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Association analysis between SNPs in the 5'-flanking region of the chicken *GRP78* gene, thermotolerance parameters, and tissue mRNA expression

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ABSTRACT. Glucose-regulated protein 78 (GRP78) is a molecular chaperone in the endoplasmic reticulum and can be induced by different kinds of environmental and physiological stress. Thus far, the role of the *GRP78* gene in thermotolerance in chickens has not been investigated. In the present study, we detected sequence variations in the 5'-flanking region of the *GRP78* gene and evaluated several thermotolerance parameters, such as T3, corticosterone, H/L ratio, and levels of $CD3^+$, $CD4^+$, and $CD8^+$ T cells, to further determine its associations at 35° and 15°C. The sequencing results revealed 10 SNPs in the 5'-flanking region of the *GRP78* gene, and seven mutations were chosen for further genotyping in a White Recessive Rock (WRR) chicken population. The SNP C.-744C>G in WRR chickens was significantly correlated with heat tolerance parameters under both conditions; it may therefore exert

a potential hereditary effect on heat tolerance, and the genotype GG may be advantageous for thermotolerance. The heart, liver, brain, and leg muscle tissues of 8-day-old WRR chickens were sampled from heat stress groups, which were defined by exposure to 1, 2, 3, and 6 h of persistent thermal stress, and a control group, which was not exposed to thermal stress. Quantitative real-time polymerase chain reaction assay indicated that the mRNA expression level of the *GRP78* gene increased gradually under heat stress, peaked at 3 h, and then decreased. We conclude that the mRNA expression of the *GRP78* gene is time- and tissue-dependent.

Key words: *GRP78* gene; Single-nucleotide polymorphism; Chicken; Heat stress; Quantitative real-time PCR

INTRODUCTION

Heat shock proteins (HSPs) are synthesized by prokaryotic and eukaryotic cells upon exposure to a cellular insult, such as severe heat shock, strong oxidants, UV irradiation, and other stressful conditions. Among the HSPs, HSP70s are one of the most conserved and important protein families (Boutet et al., 2003; Deane and Woo, 2005). Glucose-regulated protein 78 (GRP78), which belongs to the HSP70 family, is a very important and conserved chaperon in various species. As a central regulator of endoplasmic reticulum (ER) functions, the *GRP78* gene participates in ER protein folding and assembly, and regulates the unfolded protein response and specific anti-apoptotic actions. It has been shown that GRP78 acts as an anti-apoptotic chaperone that plays a key role in maintaining the proper functions of proteins and organelles (Endo et al., 2007). Jia et al. (2013) found that pretreatment with prostaglandin E1 and somatostatin alleviated ER stress by the induction of HSP70 (another member of the HSP70 family) and GRP78. Kim et al. (2013) demonstrated that GRP78 gradually increased in the mouse substantia nigra with 28-day heat exposure. Recently, GRP78 expression was found to be related to disease occurrence. A study by Winder et al. (2011) provided the first evidence that the rs391957 polymorphism of *GRP78* could be a potential predictor for clinical outcome in gastric and colorectal cancer patients. As an HSP, *GRP78* could be associated with heat stress; furthermore, we hypothesized that polymorphisms of the *GRP78* gene, especially those in the 5'-flanking region, may be involved in the regulation of its expression and even associated with thermotolerance.

In modern poultry husbandry, heat stress is a severe problem. Under high ambient temperature, chickens could suffer a series of negative impacts, such as poor feed intake, decreased weight gain, poor feed efficiency (Siegel, 1995), change in blood biochemical indicators, immunosuppression (Young, 1990), and high mortality (Yahav et al., 1995). Although prolonged and continuous selection has increased broiler tolerance to high ambient temperatures, chemical and immune regulation play very important roles in improving their thermal tolerance and adaptability. The activation of the hypothalamic-pituitary-adrenal (HPA) axis by stress is responsible for many behavioral and immune alterations (Zorrilla et al., 2001). Corticosterone secreted by the adrenal gland is the main hormone that modulates chicken physiology and behavior under heat stress conditions. It has been reported that acute heat stress increases mortality; decreases food intake, body weight gain, and feed conversion; and

increases serum corticosterone concentrations (Quinteiro-Filho et al., 2012). Both the thyroxine (T4) and 3,5,3-triiodothyronine (T3) hormones, which are secreted by the thyroid gland, are also involved in the regulation of metabolism and thermogenesis of chickens. In addition to T3 and corticosterone, other physiological parameters may also be used to evaluate thermotolerance parameters, such as heterophil to lymphocyte (H/L) ratio and the numbers of $CD3^+$, $CD4^+$, and $CD8^+$ T cells. Habibian et al. (2013) found that heat stress resulted in a significant increase in the H/L ratio. Peripheral T lymphocyte subsets are widely recognized as indicators of cell immunology status (Dietert et al., 1994).

Although many studies in humans and mice have reported that the GRP78 protein plays an important role in protecting the host from various stimuli, there are no reports showing that polymorphisms in the 5'-flanking region of the *GRP78* gene are associated with thermotolerance parameters or with its mRNA expression under heat stress. The aim of the present study was to identify some molecular markers of the chicken *GRP78* gene that may be associated with thermotolerance parameters and to analyze the chicken *GRP78* gene expression pattern among tissues.

MATERIAL AND METHODS

Experimental animals

A total of 100 individuals from eight native Chinese breeds (Wen Chang, Gu Shi, Luo Si, Silkie, Tibet, Qing Yuan, Xing Hua, and Ling Shan) as well as the White Recessive Rock (WRR) and red jungle-fowl breeds were used for sequence variation detection of the *GRP78* gene 5'-flanking region.

