Genetic polymorphism in *Hsp90AA1* gene is associated with the thermotolerance in Chinese Holstein cows

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Abstract

The heat shock protein 90 (*Hsp90*) is a copious and ubiquitous molecular chaperone which plays an essential role in many cellular biological processes. The objective of this study was to identify single nucleotide polymorphisms (SNPs) in the *Hsp90AA1* gene and to determine their association with heat stress traits in Chinese Holstein cattle breed. Direct sequencing was used to identify new SNPs. Luciferase reporter assay methods were used to assess g.-87G > C and g.4172A > G loci in the promoter activity and 3'-UTR, respectively. Quantitative real-time PCR was utilized to quantify the gene expression profile. Five SNPs were identified in 130 multiparous lactating cows: one SNP in the promoter, three SNPs in the coding region, and one in 3'-UTR were novel and reported for the first time in this study. As a result of promoter assay using dual luciferase assay system, the genotype CC showed the highest transcription activity region (13.67 ± 0.578) compared to the wild-type GG (3.24 ± 0.103). On the other hand, the result revealed that one of the selected microRNAs (dme-miR-2279-5p) was found to interact with the *Hsp90AA1* 3'-UTR sequence and to suppress the reporter activity markedly in the presence of the allele G (2.480 ± 0.136). The expression of *Hsp90AA1* in cow bearing mutant allele C was higher (4.18 ± 0.928) than cows bearing wild-type allele G (1.008 ± 0.0129) in stress season. In summary, there was an association between genetic variations in the *Hsp90AA1* and thermoresistance. This association could be used as a marker in genetic selection for heat tolerance in Chinese Holstein cattle breeds.

Keywords Chinese Holstein cattle · *Hsp90AA1* SNPs · Heat stress · Promoter activity · microRNA-mimic

Introduction

Heat stress is defined as a combination of environmental conditions that will cause the effective temperature to be higher than the temperature range of the animal's thermal neutral zone (Buffington et al. 1981). Moreover, the heat and humidity of a typical summer combine to make a very

uncomfortable environment specifically for the lactating dairy cows. Several previous studies have documented the genetic difference in thermotolerance at the physiological and cellular levels (Hansen 2004; Lacetera et al. 2006). Various cellular mechanisms are displayed to counter the stress conditions in dairy cattle including altered expression of stress candidate genes (Collier et al. 2008). The heat stress (HS) response is a highly conserved cascade of protein activation and altered gene expression in response to a variety of stressors (Collier et al. 2006). The gene expression component of this network is under heat shock transcription factor (HSF) regulation (Pirkkala et al. 2001; Page et al. 2006). The vital role that heat shock proteins (HSP) have in cytoprotection during HS is evidenced by the fact that HSP overexpression protects against hyperthermia shock during heat stroke (Lee et al. 2006). The chaperone *Hsp90* is one of the most abundant proteins in eukaryotic cells, comprising 1–2% of cellular proteins under non-stress conditions (Craven et al. 1996). There are two major cytoplasmic *Hsp90* isoforms, the inducible form (*Hsp90α*) and the constitutive form (*Hsp90β*), which have arisen by gene duplication (Chen et al. 2006). The

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presence of the *Hsp90AA1* gene in Deoni cattle (*Bos taurus*) was reported by Shergojry et al. (2012), who documented that the gene is polymorphic, and it showed a significant association with heat stress in Deoni cattle. The variations within *Hsp90AA1* gene in sheep were also reported by Marcos-Carcavilla et al. (2010), who reported that single nucleotide polymorphism (SNP) located at position – 660 in the promoter region was associated with thermal stress. In addition, polymorphism within *Hsp90AA1* that is associated with heat stress was detected within exon 3 in Sahiwal cows (Kumar et al. 2015) and exon 3 in Karan Fries (Kumar et al. 2016). The *Hsp90AA1* gene variants and association with thermotolerance in Chinese Holstein cattle breeds are still unclear. Therefore, this study was undertaken to mark the genetic variations in the *Hsp90AA1* gene at the single nucleotide level and its association with heat tolerance traits in Chinese Holstein cattle breed. Hence, these results may be useful for further genetic improvement in this breed for heat tolerance traits.

Materials and methods

Ethics statement

The study protocol was approved by the Institutional Care and Use Committee of Nanjing Agricultural University following the standard regulations for the administration of affairs concerning experimental animals (China 1988) and the standards for the administration of experimental practices (Jiangsu, China 2008).

Experimental animals and blood samples

A total of 130 Chinese Holstein cows were used in this study. These animals were maintained at the Tangquan and Xigang farms in Nanjing area, Jiangsu Province. All animals were in the same parity, physical, housing, and management conditions. Ten milliliters of blood was collected from a coccygeal

vein using 10-mL Vacutainer blood tubes (containing ethylenediamine tetraacetic acid, EDTA) as an anticoagulant and stored at – 20 °C until DNA extraction. Blood samples were collected one time from 130 cows in two Chinese Holstein dairy herds in thermoneutral season.

DNA extraction

Genomic DNA was isolated from whole blood using the E.Z.N.ATM Blood DNA Kit (Omega Bio-tek) according to the manufacturer's instructions. NanoDrop 1000 UV-Vis Spectrophotometer (Thermo-Fisher Scientific, Wilmington, DE) was used to measure DNA concentration and quality. Finally, the DNA was dissolved in 50 µL distilled water and kept at – 20 °C until used.

Primers design and PCR amplification

Five pairs of primers (P1, P2, P3, P4, and P5) were designed, based on the 5368-bp sequence for *Bos taurus Hsp90AA1* gene (NCBI–GenBank, reference sequence AC: 000178.1). Primers were designed to amplify 752, 719, 478, 720, and 997 bp (Table 1) fragments of the promoter, exon 2, exon 4, exon 8, and 3'-UTR regions, respectively, using Primer Premier 5.0 software.

