Original Article

Investigation of single nucleotide polymorphism in *TSH-β* and *CaSR* associated with body weight in Korean native chickens (Gray Brown)

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ABSTRACT This study identified single nucleotide polymorphisms (SNPs) that affect the body weight of chickens. Analysis of body weight showed that the Cornish breed had the highest body weight, and the Korean native chicken (Gray Brown) had the lowest body weight. *TSH* is composed of an α -subunit and a β -subunit, and the *TSH-β* gene encoding the β -subunit has been reported to be associated with obesity. In chickens, it is located on chromosome 26 and is reported to be associated with growth. The calcium-sensing receptor gene (*CaSR*) plays a role in the regulation of extracellular calcium homeostasis and is responsible for calcium absorption in the urinary tract, which affects the eggshell quality in poultry. It was shown that *TSH-β* was strongly correlated with weight in Cornish and Korean native (Gray Brown) chickens, particularly in those with the CC trait. However, *CaSR* showed no association with body weight in poultry; it was associated with calcium and the eggshell. Thus, selection for *TSH-β* can be used to produce individuals with more favorable traits in terms of body weight.

Keywords: *CaSR*, Cornish, Korean native chicken (Gray Brown), SNP, *TSH-β*

INTRODUCTION

The Korean native chicken (KNC) has chewy meat due to its high collagen content. The meat contains abundant sulphur-containing amino acids, such as methionine and cystine, which impart flavor; it is also favored for its juiciness and tenderness.

Since the CBD Convention in 2004, Korea has registered 15 unique animal genetic resources, 120 breeds, or endogenous breeds in the Domestic Animal Diversity

Information System (DAD-IS), an international breeding system. Forty species, including KNC, are registered (Food and Agriculture Organization of the United Nations, 2019). Since the start of the KNC restoration project, several studies have been conducted on this breed, and many meaningful results have been reported. Among these, representative studies on the genetic characteristics of KNCs include studies on the genetic composition and genetic diversity of the population using SNP markers, MS markers, or mtDNA D-loops (Hoque et al., 2009; Lee

et al., 2010; Cho et al., 2011; Lee et al., 2011; Cho et al., 2014; Seo et al., 2015a,b; Choi et al., 2017); hair color-related genetic mutations (Heo et al., 2011; Park et al., 2013; Choi et al., 2014); the frequency and developmental patterns of plumage fading (Sohn et al., 2012; Sohn et al., 2013; Bang et al., 2018); immune and stress responses (Jung et al., 2009; Hoque et al., 2011; Sohn et al., 2014; Sohn et al., 2015); and genetic parameters for productivity and economically favorable traits and practical production using breeding combinations (Sang et al., 2005; Park et al., 2011; Kang et al., 2012a,b; Kim et al., 2012a,b; Lee et al., 2013; Lee et al., 2014; Yoo et al., 2015; Jin et al., 2017).

Although there have been various studies on KNCs (Gray Brown), there has been no concrete report on their productivity or comparison of productivity between species. However, they are not economically viable because of their slow low growth rate, long breeding period, and low weight (Kwon et al., 1995; Park et al., 2010; Kang et al., 2012a,b). Therefore, improvement of growth-related traits would enhance the market value of KNCs.

Recently, extensive studies have been conducted for the development of molecular breeding techniques to improve growth-related traits in chickens. Genes such as growth hormone (*GH*) (Seo et al., 2001), growth hormone receptor (*GHR*), growth hormone secretagogue receptor (*GHSR*) (Zhang et al., 2009), insulin-like growth factor-I (*IGF-I*) (Li et al., 2009), insulin-like growth factor binding protein-2 (*IGFBP-2*) (Leng et al., 2009), insulin (*INS*) (Lei et al., 2007), leptin receptor (*LEPR*) (Choi et al., 2003), and thyroid-stimulating hormone beta subunit (*TSH-β*) (Lei et al., 2007) are associated with growth. The growth-related traits are manifested by the various actions of multiple organs.

