorphism at A*lu* I enzyme site in the 457 bp fragment in pig PRLR gene [15]. In addition, Viitala et al. discovered single nucleotide polymorphism (SNP) in PRLR signal peptide can be chosen as a genetic marker to select high productive cows [11]. Nevertheless, the researches on relationship between poultry broodiness and egg laying are limited, only restricted in gene cloning, PRLR polymorphism and sequence analysis [16]. In order to select molecular genetic markers related to egg production, single strand conformation polymorphism (SSCP) was adopted to detect SNP in the genome and the sequences of polymorphisms were compared and analyzed in combination with egg laying performance, thus it will offer the evidence for marker-assisted selection (MAS) of high-yielding strain. We have succeeded in screening out the molecular markers of high production and setting up the core group of high-yielding Wanjiang white goose.

Materials and methods

Animal selection, breeding and DNA isolation

101 adult Wanjiang white geese (Anhui Sanyuan Breeding Limited-liability Co.) of the same day-old (26 week) and similar physiological characters were picked out for research, and their production indices and parameters (including fertilization rate of eggs, hatching rate, average egg weight, and annual egg yield) were recorded as biological statistics.

Blood samples were collected from these 101 Wanjiang white geese. 1 ml blood sample was collected from wing venous of each goose and centrifuged 10 min at 1500 rpm at 4°C The serum sample and cell precipitation were restored at −70°C. Genomic DNA was extracted from frozen blood sample by conventional phenol–chloroform extraction method. DNA is preserved in TE buffer at −20°C after evaluated the purity by ultraviolet spectrophotometer.

Primer designing and PCR amplification

Five pairs of primers were designed according to PRLR sequence (NCBI Accession: DQ660982) by Primer Premier 5.0. PCR reaction system: 10× buffer 2.5 ml, Mg^{2+} (25 mM) 1.5 μl, dNTP (10 mM) 0.5 μl, Taq DNA polymerase (0.5 U/μl) 0.2 μl, upper and down stream primers (10 mM/l) 1.3 μl each, and template DNA (50 ng/μl) 2.0 μl diluted with H_2O to 25 μl. PCR amplification reaction program: Pre-denaturation for 5 min at 94°C; then 30 cycles (denaturation for 45 s at 94°C, renaturation for 30 s at annealing temperature, elongation for 1 min at 72°C); finally elongation for 10 min at 72°C. Each of the primer sequences, amplified sites, expected segments and annealing temperatures are listed in Table 1.

PCR-SSCP assay

PCR products were denaturalized at 98°C for 10 min, electrophoresed on 12% non-denaturing polyacrylamide gel at 150 V for 5–7 h followed by $AgNO_3$ staining for 20 min; Genotypes were identified according to the electrophoretogram. PCR products of different genotypes were electrophoresed on 2% agarose gel. Different genotypic individuals were selected for sequencing analysis. 50μl PCR products were purified with gels purified kits (Shanghai Huashun Co. Ltd). The purified DNA and vector pMD-18T were added into linkage system. The pMD-18T was transfected into E *coli.* DH-5α and positive clones were selected for sequencing. The positive clones were sequenced in both directions on ABI PRISMTM 377.

Analysis of gene mutation and gene structure of PRLR

The hydrophilicity and secondary structure of goose PRLR were detected with DNA*Star* Protean software. Hopp–Woods [17, 18], Garnier [19, 20] and homology-based modeling methods [21, 22] were adopted to construct three-dimensional structure of PRLR. The modeling process includes two steps: Firstly, search suitable protein crystal structure data as templates in ExPDB (the crystal image database of SWISS-MODEL). Secondly, submit the sequences to SWISS-MODEL server (http://www.expasy.org/swissmod/SWISS-MODEL.html). The server will automatically return the results of homology modeling, modification and optimizing. The 3D structure quality was detected on qualitative model energy analysis (QMEAN) website (http://swissmodel.expasy.org/qmean) and we will obtain QMEAN-score [23]. The Amino acids coding