Female WRR chickens (N = 160) were obtained from Xinguang Agriculture and Animal Husbandry Co., Ltd. Blood samples were taken from the wing vein of each bird after the birds were heat-shocked at 35°C (summer). As control samples, wing vein blood was collected from the same birds at 15°C (winter). These blood samples were used for subsequent experiments, including genomic DNA isolation and the determination of series parameters, such as H/L and levels of CD cells, T3, and corticosterone.

Measurement of related thermotolerance parameters

T3 and corticosterone levels were determined by enzyme-linked immunosorbent assay according to the manufacturer manual. The H/L ratio was determined by counting the cells at the People's Liberation Army Hospital 458 (Guangzhou, China). The levels of $CD3^+$, $CD4^+$, and $CD8^+$ T cells were determined by flow cytometry.

Heat stress experiment

Forty 1-day-old female WRR chickens were raised in a climate-controlled chamber for 1 week, and feeding conditions and management were controlled according to the normal feeding standards. On day 8, the control group was maintained at room temperature (25 ± 1°C), while the temperature of the test groups was suddenly increased from 25 ± 1°C to 40 ± 1°C. Forty broilers were distributed randomly into the heat stress test groups, which were exposed to 40 ± 1°C for 1, 2, 3, or 6 h, and a control group, which was not exposed to heat stress.

During the period of heat stress, the birds were given feed and water *ad libitum*. After heat treatment, birds in each group were sacrificed, and the heart, liver, brain, and leg muscle tissues of chickens were immediately sampled, placed in 2-mL cryopreserved tubes, and stored in liquid nitrogen. Tissue samples were used to extract total RNA. This animal experiment was approved by the Animal Ethics Committee of China.

Primer design, polymerase chain reaction (PCR) amplification, and polymorphism identification

PCR primers were designed for PCR amplification of the chicken *GRP78* gene (Gene ID: M27260) and the *GAPDH* gene. The primer pairs P1, P2, P3, and P4 were used to detect variation in the *GRP78* gene 5'-flanking region. The primer pair P5 was used to amplify a 995-bp fragment of the *GRP78* gene for further genotyping by direct sequencing. The P6 (*GRP78* mRNA primers) and P7 (GAPDH mRNA primers) primers were used for fluorescent quantitative real-time PCR (qRT-PCR). Primer sequences and information are shown in Table 1.

The PCRs for variation detection and genotyping were conducted in a 50-μL volume containing 50 ng genomic DNA, 200 μM diethylnitrophenyl thiophosphates (dNTPs), 1X buffer, 2 mM $MgCl_2$, 1 μM of each primer, and 1.5 U Taq DNA polymerase (Shanghai Biological Engineering Company, Shanghai, China). The PCR conditions for amplification were as follows: initial denaturation at 94°C for 3 min, followed by 34 cycles of denaturation at 94°C for 30 s, the appropriate annealing temperature (Table 1) for 30 s, and 72°C for 1 min/kb, and a final extension at 72°C for 5 min. PCR products were detected by 1.5% agarose gel electrophoresis and then sent to BGI (Beijing, China) for DNA sequencing. The DNASTAR software (http://www.biologysoft.com/; DNASTAR Inc., Madison, WI, USA) was used for sequence alignment.

qRT-PCR analysis

Total RNA was extracted from the heart, brain, liver, and leg muscle tissue with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following manufacturer instructions. First-strand cDNA was synthesized using the ReverTra qPCR RT Master Mix gDNA remover kit (Toyobo, Tokyo, Japan), and quantified by qRT-PCR using the Bio-Rad CFX96 system (Bio-Rad, Hercules, CA, USA) with Bestar™ Real-Time PCR Master Mix (DBI, Ludwigshafen, Germany). PCR amplification was performed at the following temperature conditions: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 20 s (signal collection), followed by the construction of a melting curve. All samples were amplified in triplicate; after amplification, the products were detected by 1.5% agarose gel electrophoresis to confirm the amplification product.

Statistical analysis

The BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) on the NCBI website was used to research splicing sequence homology. The SeqMan program of the DNASTAR software suite was used for sequence alignment, and transcription factor binding sites of the 5'-flanking region polymorphisms were predicted by the TRANSFAC database of the MOTIF website (http://motif.genome.jp).

Genotype and single nucleotide polymorphism (SNP) frequencies were analyzed by the following formula: $FA_iA_j = (A_iA_j$ / population size) x 100%, $FA_i = FA_iA_i + 1/2 \sum FA_iA_j$ ($i \neq j$). FA_iA_j is the frequency of the A_iA_j genotype; FA_i is the frequency of the A_i allele.

Hardy-Weinberg equilibrium of the SNPs was determined using the HWSIM procedure (http://krunch.med.yale.edu/hwsim/).

Association analysis of polymorphisms with thermotolerance parameters was carried out using the general linear model process of the SAS 9.0 software (SAS Institute Inc., Cary, NC, USA) and the following linear model:

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

where Y_{ij} is the phenotype value of thermotolerance parameters, μ is the population mean, G_i is the genotype or haploid effect value, and E_{ij} is the residual effect.

The heat-resistant properties of the same SNP genotype under different conditions were compared by *t*-test using the SAS 9.0 software.

qRT-PCR data analysis was undertaken by using mean values of $2^{-\Delta\Delta Ct}$ from three samples in three biological replicates. All values are reported as means ± standard error.