The thyroid plays a very important role in growth by regulating hormone metabolism in the body through the secretion of thyroid hormone (*TH*) (Ga et al., 1999). A recent report showed that thyroidectomy reduces the growth rate significantly and results in excessive accumulation of fat in the neck, back, chest, and abdominal cavity (Ga et al., 1999). The manifestation and secretion of *TH* is induced by thyrotrophin, a thyroid stimulating hormone (*TSH*), which is composed of the α -subunit and β -subunit. The *TSH-β* gene, which encodes the β -subunit, is located on chromosome 1 and is related to obesity (Nie et al., 2005; Lei et al., 2007); it is also found on chromo-

some 26 and is related to growth (Lei et al., 2007).

The growth of male chickens involves various factors and hormones, including testosterone and TH (Boertje et al., 2019). These two hormones support growth. During the growth of *Emberiza bruniceps* feathers, thyroid activity is strongly influenced by gonadal hormones (Pati et al., 1986). Breeders often administer testosterone to poultry for faster growth, longer crowing, and better feather color. Further, the g.1031 G>C marker in the intron region is related to the weight of Cornish.

The calcium-sensing receptor gene (*CaSR*) plays a role in regulating extracellular homeostasis of calcium and secretion of hormones and absorption of calcium in the urinary system (Hough et al., 2004; Cifuentes et al., 2005; Pidasheva et al., 2005; Yun et al., 2007). Decreased calcium concentration in the body leads to the secretion of parathyroid hormones by the parathyroid gland, decreasing the secretion of calcium ions from the kidneys and promoting the absorption of calcium ions in the small intestine. It also stimulates the secretion of growth hormone by the pituitary gland (Hall et al., 1985) and enhances progesterone secretion (Veldhuis and Klase, 1982). These results suggest that the calcium concentration in the body has major effects on hormones related to growth and reproduction.

The *CaSR* gene has been extensively studied in recent years. The polymorphism in the A986S variation site in the *CaSR* gene regulates calcium concentration and is related to diseases such as osteoporosis, hypertension, and obesity (Felderbauer et al., 2005; Yun et al., 2007). *CaSR* is a homodimeric complex located in the cell membrane and belongs to the class C G-protein-coupled receptor (*GPCR*) (Jensen and Bräuner-Osborne, 2007). It can sense subtle changes in extracellular calcium concentration and thus mediate PTH secretion to maintain calcium homeostasis by regulating intestinal absorption, bone storage and exchange, and renal reabsorption (Conigrave, 2016).

In addition, *CaSR* mediates various physiological and pathophysiological processes, such as ion channel activity, gene expression, inflammation, proliferation, differentiation, and apoptosis by inducing downstream signaling cascades. Chicken *CaSR* is 79 and 84% homologous with human *CaSR* at the nucleotide and amino acid levels, respectively. In situ hybridization has revealed that *CaSR* is present in the parathyroid, kidney, brain, and small intestine (Diaz et al., 1997). Yarden et al. (2000) showed

that *CaSR* expression in the parathyroid gland is inversely associated with changes in plasma calcium concentration. Chickens fed vitamin D-deficient diets with low *CaSR* expression were characterized by the highest concentration of PTH, whereas high *CaSR* gene expression levels in vitamin-D-depleted chickens were associated with low PTH content in the parathyroid gland (Yarden et al., 2000). The results indicate that the functional *CaSR* in chickens possesses characteristics similar to those of mammalian *CaSR* and may play a significant role in avian calcium homeostasis.

The regulatory capacity of this gene on calcium concentration makes it a promising candidate for the improvement of economic traits.

The KNC is recognized for its value as a genetic resource; however, trait improvement is required to increase its value as an industrial resource. Specific genetic characteristics should be identified for such trait improvement. Thus, through the investigation and analysis of genetic variations in *TSH-β* and *CaSR* genes, this study aims to provide basic molecular breeding data for the production of KNC with improved growth traits.