Final amplification reaction (50 µL) involved a PCR master mix (25 µL), forward primer (2.5 µL), reverse (2.5 µL), ddH₂O (17.5 µL), and template DNA (2.5 µL). The PCR amplification condition includes pre-denaturation at 98 °C for 30 s followed by 35 cycles, denaturation at 98 °C for 10 s, annealing at 56 °C for 30 s, extension at 70 °C for 1:30 s, and a final extension at 72 °C for 7 min. PCR products (10 µL) were visualized on 1.0% agarose gels. The remaining amplicon segments were sequenced by GenScript USA Inc. (Piscataway, NJ). Sequencing results were visualized and edited using Chromas software (v.2.22), and each edited sequence was aligned with the corresponding reference sequence of *Hsp90AA1* in *B. taurus* using DNAMAN software (v.8.0).

Table 1 Primer sequences, annealing temperature, and expected the size of HSP90AA1 and GAPDH genes

Primer	Upstream primer (5'–3')	Downstream primer (5'–3')	Annealing temperature (C°)	Expected segment size (bp)
P1	GATCCCGTGCTAGTCCTTCA	TGATCTTGTCCAGGGCCTAT	56	752
P2	AGGAGGTGGAGACTTTCGC	GCCCAAGATAAACAGCCACA	56	719
P3	TCTCAGGTGGCGTTGGTGTA	GGAACAGAATGCTGCCTCAA	56	478
P4	GAGTGTGTCTTCTGCCCGTG	GGCTGAGCGAACTACAAGG	56	720
P5	TTGTAGTTCGCTCAGCCTTG	CCACCTGCTTCCTTGACTCT	56	997
P6	AGAGCCCTTCTTTTGTCCC	TCCTCAGAATCCACCACACC	60	167
P7	TGAAGGTCGGAGTGAACGGA	TGCCGTGGGTGGAATCATAC	60	153

Quantitative traits measurement

Combined effects of interaction of ambient temperature and relative humidity was measured by temperature humidity index (THI) in order to recognize heat load acuity (Berry et al. 1964). Ambient temperature and relative humidity (RH) were recorded daily at 8:00 AM, 2:00 PM, and 10:00 PM during March (non-stress season) and July (stress season). The average of THI in March (62.06 ± 5.85) and July (81.04 ± 0.96) were taken during which quantitative parameters were recorded and used in association analysis (Table S1). THI was calculated as $THI = 0.72 (T_d + T_w) + 40.6$ (NRC 1971), where T_d is dry-bulb temperature and T_w is wet-bulb temperature. The milk yield (kg) for each cow was recorded three times per day at 7:00 AM, 1:00 PM, and 7:00 PM during the study duration as quantitative parameters. Milk samples (≥ 30 mL) were collected two times/week for somatic cell count (SCC). The SCC was detected using FOSS (FossomaticTM FM, Hillerød, Denmark) machine in the Dairy Herd Improvement Center in Nanjing Agricultural University. The results of SCC of each sample were multiplied by 1000/mL.

Gene cloning and plasmid construction

Different fragments of *Hsp90AA1* with flanking (296 bp) and 3'-UTR (309 bp) restriction sites were cloned into pGL3 and pmirGLO Dual-Luciferase vectors, respectively. *Hsp90AA1* promoter and 3'-UTR primers were redesigned to include restriction sites KpnI and XhoI in both pGL3 (308 bp) and pmirGLO (321 bp) Dual-Luciferase vectors in the 5' end of upstream and downstream primers, respectively. The promoter regions inserted into pGL3-basic luciferase reporter vector lacks eukaryotic promoter and enhancer sequences (Promega, Madison, WI) while the 3'-UTR region was inserted into pmirGLO miRNA target sites 3 of the firefly luciferase gene (luc2). The sequences of genotype GG (wild-type) and CC (mutant) plasmids for the locus g.-87 in the flanking region, and genotype AA (wild-type) and GG (mutant) plasmids for locus g.4172 were synthesized by GenScript USA Inc. and Wuhan Genecreate Biological Engineering Co., Ltd., respectively. All constructed vectors were transformed into bacteria (*Escherichia coli* DH5 α cells) and cultured for 24 h in LB media. Plasmids were isolated following E.Z.N.A. Endo-free Plasmid DNA Mini Kit II manufacturer's protocol (OMEGA Bio-tek). The quality and concentration of plasmid DNA were performed by NanoDrop 1000 UV-Vis Spectrophotometer (Thermo-Fisher Scientific, Wilmington, DE).

Cell culture and pGL3 and pLR-TK vector co-transfection

The Henrietta Lacks (Hela) human cell line was cultured in RPMI 1640 media (Gibco Life Technologies, Carlsbad,

CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) and 100 IU/mL penicillin/mg/mL streptomycin (Gibco) in a humidified incubator at 37 °C with 5% CO₂. Later, when the cells attained 80–90% confluence, they were collected and re-cultured in a 24-well plate. The transfection vectors were achieved after cells reached 70–80% confluence using LipofectamineTM 2000 reagent (Invitrogen, USA) according to the manufacturer's instructions. For constitutive expression of *Renilla* luciferase and to correct the differences in transfection and harvesting efficiency, pRL-TK vector (Invitrogen) was co-transfected as an internal control value. After 48 h, cells were lysed and transferred into a clean 1.5-mL tube. Plasmids were transfected into cultured Hela cells and designed as pGL3-G308, pGL3-C308, and pGL3-basic.

Heat shock treatment

The promoter region of *Hsp90AA1* was inserted into pGL3-basic luciferase reporter vector and then co-transfected into Hela cells. Cells were cultured in a 24-well plate, and after 24 h, cells were heat shocked at 42 °C for 1 h. Finally, the cells were washed by 1× PBS and lysed using lysis buffer.

