Genetic effect of an A/G polymorphism in the *HSP70* gene on thermotolerance in chicken

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ABSTRACT. Studying thermotolerance is important for the prevention of thermostress in chickens. This study aimed to analyze the effect of mutations in the heat shock protein 70 (*HSP70*) gene on chicken thermotolerance. The C.-69A>G SNP in the 5'-flanking region of the *HSP70* gene was genotyped in Lingshan and White Recessive Rock (WRR) chickens. Association of this SNP with thermotolerance traits revealed it to be significantly associated with CD4+/CD8+, and potentially associated with heterophil-to-lymphocyte ratio in WRR chickens exposed to thermoneutral temperature (15°C). Online prediction detected a putative myeloid zinc finger protein 1 binding factor in the C.-69A>G mutation. Under acute thermostress, mRNA levels of *HSP70* in individuals with different C.-69A>G genotypes varied in the heart, leg muscle, and liver tissues. The HSP70 protein was expressed at higher levels in individuals with the GG genotype than in those with the AA genotype. In heart and liver, protein expression of HSP70 in individuals with the GG genotype was significantly higher than in those with the AA genotype. In leg muscle, protein expression

was higher in birds with the GG genotype than in those with the AA and AG genotypes. Luciferase activity of the GG genotype was significantly higher than that of the AA genotype, suggesting that the C.-69A>G SNP regulates *HSP70* gene expression. These results indicate that the C.-69A>G SNP in the 5'-flanking region of the *HSP70* gene might affect chicken thermotolerance and that the GG genotype might be advantageous for the prevention of thermostress.

INTRODUCTION

Heat stress is one of the most challenging environmental conditions affecting commercial poultry. In the summer, high temperatures can lead to increased mortality, reduced feed intake, growth rate, and egg shell quality, as well as lower weight gain and egg production in domestic birds (Cahaner et al., 2008; Melesse et al., 2011). Consequently, substantial economic losses in the poultry industry are often observed (Rozenboim et al., 2007). Additionally, pale, soft exudative-like changes in meat quality have been observed in broilers exposed to acute or chronic heat stress, pre-slaughter (Sandercock et al., 2001; Lu et al., 2007). Like all living organisms, chickens possess mechanisms that protect them against environmental challenges. Heat shock proteins (HSPs) are a family of proteins synthesized in response to physical, chemical, or biological stresses, including heat exposure (Staib et al., 2007). HSPs are a group of evolutionarily conserved proteins that are conventionally classified according to their size, which ranges from 10 to 150 kDa (Benjamin and McMillan, 1998). HSPs act as molecular chaperones during protein assembly and disassembly (Hartl and Hayer-Hartl, 2002), protein folding and unfolding (Hartl, 1996), protein translocation (Ryan and Pfanner, 2001; Zhang et al., 2006), and the refolding of damaged proteins **(**Marques et al., 2006). HSPs play important roles in the protection and repair of cells and tissues. In addition, the overexpression of one or more *HSP* genes helps to protect cells against subsequent stress (Zhang and Sarge, 2007).

Some studies have shown that 3,5,3'-triiodothyronine (T_3) and corticosterone may be considered as physiological indices for evaluating thermotolerance traits. Hangalapura et al. (2004) showed that 1 day of cold stress significantly enhanced T_3 levels; however, there was no effect on T_3 levels when the birds were cold stressed for a long period. Bedanova et al. (2007) and Zulkifli et al. (2009) found that corticosterone plasma concentrations were elevated in broilers that were shackled and heat stressed, respectively. In addition to T_3 and corticosterone, other physiological indices (e.g., CD3+, CD4+, and CD8+T) may also be used to evaluate thermotolerance traits. Cluster of differentiation (CD) is used to identify and investigate cell surface molecules present on white blood cells, and can also be found in almost any type of cell in the body, thus providing targets for the immunophenotyping of cells. Khajavi et al. (2003) found that heat stress decreased the level of CD4+ and CD8+ expressing T cell levels, while feed restriction elevated CD4+ and decreased CD8+T cell levels.

Previous studies have shown that HSPs are expressed in a time- and tissue-specific manner, which is often variable. Using western blot analysis, Leandro et al. (2004) detected the HSP70 protein in all embryo tissues (Hubbard strain and Ross 308 strain). Brain tissue was found to contain 2-5-fold higher levels of HSP70 when compared to other tissues in either cold

or heat stressed embryos. In addition, Zhen et al. (2006) found that the expression of *HSP70* mRNA in the brain was higher than that in the liver and leg muscle. Jost et al. (2012) noted that changes in HSP70 expression varied in decapod crustaceans and whiteflies, and that HSP70 plays an important role in the thermotolerance of these species.