RESULTS

Identification of SNPs that alter transcription factor binding sites

Using the four primer pairs P1-P4 (Table 1), 10 SNPs were identified in the 2400-bp 5'-flanking region of the chicken *GRP78* gene. Analysis using the MOTIF website (http://tfbind.hgc.jp/) revealed that seven of these mutations would alter transcription factor binding sites (Table 2). These seven SNPs were further genotyped in 160 WRR chicken populations; the genotype information is provided in Table 3. The Hardy-Weinberg equilibrium test results showed that all of these SNPs were in equilibrium, indicating that these mutations have not undergone selection.

Table 1. Primer sequences and information.

Primers	Primers sequences (5'→3')	Annealing temperature (°C)	Products (bp)
P1	F: ACGGAGCCCGCACATCAC R: GGTCCAACCCGACCTCATCG	63	697
P2	F: CCGAGGCTCCCTTCTGAC R: GGGACGACGTTCTGGAAGT	62	640
P3	F: CCATCAGTGCTTTGTCTCTCTCA R: AACAGGGCTGAATCGTAGCA	62	781
P4	F: TCTGCCTCACGTAATAGCACT R: TCAGAGAAGTGGATTTGAGGC	59	717
P5	F: GAATGAGCCTGAGGCCTGG R: TTTCCATGGTAACCAACCCCG	61	995
P6	F: CCTGAGGGGGAGCGCCTGAT R: GGGGTCATTCCAGGTGCGGC	61	111
P7	F: CGTTGACGTGCAGCAGGAACACT R: CTTTGCCAGAGAGGACGGCAGC	60	110

Table 2. SNPs identified in the 2500-bp 5'-flanking region of the chicken *GRP78* gene.

Site	Allele	Variation types	Transcription factor binding sites	Note
C.-744C>G	G	Transversion	NFκB50	Nuclear factor kappa B50
C.-777G>C	C	Transversion	-	-
C.-965T>C	C	Transition	AP4	Activating enhancer binding protein 4
C.-990G>A	A	Transition	-	-
C.-1014T>C	C	Transition	ATF2	Cyclic AMP-dependent transcription factor 2
C.-1138C>T	T	Transition	E2F	E2-promoter binding factor
C.-1146G>A	A	Transition	NFκB	Nuclear factor kappa B
C.-1387G>A	A	Transition	-	-
C.-1407T>A	A	Transversion	AP1	Nuclear transcription factor activator protein 1
C.-2328G>A	A	Transversion	SP1	Uncharacterized protein

Table 3. Genotype information on seven SNPs.

Sites	Genotype (number)		HWpval
C.-744C>G	G/C	0.65/0.35	0.5392
	GG/CG/CC	0.40/0.49/0.11	
C.-777G>C	G/C	0.88/0.12	0.2313
	GG/ GC	0.76/0.24	
C.-965T>C	T/C	0.24/0.76	0.2895
	TT/ TC/ CC	0.04/0.40/0.56	
C.-990G>A	G/A	0.36/0.64	0.7917
	GG/ AG/ AA	0.12/0.48/0.40	
C.-1014T>C	T/C	0.37/0.63	1.0
	TT/ TC/ CC	0.14/0.47/0.39	
C.-1138C>T	T/C	0.06/0.94	1.0
	TC/ CC	0.12/0.88	
C.-1146G>A	G/A	0.36/0.64	1.0
	GG/ AG/ AA	0.12/0.47/0.41	

A in the initiator ATG is set to +1, and the first base upstream of A is set to -1. HWpval is the Hardy-Weinberg equilibrium test value of genotype distribution

Association analysis of polymorphisms and thermotolerance parameters of the *GRP78* gene

Seven polymorphism sites, C.-744C>G, C.-777G>C, C.-965T>C, C.-990G>A, C. -1014 T>C, C.-1138C>T, and C.-1146G>A, were chosen for association analysis with the thermotolerance parameters of WRR chicken broilers at 35° and 15°C (Tables 4 and 5). For WRR chicken broilers at 35°C, we found a significant association between the SNP C.-744C>G and corticosterone ($P < 0.05$; Table 4), but an unrelated association was detected between SNP C.-744C>G and T3 ($P > 0.05$; Table 4). Corticosterone and T3 levels were significantly higher in the CG genotype broilers than in the GG genotype broilers ($P < 0.05$; Table 4). Broilers with the CC and CG genotypes and those with the CC and GG genotypes showed no difference ($P > 0.05$; Table 4). The T3 level was higher in the TC genotype than in the CC genotype of SNP C.-965C>T ($P < 0.05$; Table 4). For the C.-990G>A site, we detected significantly lower corticosterone levels in the AA genotype broilers than in AG genotype broilers ($P < 0.05$; Table 4), while no difference was found between AA and GG genotype broilers ($P > 0.05$; Table 4). We also found that the $CD3^+$ cell level for the AA genotype was significantly higher than that of the GG genotype ($P < 0.05$; Table 4). We found significantly higher levels of $CD3^+$ cells in the CC genotype than in the TT genotype of SNP C.-1014T>C

($P < 0.05$; Table 4), while no significant difference was observed between other genotypes ($P > 0.05$; Table 4). The $CD3^+$ cell level was significantly higher in the CC genotype than in the GG genotype of SNP C.-1146A>G ($P < 0.05$; Table 4).

Table 4. Analysis of associations between seven polymorphic sites and thermotolerance parameters at 35°C.