Cell culture, pmirGLO, pLR-TK vector, and microRNA co-transfection

For the luciferase reporter assay, Hela was sub-cultured in a 24-well plate for 24 h before transfection in PRIM 1640 growth media supplemented with 10% FBS and 100 IU/mL penicillin/mg/mL streptomycin. After cells reached 70–85% confluence, they were co-transfected with pmirGLO constructed vector containing the 3'-UTR alleles A (wild-type) and G. Two miRNA-mimics, efu-miR-202 (NCBI reference sequence NR_129127.1) and dme-miR-2279-5p (NCBI reference sequence NR_047702.1), and their negative controls (anti-miRNA-mimic) were synthesized by Wuhan Genecreate Biological Engineering Co., Ltd. (China). The miRNA-mimics (efu-miR-202 and dme-miR-2279-5p) and their negative controls were also co-transfected with the constructed reported plasmid to a final concentration of 20 nmol/ μ L. The transfection was performed by using LipofectamineTM 2000 reagent (Invitrogen, USA) according to the manufacturer's instructions. The pRL-TK vector (Invitrogen) was co-transfected as an internal control value. Vectors were designed as Hsp90A-miR-202, Hsp90G-miR-202, Hsp90-NC (efu-miR-202 mimic), and Hsp90A-miR-2279, Hsp90G-miR-2279, and Hsp90-NC (dme-miR-2279 mimic) for the pmirGLO vector.

Luciferase activity assay

At 48 h after transfection, lysate cells were used to measure the ratio of transcriptional activities of firefly to Renilla luciferase in the promoter and 3'-UTR targeted sites. The relative luciferase activity was determined on a GLOMAXTM 20/20 luminometer assay system (Promega) using Dual-Luciferase reporter assay reagents (Promega) according to the manufacturer's instruments. The luciferase reporter activity in both pGL3 and pmirGLO construction plasmids was normalized to Renilla activity.

Total RNA extraction and real-time reverse transcription

To determine the expression of *Hsp90AA1* mRNAs, blood samples were collected two times from six cows for each genotype GG and CC in the thermoneutral and stress period. Total RNA was isolated using TRIzol reagent. The concentration, purity, and integrity of the RNAs were confirmed using NanoDrop 1000 UV-Vis Spectrophotometer. The total RNA was reverse transcribed using a reverse transcription level kit (TaKaRa, Dalian, China) according to the manufacturer's description. The complementary DNA (cDNA) was kept at -20°C for further use.

Real-time polymerase chain reaction

The quantification of mRNA expression was done with quantitative RT-PCR to investigate the differential expression between wild and mutant types by using Fast-Start Universal SYBR Green Master (Rox), according to the company's instruction. Primer P6 was designed by Primer Premier 5.0 software to amplify 167 bp of the *Hsp90AA1* as shown in Table 1. The reaction solution was prepared in a total volume of 20 μL comprising 10 μL SYBR, 0.5 μL each of 10 μM forward and reverse primers, 7 μL DNase/RNase-free water, and 2 μL diluted cDNA. The reaction was performed using the following amplification conditions: 10 min at 95°C , 40 cycles of 15 s at 95°C (denaturation), and 60 s at 60°C (annealing + extension). All target genotype expressions of *Hsp90AA1* gene were normalized to the endogenous reference gene GAPDH by using an optimized comparative Ct ($2^{-\Delta\Delta\text{Ct}}$) value method (Livak and Schmittgen 2001), where $\Delta\Delta\text{Ct} = \Delta\text{Ct}_{\text{target}} - \Delta\text{Ct}_{\text{GAPDH}}$. Primer P7 (GenBank accession no. NM_001034034.2) was used to amplify bovine GAPDH gene sequence (Table 1). The relative expression of *Hsp90AA1* was analyzed through differential fold change.

Statistical analysis

Allele and genotyping frequency were calculated using POPGENE 32 software package (Yeh et al. 1999), with co-

dominant methods and eventual deviations from Hardy–Weinberg equilibrium evaluated in accordance with Michael (2008). Differences were considered significant when $P < 0.05$. Furthermore, the diploid/haploid data of the single population was analyzed to get the genetic information (genotype frequency, allele frequency, heterozygosity (He), polymorphism information content PIC). Two-locus LD method was utilized to analyze the linkage disequilibrium between the SNPs and alleles in this study, according to the diploid data of the single population as described previously by Yang et al. (2012). All data are presented as means $\pm$ standard error of mean. The association of SNP genotypes with milk yield and SCC phenotypes was analyzed by analysis of variance followed by LSD all pairwise comparison test of milk yield and SCC for gene $\times$ season method as applied in the general linear model (GLM) procedure of Statistix version 8.1 (analytical software). The statistical model includes genotypes $\times$ season, milk yield, and SCC. The statistical significance of mRNA expression between genotypes was analyzed using univariate analysis of variance procedure of the SPSS 19.0. P value of < 0.05 was established as threshold for statistical significance.

Results

SNP loci identified in *HSP90AA1* gene

The complete sequence of the *Hsp90AA1* gene was amplified using five primer pairs (P1, P2, P3, P4, and P5) for Chinese Holstein cattle (Table 1). Direct DNA sequencing identified five SNP loci: one SNP (g.-87 G > C) in 5'-UTR region, three SNPs (g.605 A > G, g.1662 T > G, g.2819 G > A) in codon region, and one SNP (g.4172 A > G) in 3'-UTR region and are shown in Table 2. DNA sequence oscillograms of different genotypes are shown in Fig. 1a, and the genotypes in each SNP are compared and shown in Fig. 1b.

Genetic analysis of the genotypes and SNPs in 5'-UTR, codon, and 3'-UTR regions

Analysis of genetic variations including region and position of the mutation, genotype and allele frequencies, heterozygosity (H), polymorphism information content (PIC), and Hardy–Weinberg equilibrium (H.W.P) are shown in Table 2. On the other hand, burrows, allele correlation, and chi-square for the two polymorphisms at g.605 A > G and g.1662 T > G are shown in Table 3. The five SNPs fitted the Hardy–Weinberg equilibrium ($P > 0.05$), and all homozygous mutant alleles in SNP loci had low genotype frequencies.