MATERIALS AND METHODS

Animals and phenotypes

Genomic DNA was extracted from Cornish ($n = 192$), KNC (Gray Brown) ($n = 192$), and Rhode Island Red ($n = 192$) with different body weights (Table 1). In the breed-

ing environment, the temperature inside the windowless poultry house was maintained at 20–25°C, and the humidity was approximately 70–85%. An incandescent light bulb (60 W) is installed for the lighting time, and an automatic lighting switchgear to automatically turn off the lights for an average of 8 h per day. Feed for poultry was fed freely. For the negative control, the poultry were provided with a free supply of water passed through an ultraviolet sterilizer (Dynamics, M600, USA).

SNP genotyping

TSH-β and *CaSR* genes were retrieved from the reference sequence data (accession no. AY341265, XM_416491). The c.319 G>T SNP is a non-synonymous SNP that is substituted from serine to alanine in the 963rd amino acid position. Primer sequences were designed using the Primer3 program, and their information is shown in Table 2. For genotyping of g.1031 G>C and c.319 G>T SNPs within the *TSH-β* and *CaSR* genes, the single base extension (SBE) method was performed according to the protocol of the SNaPshot ddNTP Primer Extension Kit (Applied Biosystems, Foster City, CA).

Primer extension reactions were performed using the SNaPshot dNTP Primer Extension Kit (Applied Biosystems, Foster City, CA, USA). For primer extension, exonuclease 1 and shrimp alkaline phosphatase (SAP) were added to the reaction mixtures. Samples were cultured at 37°C for 1 h and then inactivated at 72°C for 15 min. PCR products were analyzed using the Genescan 120 LIZ standard and

Table 1. Mean and standard deviations of body weight at different ages in chicken populations

Trait	Cornish	Korea Native Chicken (Gray Brown)	Rhode Island Red
WT8	1800 ± 124.4	618 ± 74.4	805 ± 100.8
WT12	2264 ± 187.9	918 ± 107.2	1179 ± 95.5
WT16	2857 ± 191.1	1122 ± 156.5	1506 ± 135.2

WT8, body weight at 56 days of age; WT12, body weight at 84 days of age; WT16, body weight at 112 days of age.

Table 2. Information on SNP markers associated with *TSH-β* and *CaSR*

Gene	SNP	Chromosome	Position		Sequence	Tm	Product size
<i>TSH-β</i>	g.1031 G>C	26	Intron	F ¹	CGTTGTTGCGGTAGTAGGTG	60	521
				R ²	GCGTCAGGAAGAGCTCATTG		
				E ³	TGTAGTCAGTGTAGGTGCTT		
<i>CaSR</i>	c.319 G>T	1	Exon ¹	F	CAGTGCCTACCATGAGTC	59	468
				R	TGACCCACAGTTGTAACCAGA		
				E	TAAGTGAGCTGTCTAAATTG		

¹Forward primer sequence, ²Reverse primer sequence, ³Extension primer sequence.

HiDi formamide (Applied Biosystems, Foster City, CA), followed by denaturation at 95°C for 5 min. Electrophoresis was performed using an ABI PRISM 3500XL Genetic Analyzer and analyzed using GeneMapper v.4.0 software (Applied Biosystems, Foster City, CA).

Statistical analyses

Associations between body weight and individual SNPs for the 576 samples were analyzed using the following mixed analysis of covariance (ANCOVA) model using SPSS v19.0 (SPSS Inc., Chicago, IL, USA).

$$Y_{ijk} = \mu + B_i + P_j + G_k + \beta \text{age} + e_{ijk}$$

where Y_{ijk} is a phenotype, μ is the overall mean, B_i is a breed effect (i = Cornish, Korea Native Chicken [Gray Brown]), P_j is the fixed effect of calving place j , G_k is a fixed effect of genotype k , β is a regression coefficient, age is a covariate for age in days at slaughter, and e_{ijk} is a random residual assumed to have independent and identical normal distribution.

RESULTS

Genotype and allele frequency

The primer sequences of *TSH-β* and *CaSR* genes were designed using the Primer3 program from the reference data (Accession no. AY341265, XM_416491) from NCBI, and their information is shown in Table 2. For genotyping g.1031 G>C and c.319 G>T within the *TSH-β* and *CaSR* genes, the SBE method was performed according to the protocol of the SNaPshot ddNTP Primer Extension Kit (Applied Biosystems, Foster City, CA).