Table 1 The primers of goose PRLR gene designed for PCR-SSCP analysis

Primer	Upstream primer (5′–3′)	Downstream primer (5′–3′)	Annealing temperature Tm/°C	Expected segment Size/bp	Amplified site Size/bp
Primer 1	ATTCTCCTCCATTACCTT	CTGGGATTACCATTGTTG	57.5	400	27–427
Primer 2	GGTTTCCCTCCAACATCA	GTTCTAAGGCTACGCACT	56.0	305	358–663
Primer 3	AGTAAAAGCAAAAACAAC	AGTGTTATTTTACATGCA	56.6	319	468–787
Primer 4	ACATTGCCCCAGTTTTGG	GTAATACTGCTGGCTCAT	56.2	241	803–1044
Primer 5	TACTGAAACATAAAGAAA	GCCGTTGCTCCTGCTATCT	55.5	271	1024–1295

sequences were analyzed on ExPASy molecular biology server (http://www.expasy.org/tools/).

Statistical analysis

The frequencies of genes and genotypes were analyzed by Popgen 32, and the relationship between genotype and average egg laying performance was analyzed by least square variance.

Analysis model : $Y_{ij} = \mu + A_i + B_j + e_{ij}$

(Y_{ij}: egg production; μ: the average value of the breed; A_i: fixed effect; B_j: genotype effect of PRLR; e_{ij}: random residual effect).

The software SPSS V13.0 (SPSS Inc.,USA) was used to analyze association between them.

Results and analysis

PCR amplification

The designed 5 pairs of primers were adopted to amplify goose PRLR. The PCR products are consistent as expected, thus SSCP analysis was applied to decide band patterns. The amplified results are shown in Fig. 1.

SSCP detection

SSCP was applied to study the PCR products amplified with the five pairs of primers. It is shown that only products amplified by P4 have polymorphism (Fig. 2a–e). The SSCP band patterns are divided into three genotypes: AA, AB, and AC (Fig. 2f).

Results of sequence analysis

The PCR products of the three genotypes were sequenced and the different sequences were aligned in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with BLAST. It is discovered that the fragments amplified by P4 are located in PRLR exon 10. The sequence of AB genotype is the same as the original sequence (NCBI Accession: DQ660982). There are five mutations in the exon 10 of AA genotype: C → A at 840 bp, C → T at 862 bp, T → C at 875 bp, T → A at 963 bp, A → T at 989 bp. Thus, these mutations result in the following amino acid mutations: His → Asn, Thr → Ile, Asn → Lys, Thr → Ser, and synonymous mutation at 875 bp. There are five mutations in the exon 10 of AC genotype: G → T at 816 bp, A → T at 861 bp, C → T at 862 bp, T → C at 875 bp, A → G at 948 bp. Thus, they also cause amino acid mutations: Val → Phe, Thr → Phe, synonymous mutations at 875 and 948 bp (Fig. 3). The comparison results of the DNA sequencing oscillogram of the 3 genotypes are shown in Fig. 4.

Prediction of PRLR three-dimensional structure and motif analysis of exon 10

Garnier method of DNA*Star* Protean was applied to predict PRLR secondary structure. It is demonstrated that goose PRLR is mixing-type protein, in which α-helix accounts for

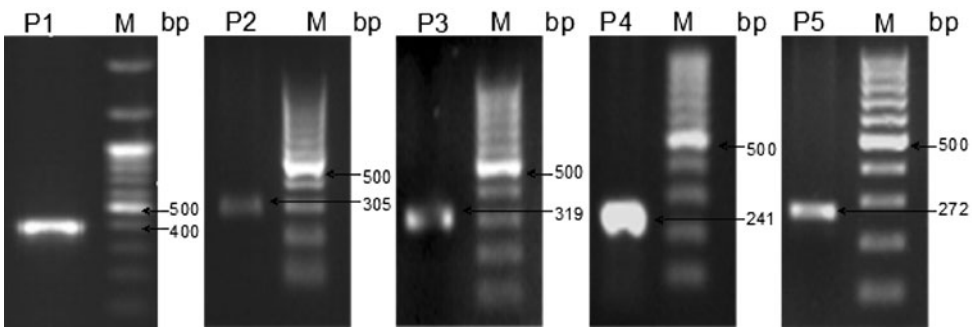

Fig. 1 PCR products of five pairs of primers *P1*: PCR products of primer 1; *P2*: PCR products of primer 2; *P3*: PCR products of primer 3; *P4*: PCR products of primer 4; *P5*: PCR products of primer 5; *M*: DNA marker