Although numerous studies have evaluated the function of HSP70, no studies have investigated the association of polymorphisms in this gene with thermotolerance traits in chickens. Therefore, the aim of the current study was to analyze the genetic effect of the single nucleotide polymorphism (SNP) C.-69A>G in the *HSP70* gene on chicken thermotolerance. In this study, we studied the C.-69A>G site in the 5'-flanking region of the *HSP70* gene and its association with thermotolerance traits under different conditions. Finally, luciferase expression assays were performed for different genotypes of this mutation.

MATERIAL AND METHODS

Animal samples and the design of thermostress experiment

A total of 330 female chickens from 2 chicken breeds, including 160 thirty-four-week-old White Recessive Rock (WRR) and 170 thirty-week-old Lingshan (LS) chickens, were obtained from Guangdong Wen's Southern Poultry Breed Company. Blood samples (5 mL) were obtained at 35° and 15°C, and were then used to isolate genomic DNA using a phenol-chloroform extraction method, and index determination of thermotolerance traits [T_3, heterophil-to-lymphocyte ratio (H/L), corticosterone, CD3+, and CD4+/CD8+], which measured heat resistance of chicken.

A total of 100 female birds, including 50 WRR and 50 LS birds, were obtained from Guangdong Wen's Southern Poultry Breed Company. At 1 day of age, chickens were housed in large coops (20 birds per coop) and the coops were placed in a controlled climate chamber and allowed 1-week to acclimatize to their new surroundings and to recover from any environmental stress. During this period, the broilers were reared under standard conditions. The relative humidity of the chamber was maintained at $60 \pm 10\%$. The room temperature (RT) was maintained at $34° \pm 1°C$ from days 1 to 3. As the chickens grew, the RT was decreased gradually and maintained at $25° \pm 1°C$ by controlled ventilation and heating until day 7. At 8 days of age, the RT sharply increased from $25° \pm 1°C$ to $40° \pm 1°C$. The temperature was routinely monitored via measurements taken from the center of each coop. The relative humidity of the chamber during the thermostress condition was maintained at $50 \pm 5\%$. The birds were given access to a commercial broiler feed and water *ad libitum* throughout the period of thermostress. Upon termination of thermal treatment, blood samples (2 mL) were repackaged into blood collection tubes for genomic DNA isolation and each bird was sacrificed by decapitation. Following exsanguination, the birds were manually eviscerated, and the heart, liver, and leg muscle were quickly dissected and placed into 2-mL tubes. The tubes were placed in liquid nitrogen, and then stored at -80°C until subsequent extraction of total RNA. The experiment was undertaken in accordance with, and approved by, the Animal Care Committee of South China Agricultural University (Guangzhou, China) (approval ID: SCAU#0011).

Measurements of thermotolerance traits

T_3 and corticosterone were measured by enzyme-linked immunosorbent assay

(ELISA) following the manufacturer instructions. The H/L ratio was counted by the People's Liberation Army Hospital 458 (Guangzhou, China), and the expression levels of CD3+, CD4+, and CD8+T were analyzed using flow cytometry.

Primer design, PCR amplification, and polymorphism identification

PCR primers for the *HSP70* (GenBank accession No. J02579) and *β-actin* genes were designed based on reference sequences. Information on these primers is presented in Table 1. P1 primers were used to amplify 654-bp fragments of the *HSP70* gene. P2 (*HSP70* mRNA primers) and P3 (*β-actin* primers) primers were used for fluorescent quantitative real-time PCR (qRT-PCR).

Table 1. Primers used in this study.

	Primers sequences (5'-3')	Annealing temperature (°C)	Products (bp)
P1	F: ATTGTCCTTAGCGTTCT	62	654
	R: TCTTCATCTCACCCTTGTAC		
P2	F: GTAGAAGAGTTCAAGCGTAAG	57	166
	R: TGGAGGTGTAGAAGTCAATG		
P3	F: CTCCCCCATGCCATCCTCCGTCTG	57	179
	R: GCTGTGGCCATCTCCTGCTC		
A-69G	F: CGGggtaccCGATCTGGCTGCAATCTACGGGA	55	310
	R: CCGctcgagGATGATAGATTCCTCTTGGTCAG		

Underlined letters represent protective bases. Lower-case letters represent restriction enzyme sites.