Table 2 Genotypes, alleles frequencies, and summary of genetic variation statistics for all five loci at the *HSP90AA1* gene

Position (bp)	Genotype	Gene frequency	Allele frequency	He ^a	PIC ^b	H.W.P. ^c
g.-87 G/C	GG 67	0.52	0.71	0.412	0.327	0.422
	GC 50	0.38				
	CC 13	0.10				
g.605 A/G	AA 71	0.55	0.75	0.375	0.305	0.320
	AG 53	0.41				
	GG 6	0.04				
g.1662 T/G	TT 67	0.55	0.75	0.375	0.305	0.320
	TG 53	0.41				
	GG 6	0.04				
g.2819 G/A	GG 78	60	0.76	0.365	0.298	0.738
	GA 43	0.33				
	AA 9	0.07				
g.4172 A/G	AA 75	0.58	0.76	0.365	0.298	0.849
	AG 48	0.37				
	GG 7	0.05				

^a Heterozygosity^b Polymorphism information content^c Hardy–Weinberg test *P* value**Fig. 1** Oscillograms (a) and alignment (b) of each genotype showing the mutations in the fragmentary amplification by P1 and P2. The oscillograms indicated two mutations in the promoter and two mutations in the coding regions. Alignment showing substitutions of bases (the bases in the red color), and the locus of the bases in *HSP90AA1* gene was marked with digits

Table 3 Analysis of linkage disequilibrium between alleles in the loci g.605 A > G and g.1662 T > G in the codon region

Locus	Allele	Locus	Allele	Burrows	Correlation	χ^2	Probability
g.605 A → G	A	g.1662 T → G	T	0.0745	0.4708	35.91	0.000
	G		G	0.0745	0.4708	35.91	0.000

Number of significant ($P < 0.05$) linkage disequilibria = 4. All χ^2 tests have one degree of freedom

Haploid/diploid and linkage disequilibrium analysis

Based on the classification of PIC (PIC value < 0.25 , low; $0.25 < \text{PIC value} < 0.5$, intermediate; and PIC value > 0.5 , high polymorphism), all the five SNPs were found with moderate polymorphism and heterozygosity. Genetic linkage analysis (Table 3) showed that alleles in the codon region g.605 A > G in exon 2 and g.1662 T > G in exon 4 were in highly significant linkage ($P < 0.001$).

Hsp90AA1 promoter activity

To explore whether the g.-87 G > C SNP could affect the promoter activity of the bovine *Hsp90AA1*, 296 bp of genomic DNA between -237 and +59 of each wild-type allele G and mutant allele C were cloned into a promoterless pGL3-basic vector (Promega, USA) KpnI and XhoI restriction sites (Fig. 2a, b) to construct pGL3-G308 and pGL3-C308 plasmids, which were subsequently transferred into Hela (Sigma USA). The promoter activity of each construct was evaluated by measuring relative luciferase efficacy of pGL3-G308 (3.24 ± 0.103) and pGL3-C308 (13.67 ± 0.578) in comparison to pGL3 basic vector (control). Four measurements were conducted for each sample and luciferase activity of the mutant plasmid was increased 297% when compared to the wild-type constructed plasmid (Fig. 2c).

Luciferase-based heat shock promoter assays

Co-transfected cells were heat shocked at 42 °C for 1 h to underline the effect of the polymorphism in the promoter region of *Hsp90AA1* on the heat tolerance. Then, we measured the luciferase reporter activity of the quadruplicate transfections 24 h immediately after heat shock treatment (0 h) and after 3 h in a luminometer. The heat-induced activity of wild-type and mutant promoter was found by comparing the ratio of firefly luciferase activity over Renilla luciferase activity in heat shock and control samples. The relative luciferase activity was highly significant ($P < 0.01$) in the presence of the allele C in both 0 h (13.28 ± 1.139) and 3 h (23.31 ± 0.988) in comparison to the allele G in 0 h (8.68 ± 0.461) and 3 h (5.05 ± 0.882) shown in Fig. 2d.

The effect of the genetic variation in the promoter region of the *Hsp90AA1* on the mRNA expression levels

To confirm whether the genetic variation in the promoter region of the *Hsp90AA1* gene can affect the level of the mRNA expression, we performed quantitative RT-PCR. The results showed that the value of mRNA fold of cows bearing the CC mutant is 4.179 ± 0.928 in the stress season which is significantly higher ($P < 0.01$) than the CC mRNA fold in non-stress season (0.753 ± 0.075). Moreover, animals bearing the CC mutant genotype of promoter SNP showed significantly ($P < 0.01$) higher *HSP90AA1* mRNA expression compared to those with the GG wild genotype (1.008 ± 0.129) in stress season (Fig. 2e). The CC mutant genotype mRNA was increased 3.171-fold from GG wild type in stress season. All expression values of wild and mutant types in non-stress and stress seasons are shown in Table S5.

Association analysis between five SNPs in *Hsp90AA1* and animal performance

Data of the effect of different SNPs on the animal phenotype were analyzed for association study using general linear model (GLM) procedure of Statistix software (version 8.1). The statistical analysis revealed a significant effect ($P < 0.05$) of genotypes of the five SNPs of *Hsp90AA1* gene on animal performance between hot and cool periods. However, genotypes CC (g.-87 G > C), GG (g.605A > G), GG (g.1662 T > G), GG, GA, AA (g.2819G > A), and AA, AG, GG (g.4172A > G) showed an insignificant difference ($P > 0.05$) in milk yield and SCC between two seasons (Figs. 3a, b; 4a, b; and 5a, b). However, genotypes GG in loci g.2819 (Fig. 4b) and g.4172 (Fig. 5b) showed a significant difference ($P < 0.05$) in the SCC between the two seasons (Figs. 5 and 6). All statistical association values of interaction between seasons and genetic types with milk yield and SCC traits in the Chinese Holstein cattle are summarized in Tables S2, S3, and S4.