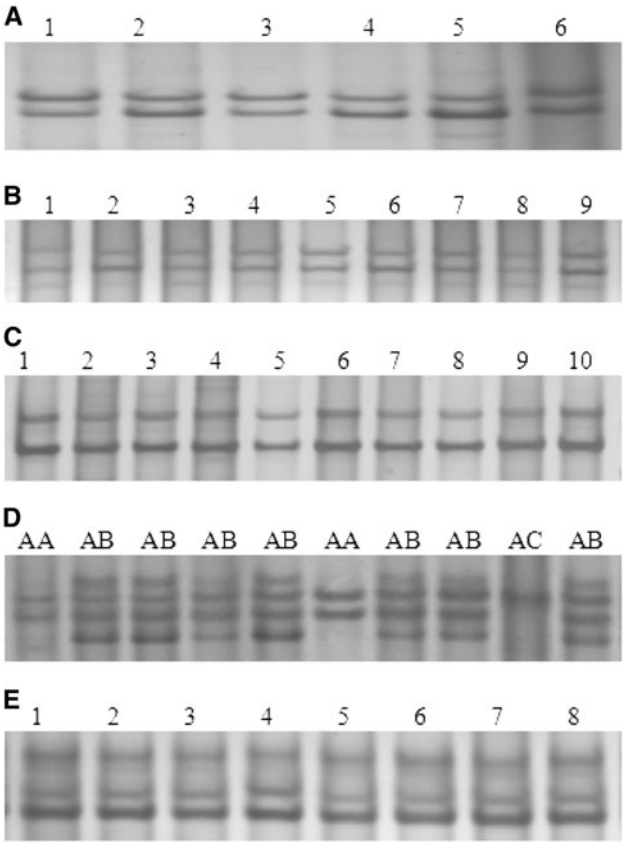

Fig. 2 PCR-SSCP patterns of three loci in Wanjiang white goose PRLR gene. **d** The P4 locus patterns in Wanjiang goose samples; three genotypes AA, AB and AC were detected at this locus, and the lanes are labeled accordingly. **a**, **b**, **c**, **e** The P1, P2, P3 and P5 locus pattern; no polymorphism was detected at this locus after electrophoresis

33.2%, β-pleated sheet 38.4%, Turn 15.9%, and Coil 12.5% (Fig. 5a). The hydrophilic region of goose PRLR distributed evenly and densely, thus PRLR protein is hydrophilic. 3D structure of goose PRLR proteins were predicted and listed in Fig. 5b. The QMEAN-score is 0.6 (Z-score: −1.45) (Fig. 5c). It is indicated that the target sequences quite match the homologous templates on the server, so the model is reliable [24]. Different genotypic motifs were predicted on the website (http://myhits.isb-sib.ch/cgi-bin/motif_scan) and the outcomes proved that there are an *N*-glycosylation site (NQSR), three casein kinase II phosphorylation sites of SIIE, SKTE, and SLMD in AA genotype; three casein kinase II phosphorylation sites of SIIE, SKTE, and TLMD in AB genotype; three casein kinase phosphorylation sites of SIFE, SKTE, and TLMD in AC genotype (Fig. 3). Isoelectric points of the three genotypes change slightly: AA 5.70, AB 5.65, AC 5.68. In addition, the mutations lead to many changes in secondary structure (Fig. 5a): Turn changes into β-pleated sheet (His → Ile), Coil changes into β-pleated sheet (Asn → Lys, Thr → Ser) in AA genotype; β-pleated sheet changes into α-helix (Val → Phe), Turn and Coil change into β-pleated sheet (Thr → Phe) in AC genotype. However, the predicted 3D structures of three genotypes are the same to each other.

Genetic diversity of exon 10 of PRLR gene

The frequencies of genotypes and alleles of PRLR exon 10 of Wanjiang white goose are listed in Table 2. It is indicated that A allele is preponderant gene due to its moderate polymorphism and high heterozygosity in the group ($0.5 > PIC > 0.25$).

Analysis of the correlation between PRLR polymorphism and egg laying performance

The least square variance and standard error of egg productivity among different genotypes are shown in Table 3. It is evident that the polymorphism is significantly correlated with egg yields, and the amount of AB genotype (64.071 ± 0.685) is obviously higher than those of AA (44.714 ± 2.232) and AC (39) genotypes ($P < 0.05$).