Site	Genotype	Parameter				
		T3 (ng/mL)	Corticosterone (ng/mL)	H/L	$CD3^+$ (%)	$CD4^+$ $CD8^+$
C.-744C>G	GG	13.92 ± 2.46(56)[b]	32.76 ± 5.19(50)[b]	0.32 ± 0.02(42)	3.62 ± 0.23(34)	3.54 ± 1.34(34)
	CG	20.73 ± 2.29(65)[a]	49.49 ± 4.91(60)[a]	0.33 ± 0.02(57)	3.34 ± 0.25(30)	3.83 ± 1.12(30)
	CC	15.77 ± 4.93(16)[ab]	27.47 ± 10.05(16)[ab]	0.30 ± 0.04(10)	2.65 ± 0.48(8)	9.15 ± 2.90(8)
C.-777G>C	GG	17.82 ± 1.93(104)	41.23 ± 3.93(96)	0.33 ± 0.02(81)	3.40 ± 0.18(59)	3.77 ± 0.99(59)
	GC	16.29 ± 3.43(33)	34.30 ± 7.03(30)	0.31 ± 0.03(28)	3.36 ± 0.39(13)	5.10 ± 1.57(13)
C.-965T>C	TT	17.24 ± 8.71(5)[ab]	26.04 ± 17.07(5)	0.26 ± 0.07(3)	3.09 ± 0.79(3)	1.38 ± 5.09(3)
	TC	21.63 ± 2.65(54)[a]	47.61 ± 5.34(51)	0.32 ± 0.02(46)	3.04 ± 0.25(29)	5.30 ± 1.34(29)
	CC	14.58 ± 2.20(78)[b]	34.58 ± 4.56(70)	0.33 ± 0.02(60)	3.67 ± 0.22(40)	3.50 ± 1.10(40)
C.-990G>A	GG	16.20 ± 4.75(17)	30.64 ± 9.28(17)[ab]	0.30 ± 0.04(12)	2.65 ± 0.48(8)[b]	7.93 ± 2.71(8)
	AG	20.20 ± 2.43(65)	48.29 ± 4.98(59)[a]	0.33 ± 0.02(56)	3.28 ± 0.24(31)[ab]	3.91 ± 1.15(31)
	AA	14.60 ± 2.64(55)	33.65 ± 5.31(50)[b]	0.32 ± 0.02(41)	3.73 ± 0.23(33)[a]	3.54 ± 1.35(33)
C.-1014T>C	TT	19.10 ± 4.51(19)	32.12 ± 8.76(19)	0.30 ± 0.04(14)	2.66 ± 0.45(9)[b]	8.30 ± 2.37(9)
	TC	19.18 ± 2.46(64)	46.95 ± 5.06(57)	0.33 ± 0.02(54)	3.30 ± 0.25(30)[ab]	3.60 ± 1.17(30)
	CC	14.83 ± 2.67(54)	33.86 ± 5.40(50)	0.32 ± 0.02(41)	3.73 ± 0.23(33)[a]	3.54 ± 1.34(33)
C.-1138C>T	TC	13.89 ± 4.77(17)	33.88 ± 9.64(16)	0.35 ± 0.04(11)	2.67 ± 0.56(6)	5.66 ± 2.55(6)
	CC	17.96 ± 1.79(120)	40.34 ± 3.68(110)	0.32 ± 0.01(98)	3.46 ± 0.17(66)	3.96 ± 0.89(66)
C.-1146G>A	GG	16.20 ± 4.77(17)	27.74 ± 9.29(17)	0.30 ± 0.04(12)	2.65 ± 0.48(8)[b]	7.93 ± 2.71(8)
	AG	19.79 ± 2.48(63)	45.23 ± 5.03(58)	0.33 ± 0.02(54)	3.20 ± 0.25(29)[ab]	4.05 ± 1.18(29)
	AA	15.25 ± 2.60(57)	36.95 ± 5.36(51)	0.32 ± 0.02(43)	3.78 ± 0.23(35)[a]	3.39 ± 1.31(35)

Data are reported as least-square means ± standard errors (SE). Differences between data with different superscript lowercase letters within a column are significant ($P < 0.05$). Numbers in parentheses denote the number of genotypes.

At 15°C, SNP C.-744C>G, which is located in a nuclear factor kappa B50 (NFκB50) binding site as revealed by transcription factor binding site analysis, was significantly associated with $CD3^+$ cell levels ($P < 0.05$; Table 5), with a lower $CD3^+$ value observed for the CG genotype than the CC genotype. Additionally, the corticosterone level in the CG genotype was significantly lower than that seen in the CC genotype at this site. A mutation at C.-965C>T was significantly associated with $CD4^+/CD8^+$ cell ratio ($P < 0.05$; Table 5), with a higher $CD4^+/CD8^+$ value for the TC than the CC genotype ($P < 0.01$; Table 5), but no significant difference was found between TT and the other two genotypes ($P > 0.05$; Table 5). The SNP C.-990A>G was significantly associated with $CD3^+$ ($P < 0.05$; Table 5), with a higher $CD3^+$ value for the AA genotype than for the other two genotypes; alternatively, the corticosterone content for GG was higher than that for the AG genotype ($P < 0.05$; Table 5). We also found a significant association between the SNP C.-1014C>T and $CD3^+$ cell levels ($P < 0.05$; Table 5); the $CD3^+$ cell level was significantly higher in the CC genotype than in the TT and TC genotypes ($P < 0.05$; Table 5), whereas the corticosterone content was higher for the TT genotype than for the TC genotype at this site ($P < 0.05$; Table 5). The SNP C.-1146A>G was significantly associated with $CD3^+$ cell levels ($P < 0.05$; Table 5); the $CD3^+$ cell level was significantly higher for the AA genotype than for the other two genotypes ($P < 0.05$; Table 5), whereas the corticosterone content was higher for the GG genotype than the AG genotype ($P < 0.05$; Table 5).