Hsp90AA1 miRNA-mimic activity

To identify miRNAs that likely target the vicinity of the g.4172 A > G polymorphism in the *Hsp90AA1* 3'-UTR, first the website miRBase (<http://www.mirbase.org/>)

Fig. 2 Association analysis between SNP g.-87 G>C genotypes and promoter activity before heat shock (c) and after heat shock (d) in Hela cells, and assessment of relative mRNA expression of individual bearing genotypes GG and CC in non-stress and stress seasons (e). (a) and (b) are a plasmid and plasmid digestion. Luciferase activities were measured by using the Luciferase Assay System

Fig. 3 Correlation between SNP G>C g.-87 and milk yield (a) and somatic cell count (b) between seasons. Data were analyzed by analysis of variance with a general linear model using comparative means in Statistix (version 8.1). $P < 0.05$ considered significant

Fig. 4 Association between SNPs A > G g.605, T > G g.1662, and G > A g.2819 genotypes with milk yield (a) and somatic cell count (b) between seasons. Three SNPs were identified using sequencing

technique by GenScript USA Inc. (Piscataway, NJ). Data were analyzed by analysis of variance with a general linear model using comparative means in Statistix (version 8.1). $P < 0.05$ considered significant

(Kozomara and Griffiths-Jones 2014) was used, and that yielded two candidate miRNAs, miR-202 and miR-2279-5p, of which their seed sequences are complementary to the *Hsp90AA1* mRNA sequence around the g.4172 polymorphism (Fig. 6). In addition, 309 bp of genomic DNA between +4027 and +4336 was cloned into a pmirGLO

vector (Fig. 7a), and the plasmids were digested and ligated using KpnI and XhoI enzymes (Fig. 7b). The co-transfected miRNA-mimics with constructed vectors bearing the g.4172 A or G allele into Hela cell in comparison with a miRNA-mimic negative control were performed to determine the predicted targeting of efu-miR-202 and

Fig. 5 Correlation between SNP A > G g.4172 genotypes and milk yield (a) and somatic cell count (b) between seasons. Data were analyzed by analysis of variance with a general linear model using comparative means in Statistix (version 8.1). $P < 0.05$ considered significant

Fig. 6 Polymorphism in g.4172 in 3'-UTR of *Hsp90AA1* is predicted to be targeted by efu-miR-202 (a) and dme-miR-2279-5p (b). miRNAs with a seed sequence base pairing on g.4172 A/G were identified with microRNA database: miRBase website (<http://www.mirbase.org/>). The position of mutation; A and G bases were given in red color

dme-miR-2279 to the 3'-UTR of *Hsp90AA1*. A significant increase ($P < 0.01$) of the luciferase activity with the reporter bearing g.4172A (3.380 ± 0.099) was observed, compared to that bearing g.4272G (2.480 ± 0.136). Furthermore, the Renilla expression of reporter vector bearing g.4172G allele was found to be significantly lower ($P < 0.01$) than its negative control (3.365 ± 0.033) in HeLa cells, indicating that the miR-2279 might be regulating the reporter expression (Fig. 7d). In contrast, miR-202 mimic co-transfection did not exhibit any significant change ($P < 0.05$) to the luciferase activity between reporter bearing g.4172A or G allele and their negative controls (Fig. 7c). The experiment was independently repeated twice, and each sample was evaluated in quadruplicate.

Discussion

Heat stress poses an adverse impact on reproduction and production of all domestic livestock, especially dairy animals of high genetic merit (Sailo et al. 2015). Dairy cows have developed a wide range of different strategies for adaptation to minimize environmental hazards (Bedulina et al. 2013). When cells are exposed to conditions of proteotoxic stress (e.g., heat shock), the expression of HSP families is induced (Morimoto 1998). The intensity of expression of *Hsp90* was found to be different in response to acute heat stress and has previously been reported in chicken (Wan et al. 2017), bay scallops (Yang et al. 2015), and bovines (Hu et al. 2016).

However, there were no comparative studies on possible polymorphisms in the *Hsp90AA1* and their association with heat tolerance in Chinese Holstein cows. We screened SNPs and their polymorphism in *Hsp90AA1* to confirm whether cellular thermotolerance is associated with the polymorphisms in the *Hsp90AA1* gene.

In the present work, a total of five SNP sites were identified in *Hsp90AA1* gene; one in the promoter (g.-87G > C), three in the coding (g.605 A > G, 1662 T > G, 2819 G > A), and one in 3'-UTR (g.4172 A > G) regions. Data analysis revealed that all genotype distributions in all the five loci were in accordance with Hardy-Weinberg equilibrium, as well as showed moderate PIC and heterozygosity. Our results showed that both genotype and allele frequencies remain constant in Chinese dairy herds. Additionally, our findings affirm that Chinese Holstein population might have moderate allelic variation and half of the individuals under study carry two different alleles from *Hsp90AA1* approximately. Furthermore, we detected a complete significant linkage ($P > 0.01$) between alleles in loci sites g.605 A > G and g.1662 T > G. This clarifies the association between the frequency of alleles at both loci, and these loci are extremely close together and virtually never separated by crossing over. It was previously reported that polymorphisms in *Hsp90AA1* could be associated with heat tolerance, where several studies were conducted to find a link between the thermal-stress-related phenotypes and genotypes. Kumar et al. (2015) reported that polymorphism in exon 3 of *Hsp90AA1* was associated with respiratory rate (RR) and rectal temperature (RT) in Sahiwal cows. Moreover, the polymorphisms in *Hsp90AA1* were found to be associated with heat tolerance coefficient (HTC) in Thai cattle (Charoensook et al. 2012), Frieswal cattle in India (Deb et al. 2013), and sheep breeds from different countries (Marcos-Carcavilla et al. 2010).