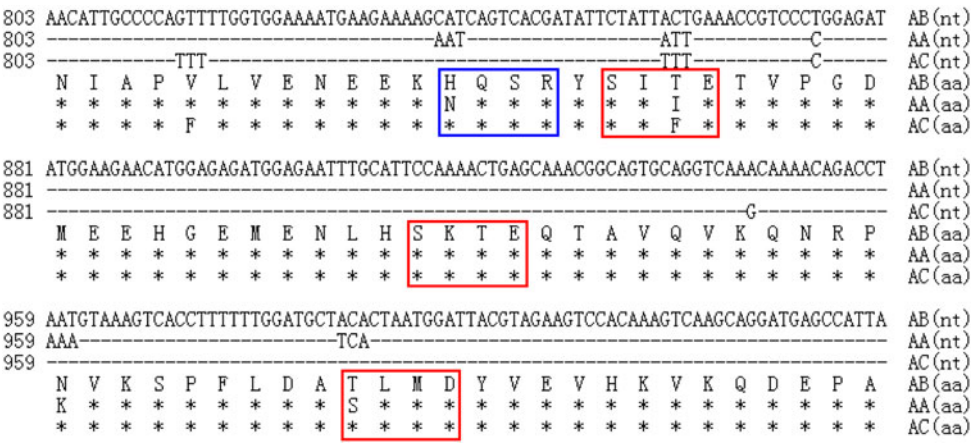

Fig. 3 Amino acid sequence analysis of PCR-SSCP pattern in goose PRLR gene. *nt* is nucleotide sequence, *aa* is amino acid sequence. *Blue box* is *N*-glycosylation site, *Red box* is casein kinase II phosphorylation site