PCR was performed in 25-μL volumes containing 50 ng genomic DNA, 200 μM diethylnitrophenyl thiophosphates (dNTPs), 1X buffer, 2 mM $MgCl_2$, 1 μM each primer, and 1.5 U Taq DNA polymerase (Shanghai Biological Engineering Company, China). The following PCR protocol was used to amplify the *HSP70* gene: initial amplification by incubating the PCR mixture at 94°C for 3 min, following by 32 cycles of incubation at 94°C; annealing temperatures of 62°C for 30 s and 72°C for 30 s; and a final incubation at 72°C for 4 min. PCR products were subjected to 1.2% agarose gel electrophoresis and visualized using a TFM-40 Ultraviolet Transilluminator (UVP Company, Cambridge, UK) by ethidium bromide staining. DNA sequencing was performed by the dideoxy chain-termination method using dye terminator cycle sequencing in an Applied Biosystem model 3730 sequencer. Sequence analysis was conducted using the DNASTAR V 3.0 software (http://www.biologysoft.com/; Steve ShearDown, 1998-2001 version reserved by DNASTAR Inc., Madison, WI, USA).

RNA extraction and cDNA synthesis

The heart, liver, and leg muscle tissues were collected from 100 eight-day-old broilers. Total RNA was extracted with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer instructions and then treated with DNase (Promega, Madison, WI, USA). The DNase reaction included 1 μg total RNA, 1 U RNase-free DNase, 1 μL 10X reaction buffer, and 7 μL nuclease-free water. The mixture was incubated at 37°C for 30 min, followed by denaturation at 65°C for 10 min, and snap cooled on ice for 2 min. The quality and purity of the RNA were confirmed by agarose gel electrophoresis and spectrophotometry. cDNA was synthesized in a final volume 20 μL including 1 μg total RNA, 1X MMLV Buffer, 1 mM each dNTP, 2.5 μM oligo (dT)18, 0.5 μL (40 U/μL) RNase inhibitor, and 100 U MMLV SuperScript

III reverse transcriptase (Invitrogen). Reverse transcription was performed for 40 min at 42°C, followed by heating for 5 min at 95°C, and samples were then cooled on ice.

qRT-PCR

qRT-PCR was performed with a CFX96 Real-Time PCR Detection System (Bio-Rad, USA) using SYBR Green PCR Master Mix. The obtained cDNAs were used as templates for qPCR amplification with the primers P2 and P3 (Table 1), and the chicken *β-actin* gene was used as an internal control. Each reaction mixture contained 10 µL SYBR Green PCR Master Mix, 2 µL each primer (10 µM), 4 µL ultrapure RNase-free water, and 2 µL cDNA in a final volume 20 µL. Standard amplification conditions were as follows: 95°C for 3 min; 40 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 40 s. Fluorescent signals were collected after the extension at 72°C in each cycle. The entire experiment was repeated at least three times. After amplification, dissociation curve analysis was conducted to ensure that only one product was obtained. The product was then sequenced to confirm amplification of the correct sequences.

Protein determination and electrophoresis

Tissues samples (1 g) were homogenized in 50-mL polypropylene centrifuge tubes using 10 mL lysis buffer (20 mM Tris-HCl, pH 7.5; 0.9% NaCl; 2 mM β-mercaptoethanol). Samples were homogenized three times (30 s each) using an Ultra-turrax homogenizer at 20,000 rpm and placed in an ice-bath for 30-s intervals. The lysate was centrifuged at 31,000 *g* for 30 min at 4°C. The supernatant was transferred to 15-mL polypropylene tubes and manually homogenized 10 times using a Potter-Elvehjem homogenizer. Two 300-mL aliquots were separated for total protein determination and electrophoresis. Three hundred microliters of sample buffer [125 mM Tris-HCl, pH 6.8; 40% glycerol; 8% SDS (sodium dodecyl sulfate); 0.002% bromophenol blue] and 40 µL β-mercaptoethanol were added to the electrophoresis samples, which were boiled for 2 min and stored at -20°C until electrophoresis. The concentration of protein in supernatant aliquots was determined in quintuplicate according to the method described by Hartree (1972). A standard curve was produced using bovine serum albumin (BSA1, Sigma, Aldrich-Chemical Representações Ltda., São Paulo, SP, Brazil) in triplicate samples 0, 20, 40, 60, 80, and 100 mg (Figure 1). Thirty micrograms of total protein was loaded onto 9% polyacrylamide gels containing SDS and separated by electrophoresis (Laemmli, 1970), using the Mini-Protean II apparatus (Bio-Rad) at a constant voltage (200 V). Before loading, the samples were boiled for 2 min, and a sample of the supernatant derived from the control birds was loaded on all gels, as a reference standard. A prestained molecular weight standard (Gibco-BRL, Carlsbad, CA, USA) was used on all gels.