As shown in Table 1, Cornish chickens showed overall higher body weight than the KNC (Gray Brown) and Rhode Island Red species. At 112 days of age, the Cornish chickens were 2,857 g, which is approximately twice as large as 1,122 g for KNC (Gray Brown). The genotype and allele frequencies of the two selected SNPs were calculated for each of the three chickens (Table 3). Genotype distribution for the g.1031 G>C SNP in each breed indicated that the CC genotype was present in the Cornish (23.9%) and KNC (Gray Brown) (11.9%), and the CC genotype was present in Rhode Island Red (0%). For the c.319 G>T SNP, the GT, and TT genotypes were identified in Cornish and KNCs (Gray Brown), except in Rhode Island Red.

Table 3. SNP frequency in each breed and candidate gene

Gene	SNP	Genotype and allele	Genotype and allele frequency (%)		
			Cornish	KR	RIR
<i>TSH-β</i>	g.1031 G>C	CC	0.239	0.119	–
		CG	0.518	0.584	–
		GG	0.243	0.297	1.000
<i>CaSR</i>	c.319 G>T	GG	0.345	0.120	1.000
		GT	0.217	0.650	–
		TT	0.438	0.230	–

Table 4. Association of g.1031 G>C and c.319 G>T SNPs with body weight in Cornish chickens

Gene	SNP	Traits (g)	Genotype LSMEAN ± SE			p-value
<i>TSH-β</i>	g.1031 G>C		CC	CG	GG	
		WT8	1915.75 ± 108.76 ^b	1895.69 ± 118.47 ^{ab}	1795.75 ± 108.76 ^a	0.019
		WT12	2374.35 ± 187.27 ^b	2169.79 ± 177.97 ^a	2097.99 ± 167.27 ^a	0.024
		WT16	3017.72 ± 210.17 ^b	2987.91 ± 195.41 ^{ab}	2796.12 ± 195.39 ^a	0.005
<i>CaSR</i>	c.319 G>T		GG	GT	TT	
		WT8	1795.57 ± 112.15	1816.93 ± 126.59	1812.43 ± 109.55	0.849
		WT12	2247.35 ± 187.17	2279.65 ± 167.67	2198.65 ± 157.77	0.788
		WT16	2807.69 ± 187.68	2887.49 ± 197.78	2903.59 ± 177.68	0.874

WT8, body weight at 56 days of age; WT12, body weight at 84 days of age; WT16, body weight at 112 days of age.

^{a,b}Means with different superscripts within the same column are significantly different ($p < 0.05$).

Table 5. Association of g.1031 G>C and c.319 G>T SNPs with body weight in Korean native chickens (Gray Brown)

Gene	SNP	Traits (g)	Genotype LSMEAN ± SE			p-value
<i>TSH-β</i>	g.1031 G>C		CC	CG	GG	
		WT8	661.84 ± 62.82 ^b	651.94 ± 71.52 ^b	588.74 ± 65.72 ^a	0.001
		WT12	954.11 ± 94.27 ^b	939.48 ± 101.17 ^b	891.21 ± 89.37 ^a	0.004
<i>CaSR</i>	c.319 G>T	WT16	1297.79 ± 142.38 ^b	1127.24 ± 151.48 ^{ab}	1091.57 ± 149.79 ^a	0.019
			GG	GT	TT	
		WT8	618.74 ± 62.12	629.94 ± 92.58	601.14 ± 78.59	0.785
		WT12	932.49 ± 99.87	912.59 ± 104.87	929.19 ± 90.87	0.984
		WT16	1219.64 ± 141.98	1127.91 ± 121.58	1297.47 ± 101.71	0.721

WT8, body weight at 56 days of age; WT12, body weight at 84 days of age; WT16, body weight at 112 days of age.

^{a,b}Means with different superscripts within the same column are significantly different ($p < 0.05$).