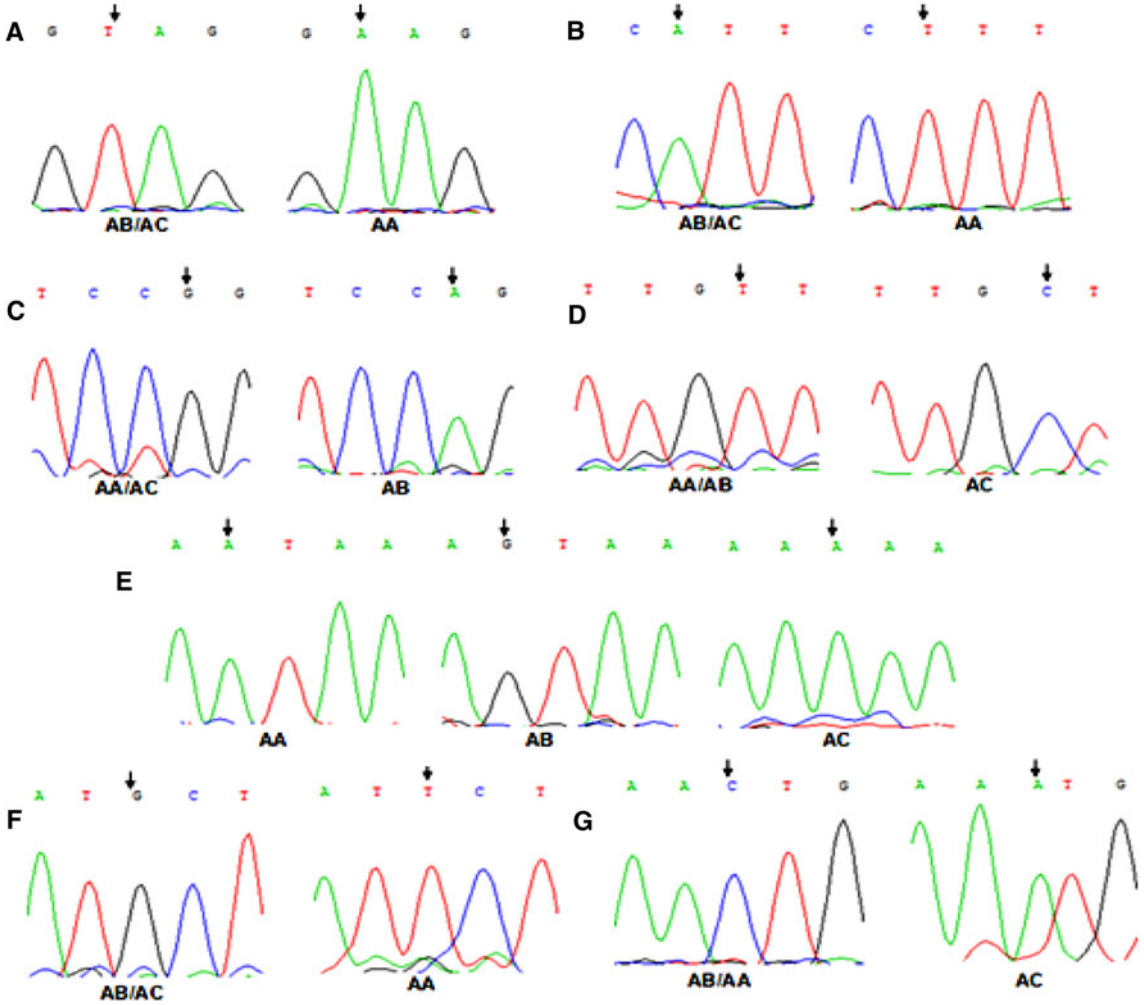

Fig. 4 Sequence comparison of AA, AB, AC genotypes of primer P4 in Wanjiang goose. **a**, **b**, **f** The differences between AB/AC genotypes and AA genotype; **c** the differences between AA/AC genotypes and AB genotype; **d**, **g** the differences between AB/AA genotypes and AC genotype; **e** the differences among the three genotypes. The changes of nucleotide positions have been marked out with *arrow*

Discussion

The relationship between goose PRLR gene polymorphism and egg productivity has never been reported. In the study, 3 genotypes including 10 SNPs were detected for the first time. Although the sample quantity is not massive, *PIC* analysis ($0.5 > PIC > 0.25$) indicated that PRLR polymorphism is abundant. High-yielding AB genotypic geese in the study could be reared to set up core groups. The mutations may impact the binding between PRL and PRLR, block signal transduction of PRL, and infect the exertion of PRL physiological effects, finally resulting in variation of production performance [25, 26]. PRLR mRNA expresses in almost all tissues, and it has been reported that PRL has more than 300 kinds of effects in different vertebrate animals. Its functions include water-salt balance regulation, growth and development, incretion, cerebrum metabolism and behavior, propagation, as well as immune regulation and protection. Up to now, most of the researches on PRLR polymorphism were focused on livestock [11–15, 27]. Although the evidences on PRLR gene mutations directly being involved in egg production are few in the poultry so far [16], current relevant studies give us some clues for further research. Dunn et al. discovered the polymorphism of PRLR gene and consider it as a molecular marker related to chicken broodiness QTL [28], Jiang et al. discovered three SNPs in PRLR gene of GreenShell and Silkie chickens, and also demonstrated the SNPs had no significant effect on broodiness and early egg productivity [29]. Nevertheless, it is suggested that mutations in exon 10 of goose PRLR is directly related to egg yielding in our research. The secreted PRL can not permeate cell membrane. The first step that PRL exerts its role is to bind with PRLR on the surface of target cell membrane. Then PRL is mediated by PRLR and signals are transducted into cells through various pathways, thus leads