Western blotting

After fractionation on SDS polyacrylamide gels, proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Amersham, Buckinghamshire, UK) using the procedure described by Towbin et al. (1979). The transfer was performed for 30 min at a constant voltage (90 V) using a mini-transblot cell (Bio-Rad). The membranes were stained with 0.5% Ponceau S in 1% acetic acid for 3 min to confirm the transfer of proteins. After washing several times with deionized water, nonspecific interaction sites were blocked

using 10 mL cold TBS buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing 5% nonfat dried milk and 0.05% Tween-20, on a shaker (~100 rpm) for 1 h at room temperature. The membranes were then incubated with monoclonal anti-HSP70 antibody (H-5157, Sigma) in 10 mL cold TBS-milk solution (1:1000 dilution) containing 0.05% Tween-20 for 1 h at room temperature with constant shaking. Four washings of 5 min each using 10 mL TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) and a 10-min wash using 10 mL cold TBS buffer were performed. The membranes were incubated with 2-mL secondary antimouse antibody conjugated to alkaline phosphatase (A-5153, Sigma) diluted in 10 mL cold TBS-milk solution (1:5000 dilution) for 1 h at room temperature with constant shaking. After rinsing with cold TBST and TBS, as described above, the color was developed for 2 min using 33 mL nitro-blue tetrazolium chloride solution (50 mg/mL in dimethylformamide) and 66 mL 5-bromo-4-chloro-3-indolylphosphate p-toluidine (50 mg/mL in 70% dimethylformamide) added to 10 mL alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl$_2$). Nonspecific binding was blocked by the addition of a solution of 30% trichloroacetic acid. The membranes were washed with deionized water and dried at room temperature, protected from light. The intensity of the bands corresponding to HSP70 was analyzed using a densitometer at 525 nm (Shimadzu CS-9301) using reflection model and zigzag scanning; HSP70 quantification was conducted according to the method described by Givisiez et al. (1999). The levels of HSP70 are reported as ng/mg of total protein (Figure 1).
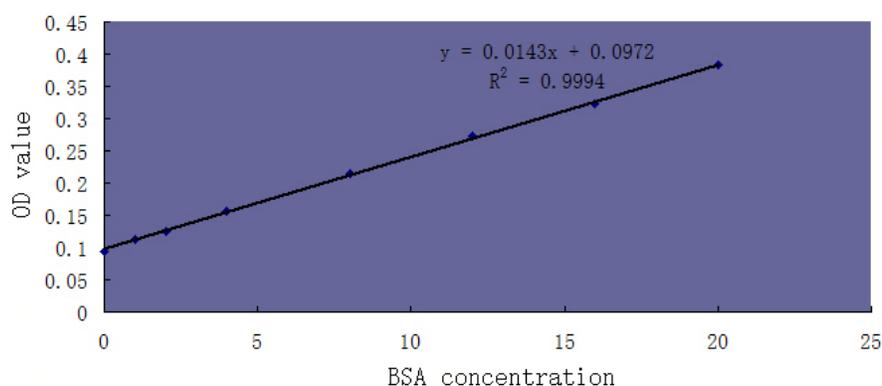

Figure 1. Bovine serum albumin (BSA) standard curve.

Luciferase expression constructs and luciferase expression assays

A pGL3-promoter vector (Promega) was double-digested with restriction enzymes and used to ligate the truncated amplification products of the 5'-flanking region of the *HSP*70 gene in order to determine the core promoter. Two expression vectors, pC.-69A>G-AA and pC.-69A>G-GG, were constructed. The A-69G primers were used to amplify the 5'-flanking region of the *HSP70* gene (Table 1). The PCR products were ligated into the pGL3-promoter vector between the *Kpn*I and *Xho*I sites. The plasmids were then transfected into chicken embryo fibroblasts at a density 1 x 10^5 cells/well. The QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used to alter SNP alleles. All constructs were verified by DNA sequencing following the manufacturer manual, the plasmids were transfected into chicken

embryo fibroblasts at a density 1 x 10^4 cells/well. A 24-well plate containing 500 μL medium containing 5% serum per well was used. A total of 1 μg plasmid pGL3 vector and 500 ng pRL-TK internal control vector (Promega) were co-transfected into cells using Lipofectamine™ 2000 (Invitrogen). After 5 h, the medium was changed to fresh medium with 10% fetal bovine serum. The cells were then incubated for 12 h, and luciferase assays were performed according to the manufacturer instructions (Promega). Luciferase activity was normalized using pRL-TK activity. Each experiment was performed six times.

Statistical analysis

Genotyping and prediction of transcription factor binding sites for the SNP C.A-69>G

DNAMAN (Lynnon Biosoft) was used for DNA contig assembly, sequence editing, and sequence translation. The identification of mutated sites was performed using the MegAlign program of the DNASTAR software (http://www.biologysoft.com/; Steve ShearDown, 1998-2001 version reserved by DNASTAR Inc.). The potential transcription factor binding sites of C.-69A>G were predicted using two bioinformatic websites, including http://motif.genome.jp and http://www.gene-regulation.com/pub/programs/alibaba2, following the setting parameters. Comparable results obtained by the two websites were selected.