Table 5. Analysis of associations between seven polymorphic sites and thermotolerance parameters at 15°C.

Site	Genotype	Parameter				
		T3 (ng/mL)	Corticosterone (ng/mL)	H/L	$CD3^+$ (%)	$CD4^+/CD8^+$(%)
C.-744C>G	GG	10.50 ± 1.67(47)	27.56 ± 3.68(47)^{ab}	0.28 ± 0.03(44)	5.72 ± 0.55(45)^{a}	26.53 ± 4.93(41)
	CG	10.55 ± 1.52(57)	23.83 ± 3.43(55)^{b}	0.29 ± 0.02(54)	3.88 ± 0.49(55)^{b}	26.15 ± 5.52(54)
	CC	17.78 ± 3.46(11)	44.26 ± 8.01(11)^{a}	0.29 ± 0.06(10)	3.33 ± 1.27(11)^{ab}	30.47 ± 11.03(10)
C.-777G>C	GG	11.24 ± 1.26(85)	27.57 ± 2.89(84)	0.27 ± 0.02(79)	4.95 ± 0.43(81)	26.71 ± 3.62(76)
	GC	11.15 ± 2.12(30)	22.83 ± 4.92(29)	0.33 ± 0.03(29)	3.43 ± 0.73(29)	27.30 ± 10.86(29)
C.-965T>C	TT	17.80 ± 5.79(4)	37.78 ± 13.31(4)	0.21 ± 0.10(4)	4.07 ± 1.99(4)	26.36 ± 11.76(4)^{AB}
	TC	12.15 ± 1.76(43)	25.22 ± 4.16(41)	0.26 ± 0.03(38)	4.41 ± 0.64(39)	38.40 ± 5.01(38)^{A}
	CC	10.24 ± 1.40(68)	26.36 ± 3.23(68)	0.30 ± 0.02(66)	4.65 ± 0.49(67)	19.30 ± 4.03(63)^{B}
C.-990G>A	GG	16.59 ± 3.33(12)	41.19 ± 7.55(12)^{a}	0.27 ± 0.05(11)	3.12 ± 1.12(11)^{b}	30.47 ± 11.03(11)
	AG	10.54 ± 1.53(57)	23.23 ± 3.53(55)^{b}	0.29 ± 0.02(54)	3.93 ± 0.49(55)^{b}	26.15 ± 5.52(54)
	AA	10.65 ± 1.70(46)	26.21 ± 3.86(46)^{ab}	0.27 ± 0.03(43)	5.81 ± 0.55(44)^{a}	26.53 ± 4.93(40)
C.-1014T>C	TT	15.48 ± 3.09(14)	40.84 ± 6.96(14)^{a}	0.30 ± 0.05(13)	3.01 ± 1.04(13)^{b}	30.47 ± 11.03(13)
	TC	10.46 ± 1.55(56)	22.31 ± 3.55(54)^{b}	0.29 ± 0.02(52)	3.98 ± 0.50(53)^{b}	26.15 ± 5.52(52)
	CC	10.84 ± 1.72(45)	26.69 ± 3.88(45)^{ab}	0.27 ± 0.03(43)	5.81 ± 0.55(44)^{a}	26.53 ± 4.93(40)
C.-1138C>T	TC	12.26 ± 3.11(14)	35.17 ± 7.05(14)	0.25 ± 0.05(13)	3.38 ± 1.05(14)	16.62 ± 13.15(14)
	CC	11.07 ± 1.16(101)	25.11 ± 2.65(99)	0.29 ± 0.02(95)	4.72 ± 0.40(96)	27.50 ± 3.51(91)
C.-1146G>A	GG	16.59 ± 3.33(12)	41.19 ± 7.53(12)^{a}	0.27 ± 0.05(11)	3.12 ± 1.12(11)^{b}	30.47 ± 11.03(11)
	AG	10.66 ± 1.54(56)	22.08 ± 3.55(54)^{b}	0.29 ± 0.02(52)	3.91 ± 0.50(53)^{b}	26.15 ± 5.52(52)
	AA	10.51 ± 1.68(47)	27.48 ± 3.80(47)^{ab}	0.28 ± 0.03(45)	5.75 ± 0.54(46)^{a}	26.53 ± 4.93(42)

Data are reported as least-square means ± standard errors (SE). Differences between data with different superscript lowercase letters within a column are significant ($P < 0.05$), and differences between data with the different superscript capital letters are very significant ($P < 0.01$). Numbers in parentheses denote the number of genotypes.

Comparison of physiological and immune indices of the same genotype of *GRP78* gene SNPs at different temperatures

The seven polymorphism sites C.-744C>G, C.-777G>C, C.-965T>C, C.-990G>A, C.-1014T>C, C.-1138C>T, and C.-1146G>A were used to compare thermotolerance indices of the same genotype at different temperature (**Table S1**). Compared with the levels seen at 15°C, corticosterone content, T3 level, and H/L ratio were significantly elevated, and the $CD4^+/CD8^+$ T ratio and $CD3^+$ T cell level were significantly lower within the same genotype at all sites at 35°C (**Table S1**).

mRNA expression levels of the *GRP78* gene in WRR chickens

Figure 1 shows that the levels of *GRP78* mRNA expression in the liver, brain, and heart were remarkably higher after 1 h of thermal stress than in the control group in WRR chicken broilers ($P < 0.01$; Figure 1A, B, C, and D). The *GRP78* gene mRNA expression in all tissues increased first and then decreased with continued thermal stress, reaching its peak at 3 h; this pattern was remarkably different from that seen in control WRR chickens (Figure 1A, B, C, and D). The levels of *GRP78* mRNA began to recover or had already returned to normal levels after 6 h of heat exposure (Figure 1A, B, C, and D). At 6 h, we also found remarkable differences in *GRP78* mRNA expression in the leg muscle of WRR chickens compared with the control group ($P < 0.01$; Figure 1C) and no differences in other tissues ($P > 0.05$; Figure 1A, B, and D).