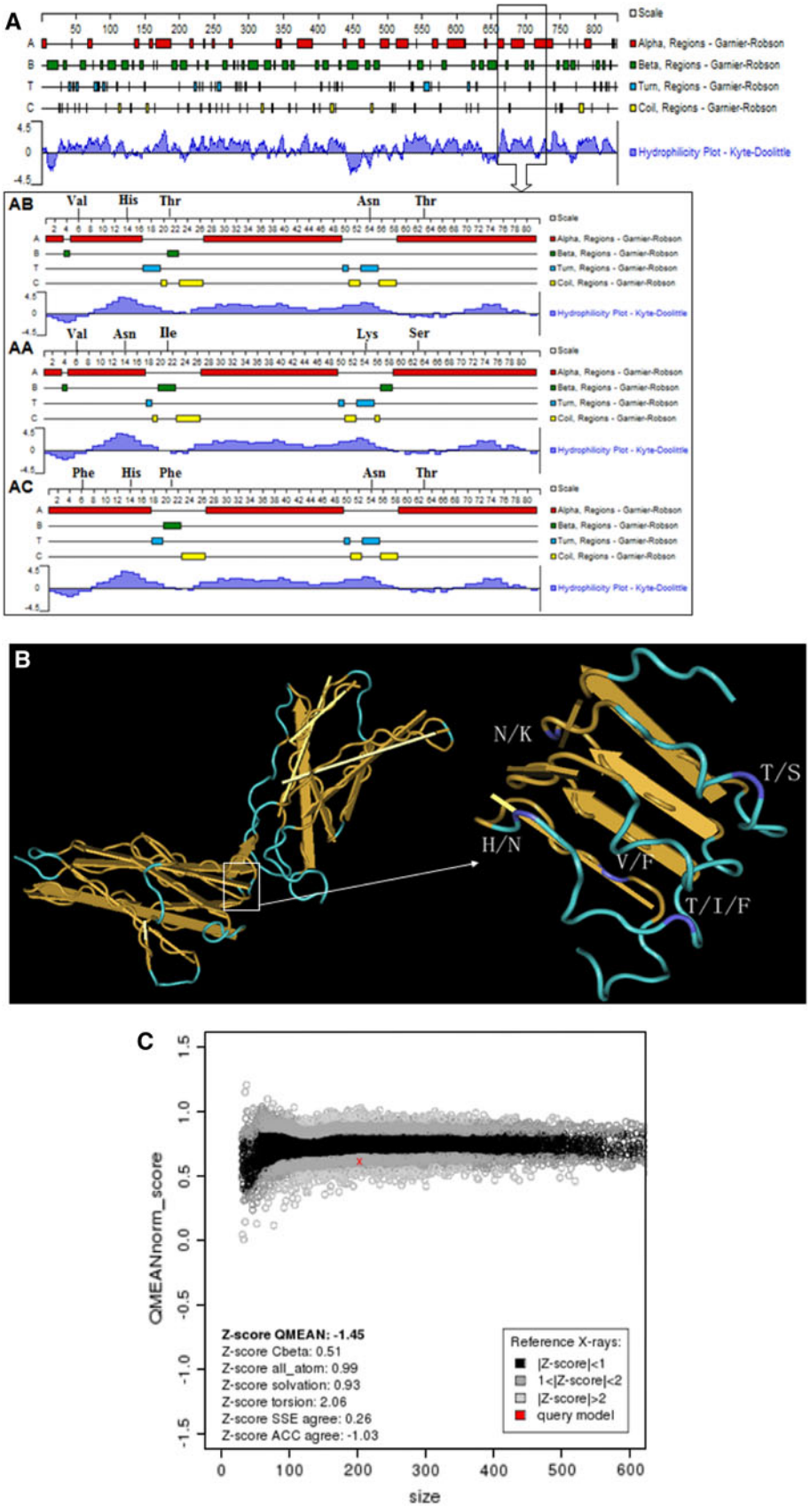

Fig. 5 Three-dimensional structure of goose PRLR prediction and assessment. **a** Prediction of the secondary structure of PRLR, and analysis of hydrophilicity. **b** Three-dimensional structure of goose PRLR gene; **c** The results from QMEAN. QMEAN score is a composite score consisting of a linear combination of six terms. The pseudo-energies of the contributing terms are given below together with their *Z*-scores with respect to scores obtained for high-resolution experimental structures of similar size solved by X-ray crystallography: *C* beta interaction energy: 1.00 (Z-score: 0.51); All-atom pairwise energy: −70.97 (Z-score: 0.99); solvation energy: −4175.50 (Z-score: 0.93); torsion angle energy: −14.14 (Z-score: 2.06); secondary structure agreement: −13.9% (Z-score: 0.26); solvent accessibility agreement: 84.7% (Z-score: −1.03); total QMEAN-score: 0.600 (Z-score: −1.45) (estimated model reliability between 0–1)

Table 2 Genotype and allele frequencies of exon 10 of PRLR gene

Genotype	Genotype frequency	Allele	Allele frequency	Polymorphism information content
AA	0.139(14)	A	0.552	
AB	0.815(86)	B	0.408	0.4243
AC	0.010(1)	C	0.040	

Note: The numbers in the brackets are the individuals that belong to respective genotypes

Table 3 Least squares mean and standard error for litter size of different genotypes of exon 10 of PRLR gene in Wangjiang white goose

Genotype	Number of samples	Average number of egg production
AA	14	44.714 ± 2.232^{a}
AB	86	64.071 ± 0.685^{b}
AC	1	39.000

Note: Least squares means with the different superscripts for the same pair of primers differ significantly ($P < 0.05$)

to series of physiological responses. It is possible that mutations in exon 10 can influence the exertion of PRL physiological effects, resulting in the variation of animal production and follicular propagation pattern. Further researches are needed on this possibility.