Gene frequency, genotype frequency, and Hardy-Weinberg equilibrium

Genotypic frequency was determined as:

$$(FAiAj) = (AiAj \text{ number of individuals / samples of a population}) \times 100\% \qquad \text{(Equation 1)}$$

Gene frequency was determined as:

$$(Fai) = FAiAi + 1/2 \; \Sigma FAiAj \; (i \neq j) \qquad \text{(Equation 2)}$$

FAiAj indicates the gene frequency of AiAj on locus A; *Fai* indicates the frequency of allele Ai on locus A. The multiple comparisons for genotypic frequency of C.-69A>G in the two populations were performed using the GLM process of SAS 8.2 and Duncan's LSR. Hardy-Weinberg equilibrium testing of the C.A-69>G genotype was tested by the HWSIM procedure (http://krunch.med.yale.edu/hwsim/).

Association analyses

Association analysis I investigated SNP C.-69A>G and thermotolerance traits; association analysis II investigated SNP C.-69A>G and expression levels of *HSP70* mRNA and HSP70 protein in three tissues from thermo-stressed broilers.

The association analyses were performed using the GLM process of SAS 8.2 with the following model:

$$Y_{ij} = \mu + G_i + E_{ij} \qquad \text{(Equation 3)}$$

where, Y_{ij} is the phenotype value of thermotolerance traits, μ is the mean value, G_i is the fixed

effect of ith genotype, and E_{ij} is the residual effect.

RESULTS

Determination of gene frequency, genotype frequency, and Hardy-Weinberg equilibrium

Using the P1 primer, a 654-bp fragment, including 322 bp of the 5'-flanking region and 322 bp of the coding region, of the *HSP70* gene was obtained. One SNP, C.-69A>G, was identified in the 5'-flanking region. Online transcription factor binding site prediction identified a putative myeloid zinc finger protein 1 (MZF1) binding factor in the mutated sequence C.-69A>G (core sequence: ggagg**g**ga).

The number of LS chickens with the AA, AG, and GG genotypes was 85, 68, and 17, respectively. The number of WRR chickens with AA, AG, and GG genotypes was 134, 24, and 2, respectively. The AA genotype was observed at higher frequency in WRR chickens than in LS chickens, while the GG genotype frequency in WRR chickens was only 0.01 (Table 2). An independence test for the C.-69A>G site indicated that the genotype distribution in LS chickens was significantly different from that in WRR chickens ($\chi^2 = 43.5860$, P < 0.01). Goodness of fit revealed that the C.-69A>G site was found to be in Hardy-Weinberg equilibrium in those two populations, which suggests that these two populations either experienced no direct or indirect selection at the C.-69A>G site, or that a balanced state was achieved under selection (Table 2). Thus, the C.-69A>G was subjected to a random-mating state.

Table 2. Gene and genotype frequency, and Hardy-Weinberg equilibrium of the C.-69A>G single nucleotide polymorphism.

Site	Allele and genotype	LS (170)	LS χ^2	WRR (160)	WRR χ^2
C.-69A>G	A/G	0.70/0.30	0.3855	0.91/0.09	0.5888
	AA/AG/GG	0.50/0.40/0.10		0.84/0.15/0.01	

First nucleotide of translation start codon is designated +1; LS, Lingshan chicken; WRR, White Recessive Rock; χ^2, Hardy-Weinberg equilibrium value.

Association analysis of C.-69A>G with thermotolerance traits in WRR and LS chickens

The association of the C.-69A>G SNP with thermotolerance traits in LS and WRR chickens was analyzed under thermostress (35°C) and at normal temperature (15°C). The results showed that the C.-69A>G SNP was significantly (P < 0.05) associated with CD4+/CD8+ in WRR chickens under normal temperature conditions (15°C) (Table 3).

Table 3. Association analysis of the C.-69A>G SNP with thermotolerance traits.

Breed/condition	Thermotolerance traits				
	T_3 (ng/mL)	Corticosterone (ng/mL)	H/L	CD3+ (%)	CD4+/CD8+
LS/thermostress (35°C)	0.9020	0.2605	0.4035	0.7484	0.7420
WRR/thermostress (35°C)	0.8327	0.5901	0.6323	0.3072	0.3519
WRR/normal (15°C)	0.7536	0.5518	0.0998	0.5747	0.0132*

*P < 0.05; LS, Lingshan chicken, WRR, White Recessive Rock.