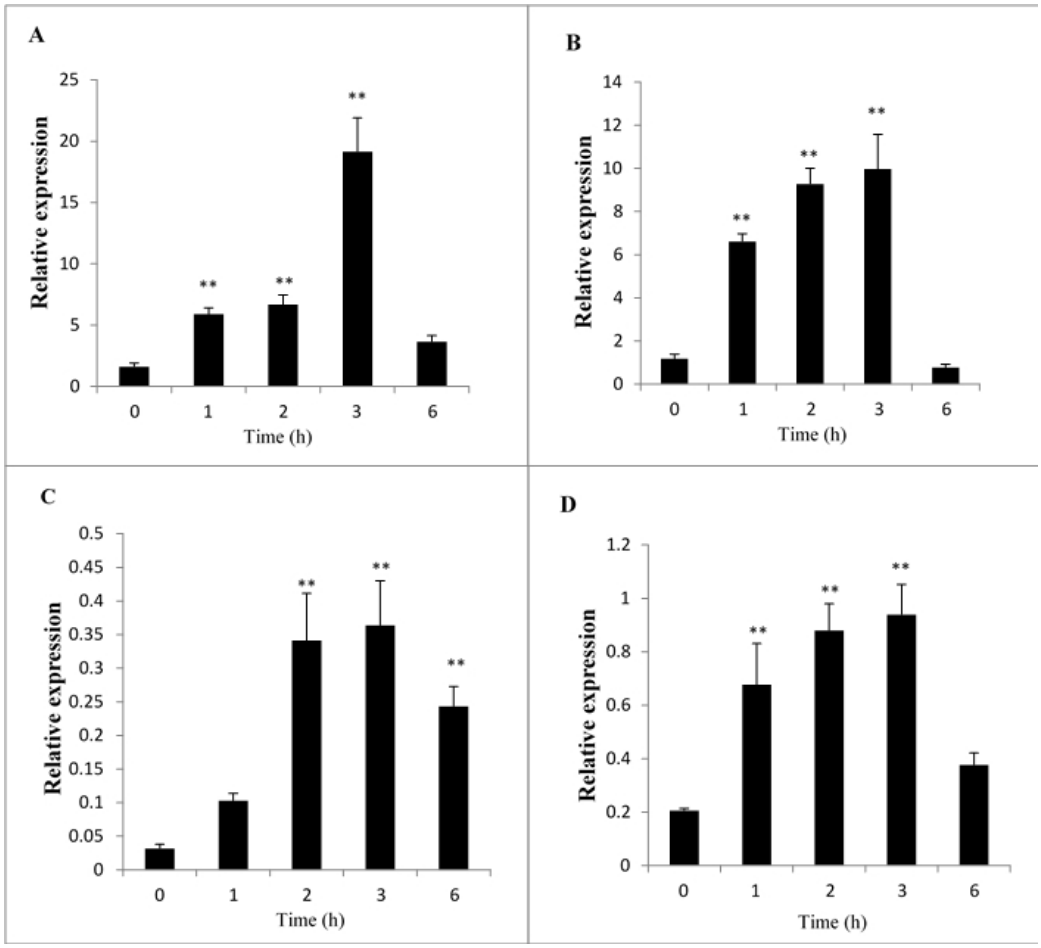

Figure 1. mRNA expression levels of the *GRP78* gene in different tissues at different times in (**A**) brain, (**B**) heart, (**C**) leg muscle, and (**D**) liver tissues. **P < 0.01 compared with the control group data. 0 h = control.

Comparison of tissue-specific *GRP78* mRNA expression in WRR chickens

We concluded that *GRP78* mRNA expression was time- and tissue-dependent. At room temperature, we found that *GRP78* mRNA expression was highest in brain tissue, and that this level was remarkably higher than that seen in WRR chicken leg muscle and liver tissues ($P < 0.01$; Table 6). Alternatively, no significant difference was found between the expression levels in leg muscle and liver tissues ($P > 0.05$; Table 6).

With continued thermal stress, *GRP78* mRNA expression remained at high levels in brain and heart tissue compared with leg muscle and liver tissues. We found no significant variation in *GRP78* mRNA expression between liver and leg muscle tissue during exposure to heat stress ($P > 0.05$; Table 6). After 1 and 2 h of thermal stress, high *GRP78* mRNA expression was observed in heart tissue, and this level was remarkably higher than that in liver and leg muscle tissues ($P < 0.01$; Table 6). The relative expression level of *GRP78* mRNA in brain

tissue was intermediate and was higher than in liver and leg muscle tissues ($P < 0.01$; Table 6), whereas no difference in expression was detected between liver and leg muscle tissues ($P > 0.05$; Table 6). At 3 h of thermal exposure, the relative expression level of *GRP78* mRNA in the brain remained high and was remarkably higher than that seen in other tissues ($P < 0.01$; Table 6). An intermediate *GRP78* mRNA expression level was seen in brain tissue and was remarkably higher than that in liver and leg muscle tissues ($P < 0.01$; Table 6), with no significant difference seen between liver and leg muscle tissues ($P > 0.05$; Table 6). After 6 h of thermal stress, the relative expression level of *GRP78* mRNA in the brain remained high and was significantly higher than that in other tissues ($P < 0.01$; Table 6), while no differences were observed among heart, liver, and leg muscle tissues ($P > 0.05$; Table 6).