Mao et al. found three abridged PRLR [(+)BoxlA, (+)BoxlB, (-)Boxl] in chicken testis [30]; moreover, Tanaka et al. discovered another two abridged PRLR in chicken testis [(+)BoxlC and (+)BoxlD]. Compared with full-length PRLR gene, they only contain intracellular regions [31]. In the study, the three-dimensional structure of PRLR is extremely similar to the extracellular domain of related templates (PDB ID: 3EW3, 3D48), thus goose PRLR is believed to be long isoform. The long isoform PRLR activates many kinases including Jak2/Stat5 (signal transducer and activator of transcription 5) [32], Src kinase [33, 34], phosphatidylinositol-3-kinase (PI3K)/AKT [35], mitogen-activated protein kinase (MAPK) [36] and Nek3-vav2-Rac1 pathways [37]. These signal events induce several PRL-responsive genes being involved in cell proliferation and differentiation [38, 39]. Several key functions for PRLR have been clarified from studies of transgenic and knockout model mice [40]. One of the obvious reproductive functions of PRL is to maintain the ovarian corpusluteum (CL) and progesterone production via binding with the long isoform PRLR [41, 42]. The ovulated follicle enter a CL through a process termed as luteinization, then the corpus luteum is to stimulate both estrogen receptor (ER) and LH receptor (LH-R) expression [43]. In our experiment, it is discovered that the mutation of PRLR leads to the transformation of amino acids, accordingly leading to the change of protein structure. It will possibly influence the combination of goose PRLR with the ligand; consequently affect the secretion and inspiration of goose estrogen and change the ovulation amount at last. We will focus on the downstream signals of PRLR in further researches.

We also analyzed PRLR exon 10 through PROSITE database Motif. It is revealed that there are three sites of phosphorylation sites of casein kinase II on each of the three genotypes, and 2 SNPs just lie on two of the phosphorylation sites of casein kinase II (SITE/SIIE/SIFE, TLMD/SLMD), respectively. It is well known that casein kinase II(CK2) belongs to Serine/Threonine protein kinases and exist widely in eukaryote bodies. It plays an important role in the regulation of gene expression [44]. Phosphorylation is one of the most important modification forms of regulation after translation [45]. It changes the protein structure, activity, and interaction with other molecules when it happens on the amino acid side chain of protein, and exerts significant roles in many biological processes, such as signal transition, gene expression, and cell division. Ahmed et al. found that CK2 is sensitive to androgen (A) regulation when they studied A regulating the PKs of prostate (RVP) growth in rat ventral [46]. The 2 mutations detected by us can probably stimulate the two sites of phosphorylation sites of casein kinase II, thus it may play an important role in activation of PRLR signal transmitting. Combining with the fact that the mutation on the two sites can cause significant decrease of egg production in AA and AC genotypes, it can be considered that the phosphorylations on this site may have ill effect on egg yielding. Besides, we found a His → Asn missense mutation in AA genotype and *N*-glycosylation site (NQSR) occurrence. This mutation may possibly induce the NQSR activation of AA genotypic PRLR. Suzuki et al. analyzed *N*-glycosylation types of chicken blood plasma IgG and its specific glycosylation site through MALDI-TOF, and discovered the glycosylation on this site can influence the translational regulation of immune protein [47]. The effect of *N*-glycosylation site in AA genotype on the expression of goose PRLR receptor is also needed to clarify the underlying implications.

In summary, although, the former interpretations present unclear facts lying in exon 10 of PRLR gene, they will provide us a meaningful clue for new variety goose breeding. Our results demonstrated that goose PRLR is an excellent candidate gene in the breeding of egg yield.

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