Association analysis between different genotypes of C.-69A>G and thermotolerance traits (T_3, H/L, corticosterone, CD3+, and CD4+/CD8+) showed that WRR chickens carrying the AA genotype have a significantly higher (P < 0.05) H/L value than chickens carrying the GG genotype under normal conditions (Table 4). Furthermore, WRR chickens with the AA genotype have significantly lower (P < 0.01) CD4+/CD8+ levels than chickens with the AG genotype under normal condition (Table 4). However, no association was found under the thermostress condition (35°C) in those two population.

Table 4. Association of different genotypes of the C.-69A>G SNP with thermotolerance traits.

	Genotype	Thermotolerance traits				
		T_3 (ng/mL)	Corticosterone (ng/mL)	H/L	CD3+ (%)	CD4+/CD8+
LS/thermostress (35°C)	AA	17.21 ± 1.99 (75)	22.68 ± 3.33 (79)	0.32 ± 0.02 (73)	3.20 ± 0.24 (37)	19.55 ± 1.53 (37)
	AG	17.04 ± 2.17 (63)	16.22 ± 3.67 (65)	0.33 ± 0.02 (60)	3.27 ± 0.26 (30)	19.13 ± 1.70 (30)
	GG	14.96 ± 4.60 (14)	11.67 ± 7.64 (15)	0.37 ± 0.04 (15)	3.63 ± 0.51 (8)	16.74 ± 3.30 (8)
WRR/thermostress (35°C)	AA	17.60 ± 1.73 (133)	40.66 ± 3.71 (122)	0.28 ± 0.02 (110)	3.34 ± 0.17 (67)	21.53 ± 1.79 (67)
	AG	17.20 ± 4.07 (24)	37.89 ± 8.53 (23)	0.30 ± 0.04 (18)	3.83 ± 0.52(7)	15.58 ± 5.55 (7)
	GG	9.03 ± 14.11 (2)	11.52 ± 28.94 (2)	-	5.15 ± 1.38 (1)	5.64 ± 14.69 (1)
WRR/normal (15°C)	AA	12.40 ± 1.20 (110)	27.41 ± 2.66 (106)	0.32 ± 0.01 (105)[a]	4.79 ± 0.39 (105)	4.62 ± 3.19 (105)[B]
	AG	12.08 ± 3.05 (17)	29.86 ± 6.45 (18)	0.30 ± 0.03 (17)[a b]	3.73 ± 0.94 (18)	29.50 ± 7.71 (18)[A]
	GG	5.66 ± 8.89 (2)	7.57 ± 19.35 (2)	0.12 ± 0.09 (2)[b]	5.01 ± 2.81 (2)	2.150 ± 23.12 (2)[AB]

[a,b]Values within a row with no common superscript are different significantly at P < 0.05; [A,B]Values within a row with no common superscript are significantly different at P < 0.01. LS, Lingshan chicken; WRR, White Recessive Rock. Numbers in parentheses mean tested chicken of each genotype.

Association between C.-69A>G genotypes and *HSP70* expression

In the heart, leg muscle, and liver tissues, the mRNA level of *HSP70* varies with different genotypes of C.-69A>G (Figure 2). In both the heart and leg muscle, *HSP70* mRNA was expressed at higher levels in individuals with the GG genotype than in those with AA and AG genotypes; however, this difference was not significant. mRNA expression of *HSP70* in livers from individuals with the AA genotype was significantly (P < 0.05) higher than in livers from those with the AG genotype.
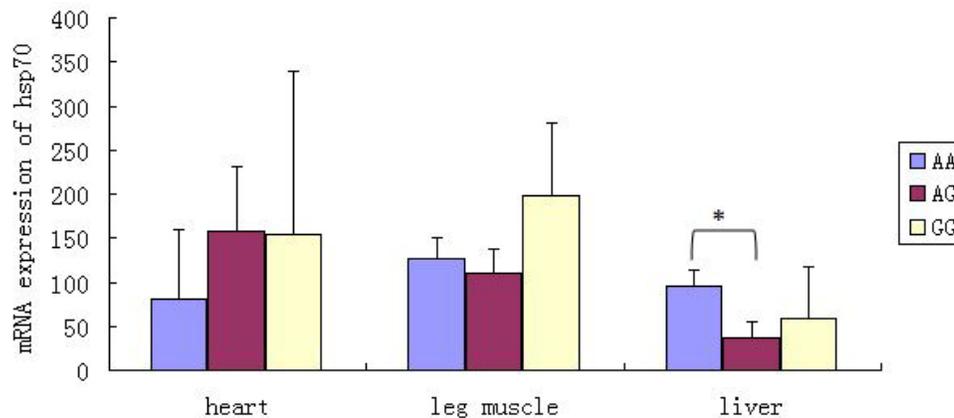

Figure 2. mRNA levels of *HSP70* in individuals with different genotypes of the C.-69A>G single nucleotide polymorphism. *P < 0.05.