The correlation between different genotypes with milk yield and SCC in stress and non-stress seasons showed that cows bearing GG genotype in both g.605 and g.1662 loci have no significant difference in milk yield and SCC between the two seasons (Fig. 4a, b), while only the wild-type allele G in g.2819 locus has higher SCC in stress season compared to non-stress season (Fig. 4b). Our results might confirm the superiority of the genotypes GG in SNPs g.506 and g.1662 (in completing linkage) to heat tolerance among other genotypes at the same loci. Moreover, all three genotypes at the g.2819 locus may be associated with heat tolerance. This indicates that the bovine *Hsp90AA1* gene may have an essential role in the adaptation of Chinese Holstein cows to heat stress.

The genetic variations in *Hsp90* genes have been identified to be associated with heat regulation traits in cattle (Charoensook et al. 2012; Shergojry et al. 2012). Few studies have been conducted to investigate the association between heat tolerant and SNPs in 5'- and 3'-UTRs in the heat shock proteins (Lacetera et al. 2006; Erhardt and Weimann 2007;

Fig. 7 A 309 bp of *HSP90AA1* with A allele or G allele in the 3'-UTR region inserted into pmirGLO miRNA target sites between restriction sites KpnI and XhoI (**a**). the plasmid was digested and visualized (**b**). miRNA mimics (20 nM) of miR-202, miR-2279, and a negative control (NC) were co-transfected with pmirGLO Dual-Luciferase vectors and an internal transfection control pRL-TK vector into Hella cells. After 48 h of transfection, luciferase activities (firefly luciferase activity was normalized to Renilla luciferase expression) in miR-202 or miR-2279-

transfected cells were measured and compared to those in negative control-transfected cells. Transcripts with miR-202 seed sites (white) did not affect the luciferase reporter activity (**c**); however, transcripts with miR-2279 seed sites can specifically repress luciferase reporter activity. The luciferase expression registered with G mutant allele (white) was lower than those observed with control (black) and A wild-type allele (**d**). Representative data from triple independent experiments are shown with mean $\pm$ SE

Basiricò et al. 2011). Previous studies reported that the changes in nucleotide sequence in the 5'-UTR regions of a *Hsp90AA1* gene could influence the level of expression and adaptation to different environmental conditions in sheep (Marcos-Carcavilla et al. 2010; Salces-Ortiz et al. 2013). Yang et al. (2015) found genetic variations in the promoter of *Hsp90* gene associated with heat tolerance in bay scallops. However, quantitative studies of this promoter activity in transfection cells (in vitro) in cattle (general) and in Chinese Holstein cows (particularly) have not been reported to date. In the present study, analysis of the allelic and genotypic frequencies showed that the g.-87G > C SNP was in Hardy-Weinberg balance, and the genotype frequency of the wild-type GG is greater than mutant CC.

The promoter activity assay was conducted in order to try and find out whether there is an association between genetic variation in the promoter of *Hsp90AA1* gene and some

phenotypic traits. This was achieved by luciferase activity assay to confirm whether the wild allele G or mutant allele C can affect the promoter activity of the *Hsp90AA1* in Chinese Holstein cows. It was observed that the luciferase activity in both recombinant vectors pGL3-G308 and pGL3-C308 was significantly higher than the control (pGL3-basic vector). Furthermore, the promoter activity of pGL3-C308 was highly significant ($P < 0.01$) compared to pGL3-G308 (Fig. 2c). This result was confirmed by the heat shock experiment, as the relationship between SNP in the promoter of the *Hsp90AA1* gene and the heat stress was examined. The results also demonstrated that the activity of the reporter luciferase gene increased in a highly significant manner in the presence of the mutant allele C compared to the wild-type allele G (Fig. 2d). These results indicate that the genetic variation in the promoter region might play an essential role in the heat tolerance by inducing expression of *Hsp90AA1* gene.

To verify whether the genetic polymorphism in promoter can contribute to induction of *Hsp90AA1* gene in response to thermal stress, we examined the expression level of *Hsp90AA1* mRNA for cows bearing the wild-type G allele and those bearing mutant allele C in thermoneutral and thermal stress seasons. This study revealed a significant expression of CC mutant genotype during stress period compared to the non-stress season. Additionally, the expression level of CC mutant was higher than wild-type GG during combined effect of heat and humidity (Fig. 2e). Results confirmed those obtained by the promoter activity assay for both genotypes GG and CC in vitro. Additionally, both results of promoter activity and qRT-PCR provided additional support for the association between this SNP marker and milk production traits. The findings deduced lack of significant difference ($P > 0.05$) in the performance of individuals bearing CC genotype between seasons (Fig. 2e). Our findings further showed that the CC mutant genotype has no significant difference milk yield (550.37 ± 31.185) and SCC (22.957 ± 2.615) during thermoneutral period compared to milk yield (480.87 ± 31.102) and SCC (29.367 ± 8.778) in the stress season. Based on the results, it can be concluded that the mutant allele C could affect the promoter activity of the *Hsp90AA1* gene. In other words, the present finding might support the idea that the bovine *Hsp90AA1* promoter can drive efficient heat tolerance. Consequently, polymorphisms in bovine *Hsp90AA1* 5'-UTR could modify the inducible expression of this gene in response to heat stress, and it could be considered as the heat sensitivity/tolerance-related SNP marker.

It has been established that miRNAs are post-transcriptional regulators that manage mRNA stability and the translation competence as well as has the potential of regulating multiple protein coding by binding to the imperfect complementary sequence in their target genes (Jin et al. 2013). This means that the actions of miRNAs are not gene specific, but sequence specific, so they can operate on any genes comprising motifs matching their seed sites. Therefore, verification of interaction between the miRNA and its targeting seed sites in the mRNAs is **necessary** for understanding miRNA functions. In the present study, we identified SNP g.4172 A > G in the bovine *Hsp90AA1* 3'-UTR by DNA sequencing. The allele frequencies and genotypic distribution analysis revealed that the wild-type AA has higher genotypic frequencies (0.58) in comparison with mutant GG genotype. Furthermore, the polymorphism information content and He were moderate, and genotypes met Hardy-Weinberg equilibrium (Table 2).