Effect of SNP genotype in body weight

We analyzed body weight depending on the g.1031 G>C and c.319 G>T SNPs in three chicken breeds. The g.1031 G>C SNP was associated with a variety of body weights in Cornish and KNCs (Gray Brown) ($p < 0.05$, Tables 3 and 4). In particular, the g.1031 G>C SNP was associated with all the phenotypes at 56 days of age, 84 days of age, and 112 days of age. For Rhode Island red, only one genotype (GG) was identified and excluded from the analysis.

As shown in Table 4, body weight in the g.1031 G>C SNP was observed to be higher at 1,915 g, 2,374 g, and 3,017 g in the genotypes of CC ($p < 0.05$). A similar trend was found in the KNC (Gray Brown), showing a significant difference of 661 g, 954 g, and 1,297 g compared to the CC genotype (Table 5). In the *CaSR* gene, it was confirmed that there was no significant difference in body weight because it is a gene that affects calcium or eggshell.

DISCUSSION

The thyroid is an organ that plays an important role in growth by regulating hormones in the body. Thyroid-stimulating hormone (*TSH*), a hormone that stimulates the thyroid, is secreted by the anterior pituitary. This consists of the α -subunit and β -subunit; the α -subunit has amino acid sequences, which correspond to gonadotropins such as *LH*, *FSH*, and *hCG*.

Various characteristics of the β -subunit have been reported to influence the specific activity of *TSH* (Cho, 2007). The *TSH-β* gene in chickens is located in the 3.9 Mbp area of chromosome 26, and QTL D/B information displays various weight-related QTLs (<http://www.animal-genome.org>). According to a report by Nadaf et al. (2009), a QTL related to body weight at 84 days of age is located

in the 2 to 4 Mbp area of chromosome 26, and this QTL area includes the *TSH-β* gene (3.9 Mbp). Nearby, there is also a QTL (2.4 to 3.4 Mbp) related to body weight at 56 days of age.

Therefore, it is considered that the *TSH-β* gene has a very close connection with growth traits. There are many different genes and genetic variations in specific traits, and these genetic structures have diverse effects on specific traits. Moreover, genetic structures of genetic variations can be identified through linkage disequilibrium (LD) analysis, and the characteristics of this LD block structure can be classified depending on the variety (Gautier et al., 2007; Amaral et al., 2008; Megens et al., 2009).

When analyzing the variation region of the *TSH-β* gene in this study, genetic characteristics depending on the varieties were revealed. It was identified that all the analyzed individuals of Rhode Island Red had a GG genotype and that Cornish and KNC had a significant correlation ($p < 0.05$) between genotype and weight.

According to a report by Seo et al. (2013), g.1031 G>C applied in this study was measured by weight. Further, comparative analysis showed a correlation with body weight only at the age of 150 days in Cornish chickens, and there was no correlation in conventional chickens. However, based on the results, Cornish and native chickens showed a strong correlation between the g.1031 G>C marker and body weight. Although the correlation analysis results for the Cornish chickens were the same, different results were obtained for conventional chickens in this study as these were KNCs (Gray Brown) restored in Gyeongsangbuk-do. Although it can be broadly classified as the same breed as conventional chickens, the phenotype and genetic characteristics of the native chicken breed used in this study are different from those of the

native chicken provided by the National Institute of Live-stock Science. Further, in the previous studies analyses were carried out at 150 days and 270 days. In this study, analyses were carried out at 56 days, 84 days, and 112 days; this may have contributed to the difference in the results obtained.

It is believed that the *TSH-β* gene, particularly the g.1031 G>C variation region, is closely correlated with growth traits. Furthermore, it is anticipated that G, an allele, can be used as a biological marker for the selection of growth-related and other economically favorable traits. Although *TSH-β* is a gene closely linked with growth traits, there have not been many studies on its economic use. Therefore, varied approaches and studies on the utilization of *TSH-β* are expected to play a crucial role in the improvement of economic traits in KNCs.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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AUTHOR CONTRIBUTIONS

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