Association of HSP70 protein expression with different C.-69A>G genotypes

The HSP70 protein was expressed at significantly ($P < 0.05$) higher levels in hearts from individuals with the GG genotype than in hearts from those with the AA genotype (Figure 3). In the leg muscle, HSP70 protein expression was slightly higher in birds with the GG genotype than in individuals with the AA and AG genotypes, although this difference was not significant (Figure 3). In the liver, the level of HSP70 protein in individuals with the GG genotype was significantly higher than in individuals with the AA and AG genotypes ($P < 0.01$ and $P < 0.05$, respectively) (Figure 3). Overall, the expression of HSP70 in individuals carrying the GG genotype was higher than in those carrying the AA genotype.
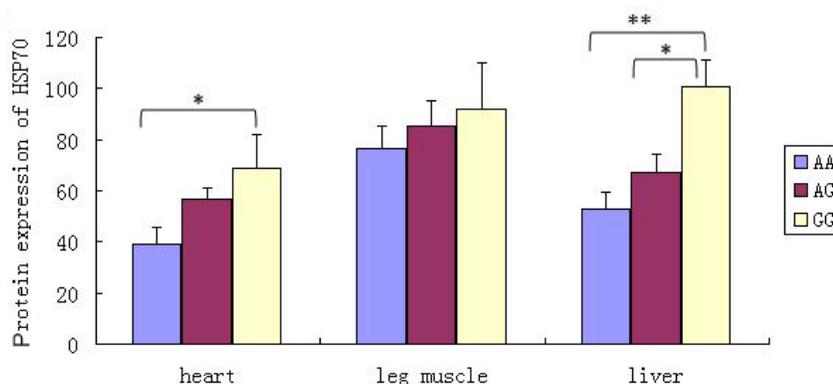

Figure 3. Protein levels of HSP70 in individuals with different genotypes of the C.-69A>G SNP. *$P < 0.05$; **$P < 0.01$.

Luciferase expression assays

Results from luciferase expression assays investigating different C.-69A>G genotypes revealed that expression of the *HSP70* gene was significantly different ($P < 0.01$) between birds with AA and GG genotypes, and in the GG genotype it was 1.37-fold affected *HSP70* gene expression (Figure 4).
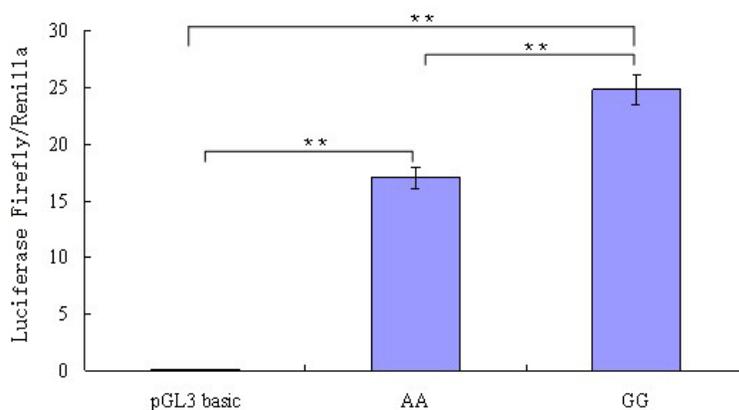

Figure 4. Luciferase expression assays for different genotypes of the C.-69A>G SNP. **$P < 0.01$.

DISCUSSION

In recent years, research has shown that SNPs in both the coding and non-coding regions of genes play an important role in regulating their function, and our understanding of gene structure has expanded. SNPs in non-coding regions can regulate the binding activity of transcriptional factors by altering the structure of some transcriptional domain binding sites (Jeng et al., 2008; Shibata et al., 2009; Ucisik-Akkaya et al., 2010). Using statistical analysis, Mukai et al. (2005) found that SNP in the 5'-untranslated region of the *CD40* gene had some influence on its expression, and Ordovás et al. (2008) found the g.763G>C SNP in the 5'-untranslated region of the bovine *FASN* gene affects its promoter activity. Moreover, Sun et al. (2010) found that the rs11868112 SNP locus on the 26-kb gene upstream of the *RPTOR* gene altered the RAR and POU2F1 transcription factor binding sites, which led to a decrease in gene expression (as determined by RT-PCR and ChIP methods) in response to climatic adaptations. In this study, a luciferase assay was used to show that the GG genotype was present at significantly higher levels than the AA genotype in the C.-69A>G site of the *HSP70* gene (P < 0.01).