To interpret the miRNA function, we examined the interaction between miRNA and its targeting seed sites in the mRNAs by using miR-mimic assay. The prediction of the miRNA target site revealed that efu-miR-202 in the presence of the A allele and dme-miR-2279 in the presence of the G allele could bind to the 3'-UTR region of the *Hsp90AA1* gene.

In molecular biology, miR-202 and miR-2279 are short RNA molecules that regulate the expression levels of other genes by several mechanisms. In the present study, we used dual luciferase reporter assay to determine the functional importance of g.4172A > G transition. Compared to the allele A and negative control, dem-miR-2279 significantly suppressed the luciferase expression in the presence of allele G ($P < 0.01$). In contrast, efu-miR-202 did not significantly affect luciferase expression ($P > 0.05$) in Hela cells in vitro (Fig. 7c, d). Trott et al. (2014) reported that polymorphism in 3'-UTR may be located within or in the vicinity of the miRNA binding site and could interfere with miRNA function and lead to differential gene expression level, thereby affecting the animal performance traits. These findings are supported by evidence of animal performance under different environmental conditions. In the present study, individuals bearing allele G showed significantly higher ($P < 0.05$) SCC during stress periods (199.500 ± 30.5000) compared with non-stress periods (54.250 ± 21.2500). On the other hand, individuals bearing allele A have no significant difference ($P > 0.05$) in SCC during the different seasons (Fig. 5b). This may be caused by the miR-2279-mimic down-regulation of the *Hsp90AA1* gene expression in animals bearing g.4172G allele which in turn elevated the amount of SCC in milk during stress periods. Moreover, we suggested that miR-2279 direct binding to the *Hsp90AA1* 3'-UTR region might mediate differential modulation of *Hsp90AA1* expression, which in turn alters the density of this gene in cells.

Conclusion

The genetic variations within cattle with respect to heat stress adaptations may provide efficient tools to select productive and thermotolerant animals. Our results suggest that 5'- and 3'-UTRs of the *Hsp90AA1* gene were polymorphic and had significant associations with milk production trait. The role of *Hsp90AA1* in regulating thermotolerance proposes that there is an opportunity to study the association between polymorphisms within the *Hsp* genes, physiological response to heat stress, and production traits in dairy cattle. In consequence, the genetic polymorphisms observed in *Hsp90AA1*'s association with animal performance traits reveal their importance as potential genetic markers and could be applied in the future molecular-assisted selection programs in Chinese Holstein cattle breed.