Table 6. Comparison of tissue-specific *GRP78* mRNA expression in White Recessive Rock chickens.

Tissue	Period of exposure to thermal stress				
	0 h	1 h	2 h	3 h	6 h
Brain	1.63 ± 0.29^{A}	5.89 ± 0.53^{A}	6.67 ± 0.80^{Ab}	19.15 ± 2.74^{A}	3.63 ± 0.52^{A}
Heart	1.18 ± 0.21^{A}	6.62 ± 0.36^{A}	9.28 ± 0.72^{Aa}	9.98 ± 1.61^{B}	0.77 ± 0.16^{B}
Leg muscle	0.03 ± 0.01^{B}	0.10 ± 0.01^{B}	0.34 ± 0.07^{B}	0.36 ± 0.07^{C}	0.24 ± 0.03^{B}
Liver	0.21 ± 0.01^{B}	0.68 ± 0.15^{B}	0.88 ± 0.10^{B}	0.94 ± 0.11^{C}	0.38 ± 0.05^{B}

Data are reported as least-square means ± standard errors (SE). Differences between data with different superscript lowercase letters within a column are significant ($P < 0.05$), and differences between data with the different superscript capital letters are very significant ($P < 0.01$).

DISCUSSION

The HSP70 family is a family of highly conserved proteins that bodies produce and can maintain cells under many stringent conditions. A previous study by Tomoda et al. (2012) showed that SNPs in the *GRP78* gene were significantly associated with the development and recurrence of hepatocellular carcinoma in Japanese patients infected with the hepatitis C virus. Zhu et al. (2013) showed that the *GRP78* promoter haplotype and diplotype carrying rs391957, which is 415 bp, allele G and genotype GG were strongly associated with hepacellular carcinoma risk, and rs391957 was also shown to increase the affinity of the transcriptional activator Ets-2, resistance to apoptosis, as well as cell instability in stressful microenvironments.

Although many studies have identified GRP78 as one of the most important responders to disease-related stress, there are few reports of genetic variations that show an association with thermotolerance in chicken breeds. Chen et al. (2013) indicated that SNP C.-141G>A in the 5'-flanking region of the *Hsp90b* gene in chickens had some effect on thermotolerance traits, and this might be a potential molecular marker of thermotolerance. Liu et al. (2011) found that a novel SNP of the *ATP1A1* gene is associated with heat tolerance traits in dairy cows. In this study, 10 SNPs were identified in a 2400-bp 5'-flanking region of the chicken *GRP78* gene, and seven of these polymorphism sites caused transcription factor binding sites to disappear, occur, or change.

Heat stress in the summer is a serious issue that affects the production performance of chickens. Evaluation of heat tolerance in poultry is the basis for studying heat tolerance mechanisms and breeding heat-tolerant varieties. Our goal was to evaluate the association of polymorphisms in the 5'-flanking region of the *GRP78* gene with thermotolerance parameters

in order to find a molecular marker to evaluate and improve chicken heat stress adaptation.

We examined five physical and immune parameters, including corticosterone, T3, H/L, $CD3^+$ T cells, and $CD4^+/CD8^+$ ratio, at 35° and 15°C in WRR chickens.

Corticosterone is a kind of glucocorticoid that is secreted by HPA. Several studies found that stress increased corticosterone levels, which subsequently increased corticosterone secretion. Corticosterone can be treated as a physiological index to precisely evaluate the acute stress state of poultry (Post et al., 2003; Star et al., 2007). In addition, the rate of decrease in immune indices showed a significantly positive correlation with the rate of plasma corticosterone increase and was negatively associated with heat stress survival time at high temperatures.

Qi et al. (2012) also found the reduction of inflammatory cells in V-infected lungs because of higher GC levels that inhibited anti-virus immune response. T3 is produced by thyroid hormone through the effects of deiodinase and plays an important role in regulating metabolism and thermogenesis in chickens. Sinurat et al. (1987) indicated that the plasma T3 concentration decreased, but T4 concentration increased during exposure to high temperature. Heat stress resulted in deterioration of immune function and increased disease susceptibility in chickens.

When threatened by external factors (such as pathogen invasion and heat stress), lymphocyte and monocyte levels in blood decreased, and heterophilic granulocytes increased, resulting in an increase in the H/L ratio (Stevenson and Taylor, 1988). Numerous studies have confirmed that the H/L ratio can be an effective indicator of stress response and resistance (Campo and Davila, 2002).

CD3 is a marker for mature T-lymphocytes; therefore, the determination of peripheral blood $CD3^+$ T cells may be of importance in evaluating immunodeficiency. The ratio of $CD4^+$ and $CD8^+$ T cells are also important indices for evaluating immune status. During the process of antigen recognition, $CD3^+$ T cells conduct the signal, $CD4^+$ T cells assist in cell immunity and humoral immune response, and $CD8^+$ T cells are the main effector cells. In this study, corticosterone and T3 levels as well as H/L ratio were slightly elevated, and $CD4^+/CD8^+$ T cells and $CD3^+$ T cells showed a significant decrease within the same genotype in all sites at 35°C compared with the levels at 15°C. According to the study of Qi et al. (2012), we speculated that the corticosterone content was significantly increased after heat stress, which might be a reason for the decrease of the $CD4^+/CD8^+$ T cell ratio and $CD3^+$ T cell level.