Thermostress is one of the most challenging environmental conditions affecting commercial poultry (Maak et al., 2003; Cahaner et al., 2008; Melesse et al., 2011). The use of modern molecular breeding technology to identify genetic markers related to thermotolerance could allow for the possibility of direct gene selection. The potential transcription factor binding sites of C.-69A>G were predicted with a standard of 85 points via a TRANSFAC search on a bioinformatic website (http://motif.genome.jp). We found that variation in the C.-69A>G SNP may have some effect on the regulation of *HSP70* gene expression and could have an impact on the function of the HSP70 protein. Substitution of the G allele at the C.-69A>G site resulted in the formation of an MZF1 binding site, which is involved in the transcriptional regulation of multiple genes, and is related to the development of the bone marrow system (Hromas et al., 1995). The frequency of the G allele was much higher in the LS chicken population than the WRR population, because the transcription factor binding site (MZF1) promoted expression of the *HSP70* gene, and thus enhanced LS chicken thermotolerance.

The heart and liver are organs associated with the circulation and blood, nervous system regulation, and the functional recovery from stress damage. Under conditions of thermostress, HSP70 synthesis is increased in order to protect the circulatory system from serious injury (Hartl, 1996; Gu et al., 2012). However, changes in gene expression for other reasons should also be considered, as they may not be associated with changes in the activity of gene products. Although the qRT-PCR results showing *HSP70* expression were not consistent with the results obtained by luciferase expression assays, the predicted transcription factor binding sites and results obtained by western blotting were consistent with the luciferase results. This discrepancy could be explained in two ways: 1) the sample quantity was too small, or 2) the HSP70 protein develops a biological function in the thermostress reaction. Therefore, the effect of each genotype on the expression of HSP70 can be more easily verified by determining protein and mRNA levels.

Computer analysis suggests that the 5'-flanking region of the chicken *HSP70* gene contains several binding sites for putative transcription factors such as the MZF1. MZF1 is known to be involved in the development of cancer. Based on the presence of MZF1 binding sites within the promoter regions of several nuclear genes that regulate mitochondrial biogenesis (Yan et al., 2006), it is suggested that MZF1 may also play a role in regulating

the level of *HSP70* gene expression. The results of the luciferase expression assays and the predicted transcription factor binding sites suggest that the GG genotype of the C.-69A>G SNP possesses an MZF1 binding site, which led to increased expression of the GG genotype over the AA genotype. Therefore, the C.-69A>G SNP is thought to affect the *HSP70* gene expression.

Under artificially controlled conditions, genetic equilibrium will be disrupted, affecting gene or genotype frequencies. Thus, inherited characteristics, methods of selection, and hybridization, which are important measures in animal breeding, will be altered. Surveying variation in gene and genotype frequencies in breeds that possess different traits could lead to the selection of some sites related to traits of interest (Mashaly et al., 2004). In this study, two breeds with different levels of thermotolerance were used to assess variations in gene and genotype frequencies of the C.-69A>G SNP. The LS chicken is a local breed in China, which is considered to be thermotolerant, while the WRR chicken is a breed thought to possess poor thermoresistance. The frequency of the GG genotype of the C.-69A>G SNP in LS chickens was much higher than that in the WRR chickens. Moreover, the distribution frequencies of the SNP C.-69A>G genotypes were highly diverse ($P < 0.01$) in LS and WRR chickens. This indicated that the C.-69A>G SNP in two populations with different levels of thermotolerance may have undergone artificial selection during the process of selection and cultivation, with the aim of advancing thermotolerance levels.

In the current study, the C.-69A>G SNP was genotyped in LS and WRR chickens, and these two breeds were used to investigate physiological and immunological traits under conditions of thermostress. Only WRR chickens were used to detect the same traits under the cited condition. Furthermore, association of the C.-69A>G site with chicken thermotolerance traits was studied. Some previous studies have shown that H/L is an important trait of cell-mediated immunity, and could serve as an index for the study of stress responses and resistance (Campo and Davila, 2002). Results of the association analysis showed that the C.-69A>G site was significantly associated ($P < 0.05$) with CD4+/CD8+, and a potential association was found between the C.-69A>G SNP and H/L. Furthermore, there was no difference between LS and WRR chickens with regard to T_3 levels regardless of line and age, which is consistent with the results reported by Maak et al. (2003).

In summary, the results in this study suggest that the C.-69A>G SNP in the 5'-flanking region of the *HSP70* gene affects chicken thermotolerance traits in WRR chickens exposed to thermoneutral temperature (15°C), and that the GG genotype might be advantageous for the prevention of thermostress. Thus, this SNP may be a potential molecular marker for further genetic improvement of thermotolerance in chicken.

Conflicts of interest

The authors declare no conflict of interest.

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