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References

- Basiricò L, Morera P, Primi V, Lacetera N, Nardone A, Bernabucci U (2011) Cellular thermotolerance is associated with heat shock protein 70.1 genetic polymorphisms in Holstein lactating cows. *Cell stress and Chaperones* 16(4):441–448. <https://doi.org/10.1007/s12192-011-0257-7>
- Bedulina D, Evgen'ev M, Timofeyev M, Protopopova M, Garbuz D, Pavlichenko V, Luckenbach T, Shatilina Z, Axenov-Gribanov D, Gurkov A (2013) Expression patterns and organization of the hsp70 genes correlate with thermotolerance in two congener endemic amphipod species (*Eulimnogammarus cyaneus* and *E. verrucosus*) from Lake Baikal. *Mol Ecol* 22(5):1416–1430. <https://doi.org/10.1111/mec.12136>
- Berry I, Shanklin M, Johnson H (1964) Dairy shelter design based on milk production decline as affected by temperature and humidity. *Transactions of the ASAE* 7: 329–331. doi:<https://doi.org/10.13031/2013.40772>@1964.
- Buffington D, Collazo-Arocho A, Canton G, Pitt D, Thatcher W, Collier R (1981) Black globe-humidity index (BGHI) as comfort equation for dairy cows. *Transactions of the ASAE* 24(3):711–714. <https://doi.org/10.13031/2013.34325>
- Charoensook R, Gatphayak K, Sharifi AR, Chaisongkram C, Brenig B, Knorr C (2012) Polymorphisms in the bovine HSP90AB1 gene are associated with heat tolerance in Thai indigenous cattle. *Trop Anim Health Prod* 44(4):921–928. <https://doi.org/10.1007/s11250-011-9989-8>
- Chen B, Zhong D, Monteiro A (2006) Comparative genomics and evolution of the HSP90 family of genes across all kingdoms of organisms. *BMC Genomics* 7: 156. doi:<https://doi.org/10.1186/1471-2164-7-156>.
- Collier R, Collier J, Rhoads R, Baumgard L (2008) Invited review: genes involved in the bovine heat stress response. *J Dairy Sci* 91(2):445–454. <https://doi.org/10.3168/jds.2007-0540>
- Collier RJ, Stiening C, Pollard B, VanBaale M, Baumgard L, Gentry P, Coussens P (2006) Use of gene expression microarrays for evaluating environmental stress tolerance at the cellular level in cattle. *J Anim Sci* 84: E1–E13. doi:https://doi.org/10.2527/2006.8413_supplE1x.
- Craven R, Egerton M, Stirling, C (1996) A novel Hsp70 of the yeast ER lumen is required for the efficient translocation of a number of protein precursors. *EMBO J* 15: 2640.
- Deb R, Sajjanar B, Singh U, Kumar S, Brahmane M, Singh R, Sengar G, Sharma A (2013) Promoter variants at AP2 box region of Hsp70.1 affect thermal stress response and milk production traits in Frieswal cross bred cattle. *Gene* 532(2):230–235. <https://doi.org/10.1016/j.gene.2013.09.037>
- Erhardt E, Weimann C (2007) Use of molecular markers for evaluation of genetic diversity and in animal production. *Arch Latinoam Prod Anim* 15
- Hansen P (2004) Physiological and cellular adaptations of zebu cattle to thermal stress. *Anim Reprod Sci* 82:349–360. <https://doi.org/10.1016/j.anireprosci.2004.04.011>
- Hu H, Zhang Y, Zheng N, Cheng J, Wang J (2016) The effect of heat stress on gene expression and synthesis of heat-shock and milk proteins in bovine mammary epithelial cells. *Anim Sci J* 87(1):84–91. <https://doi.org/10.1111/asj.12375>
- Jin Y, Chen Z, Liu X, Zhou X (2013) Evaluating the microRNA targeting sites by luciferase reporter gene assay. *MicroRNA Protocols*. https://doi.org/10.1007/978-1-62703-083-0_10
- Kozomara A, Griffiths-Jones S (2014) miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res* 42(D1):D68–D73. <https://doi.org/10.1093/nar/gkt1181>
- Kumar R, Gupta I, Verma A, Verma N, Vineeth M (2015) Genetic polymorphisms within exon 3 of heat shock protein 90AA1 gene and its association with heat tolerance traits in Sahiwal cows. *Veterinary world* 8(7):932–936. <https://doi.org/10.14202/vetworld.2015.932-936>
- Kumar R, Gupta ID, Verma A, Singh SV, Verma N, Vineeth M, Magotra A, Das R (2016) Novel SNP identification in exon 3 of HSP90AA1 gene and their association with heat tolerance traits in Karan fries (*Bos taurus* × *Bos indicus*) cows under tropical climatic condition. *Trop Anim Health Prod* 48(4):735–740. <https://doi.org/10.1007/s11250-016-1016-7>
- Lacetera N, Bernabucci U, Scalia D, Basiricò L, Morera P, Nardone A (2006) Heat stress elicits different responses in peripheral blood mononuclear cells from Brown Swiss and Holstein cows. *J Dairy Sci* 89(12):4606–4612. [https://doi.org/10.3168/jds.S0022-0302\(06\)72510-3](https://doi.org/10.3168/jds.S0022-0302(06)72510-3)
- Lee W, Wen H, Chang C, Chen M, Lin MT (2006) Heat shock protein 72 overexpression protects against hyperthermia, circulatory shock, and cerebral ischemia during heatstroke. *J Appl Physiol* 100(6): 2073–2082. <https://doi.org/10.1152/japplphysiol.01433.2005>
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻ΔΔCT method. *Methods* 25(4):402–408. <https://doi.org/10.1006/meth.2001.1262>
- Marcos-Carcavilla A, Mutikainen M, González C, Calvo JH, Kantanen J, Sanz A, Marzanov NS, Pérez-Guzmán MD, Serrano M (2010) A SNP in the HSP90AA1 gene 5' flanking region is associated with the adaptation to differential thermal conditions in the ovine species. *Cell Stress and Chaperones* 15(1):67–81. <https://doi.org/10.1007/s12192-009-0123-z>
- Michael CA (2008) Simple calculator to determine whether observed genotype frequencies are consistent with Hardy–Weinberg equilibrium. [http://www.Tufts.Edu/~mcourt01/documents.Court lab-HW calculator. Xls](http://www.Tufts.Edu/~mcourt01/documents.Court%20lab-HW%20calculator.Xls)
- Morimoto RI (1998) Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes Dev* 12(24):3788–3896. <https://doi.org/10.1101/gad.12.24.3788>
- National Research Council (NRC) (1971) A guide to environmental research on animals. Natl. Acad. Sci, Washington, DC
- Page TJ, Sikder D, Yang L, Pluta L, Wolfinger RD, Kodadek T, Thomas RS (2006) Genome-wide analysis of human HSF1 signaling reveals a transcriptional program linked to cellular adaptation and survival. *Mol Biosyst* 2(12):627–639. <https://doi.org/10.1039/B606129J>
- Pirkkala L, Nykänen P, Sistonen L (2001) Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. *FASEB J* 15: 1118–1131. doi:<https://doi.org/10.1096/fj00-0294rev>.
- Sailo L, Gupta I, Verma A, Singh A, Vishwas M, Chaudhari RD, Upadhyay R, Goswami J (2015) Single nucleotide polymorphism in HSP90AB1 gene and its association with thermo-tolerance in Jersey crossbred cows. *Animal science* 9.
- Salces-Ortiz J, González C, Moreno-Sánchez N, Calvo JH, Pérez-Guzmán MD, Serrano MM (2013) Ovine HSP90AA1 expression rate is affected by several SNPs at the promoter under both basal and heat stress conditions. *PLoS One* 8(6):e66641. <https://doi.org/10.1371/journal.pone.0066641>
- Shergojry S, Ganayi B, Ramesha K, Pirzada A (2012) Single strand conformation polymorphism (SSCP) of HSP90AA1 gene in Deoni cattle. *Animal Science Reporter* 6:65–69
- Trott JF, Freking BA, Hovey RC (2014) Variation in the coding and 3' untranslated regions of the porcine prolactin receptor short form modifies protein expression and function. *Anim Genet* 45: 74–86. <https://doi.org/10.1111/age.12100>, 1.
- Wan Y, Ma C, Wei P, Fang Q, Guo X, Zhou B, Jiang R (2017) Dynamic expression of HSP90B1 mRNA in the hypothalamus of two Chinese chicken breeds under heat stress and association analysis with a SNP in Huainan chickens. *Czech Journal of Animal Science* 62: 82–87. <https://doi.org/10.17221/8/2016-CJAS>.

- Yang C, Wang L, Liu C, Zhou Z, Zhao X, Song L (2015) The polymorphisms in the promoter of HSP90 gene and their association with heat tolerance of bay scallop. *Cell Stress and Chaperones* 20(4): 297–308. <https://doi.org/10.1007/s11033-011-1175-6>
- Yang F, Li L, Liu H, Cai Y, Wang G (2012) Polymorphism in the exon 4 of β -lactoglobulin variant B precursor gene and its association with milk traits and protein structure in Chinese Holstein. *Mol Biol Rep* 39(4):3957–3964. <https://doi.org/10.1007/s11033-011-1175-6>
- Yeh F, Yang R, Boyle T (1999) POPGENE 32-version 1.31. Population genetics software. University of Alberta, Edmonton, AB Canada T6G 2H1.