Five sites, C.-744C>G, C.-965C>T, C.-990G>A, C.-1014T>C, and C.-1146A>G, were significantly associated with some of the examined physiological and immune indices. At 35°C, the corticosterone content was significantly lower in the GG genotype broilers than in the CG genotype at the C.-744C>G SNP site, and no differences were observed in corticosterone content in the GG genotype before and after heat stress. At 15°C, we also found significant association between the C.-744C>G SNP and $CD3^+$ cell levels ($P < 0.05$), and the $CD3^+$ levels were significantly higher in the GG genotype than in the CG genotype broilers. In addition, the C.-744C>G SNP was located in an NFκB50 binding site, as determined by transcription factor binding site analysis. Based on the results discussed above, the GG genotype of the C.-744C>G SNP promotes stronger thermotolerance than other two genotypes, and this site might have a potential hereditary effect on WRR chickens; individuals of the GG genotype may therefore exhibit improved thermotolerance.

At 15°C, we found a significant association between the SNP C.-965C>T site and the $CD4^+/CD8^+$ ratio, which was significantly higher in the TC genotype than in the CC genotype. However, the $CD4^+/CD8^+$ T cell ratio showed no significant differences within a genotype at 35°C. Significant association was observed between mutations at C.-990A>G and $CD3^+$ cell

levels at 15°C, but no association was detected at 35°C. The $CD3^+$ cell levels of AA genotype broilers were significantly higher than those in the GG genotype broilers under both conditions; therefore, allele A might be superior for heat stress tolerance. We also observed that $CD3^+$ cell levels were significantly associated with the C.-1014C>T SNP at 15°C but showed no association at 35°C. $CD3^+$ cell levels were significantly higher in CC genotype broilers than in TT genotype broilers under both conditions; therefore, allele C might be superior for heat stress tolerance. Moreover, significant association was observed between the C.-1146A>G SNP and $CD3^+$ cell levels at 15°C, with $CD3^+$ cell levels significantly higher in the AA genotype broilers than in the GG genotype broilers under both conditions. Consequently, allele A might be superior for heat stress tolerance. Overall, although the C.-990A>G, C.-965C>T, C.-1014C>T, and C.-1146A>G SNPs were all associated with immune indices at 15°C and caused alterations in transcription factor binding sites, no associations were demonstrated at 35°C.

When cells were exposed to various stimuli, such as heat, cold, or heavy metal stress, GRP78 overexpression immediately resulted in cell recovery and cell protection against stress (Luan et al., 2009; Stacchiotti et al., 2009). The upregulation of stress proteins is an important step for inhibiting protein aggregation and misfolding following stress (Richter-Landberg and Goldbaum, 2003). To investigate *GRP78* mRNA expression in tissues that were exposed to heat stress, fluorescent quantification using the RT-PCR assay was used to quantify *GRP78* mRNA expression, and the housekeeping gene *GAPDH* was used as a reference to normalize *GRP78* mRNA levels in order to reduce systematic errors.

In our study, at room temperature, the *GRP78* mRNA expression level was highest in the brain, intermediate in the heart and liver, and lowest in leg muscle tissue. With continued thermal stress, the *GRP78* gene mRNA expression of all tissues first increased and then decreased in WRR chickens. At 3 h, *GRP78* expression levels were much higher than at other periods of thermal stress exposure in the four tissues. Krivoruchko and Storey (2013) found that *GRP78* transcripts significantly increased in heart, kidney, and liver tissue of adult red-eared slider turtles after exposure to 5 h of anoxic submergence at 4°C and returned to aerobic control values after 20 h of anoxia. Our research indicates that the trend of the stress-induced response is similar in different tissues, which is in agreement with the results of Krivoruchko and Storey (2013).

GRP78 mRNA tissue-specific expression levels in WRR chickens were exposed to the same length of heat stress. Compared with leg muscle tissue, the *GRP78* mRNA expression levels in brain and heart tissue were high with continued thermal stress; this was related to blood supply, nervous immune system regulation, and functional recovery from stress damage. Chronic intermittent hypoxia, which might induce the ER stress, could also upregulate GRP78 transcription and expression in brain regions associated with learning and memory (Zhou et al., 2012). After 1 and 2 h of thermal stress, the *GRP78* mRNA expression level in liver tissue showed no difference, and the expression in heart tissue exceeded that in brain tissue. However, at 3 h of thermal exposure, the relative *GRP78* mRNA expression level in the brain was remarkably higher than that in other tissues. At 6 h of heat stress, the relative *GRP78* mRNA expression level in the brain was significantly higher than that in other tissues, while no difference was observed among heart, liver, and leg muscle tissues. The above results show that, upon exposure to some degree of heat stress, tissue cells develop resistance to heat.

CONCLUSIONS

The C.-744C>G SNP in the 5'-flanking region of the *GRP78* gene in WRR chickens was

significantly correlated with heat tolerance parameters under both conditions and may possess potential hereditary effects on heat tolerance; individuals with the GG genotype may show better thermotolerance compared with other genotypes. qRT-PCR assay indicated that *GRP78* mRNA expression in all tissues increased first, then decreased with continued thermal stress, and peaked at 3 h. We conclude that *GRP78* mRNA expression is time- and tissue-dependent.

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Supplementary material

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