The HSP90AA1 Gene Is Involved in Heat Stress Responses and Its Functional Genetic Polymorphism Are Associated with Heat Tolerance in Holstein Cows

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ABSTRACT

As the stress-inducible isoform of the Heat Shock Protein 90 (HSP90), the HSP90AA1 gene encodes HSP90α and plays an important role in heat stress (HS) response. Therefore, this study aimed to investigate the role of the HSP90AA1 gene in cellular responses during HS and to identify functional single nucleotide polymorphisms (SNP) associated with thermostolerance in Holstein cattle. For the in vitro validation experiment of acute HS, cells from the Madin-Darby bovine kidney (MDBK) cell line were exposed to 42°C for 1 h, and various parameters were assessed, including cell apoptosis, cell autophagy, and the cellular functions of HSP90α by using its inhibitor 17-allylamino-17-de- methoxygeldanamycin (17-AAG). Furthermore, the polymorphisms identified in the HSP90AA1 gene and their functions related to HS were in vitro validated. Acute HS exposure induced cell apoptosis, cell autophagy, and upregulated expression of the HSP90AA1 gene. Inhibition of HSP90α by 17-AAG treatment had a significant effect on the expression of the HSP90α protein (P < 0.05) and increased cell apoptosis. However, autophagy decreased in comparison to the control treatment when cells were exposed to 42°C for 1 h. Five SNPs identified in the HSP90AA1 gene were significantly associated with rectal temperature (RT; P < 0.05) and respiration score (RS; P < 0.05) in Holstein cows, in which the rs109256957 SNP is located in the 3′ untranslated region (3′ UTR) of the HSP90AA1 gene. Furthermore, we demonstrated that the 3′ UTR of HSP90AA1 is a direct target of bta-miR-1224 by cell transfection with exogenous miRNA mimic and inhibitor. The luciferase assays revealed that the SNP rs109256957 affects the regulation of bta-miR-1224 binding activity and alters the expression of the HSP90AA1 gene. Heat stress-induced HSP90AA1 expression maintains cell survival by inhibiting cell apoptosis and increasing cell autophagy. The rs109256957 SNP located in the 3′ UTR region is a functional variation and it affects the HSP90AA1 expression by altering its binding activity with bta-miR-1224, thereby associating with the physiological parameters of Holstein cows.

Key Words: HSP90AA1, heat stress, cell apoptosis, cell autophagy, genetic variation

INTRODUCTION

The severity of global warming is becoming evident in modern times and has caused serious economic losses, animal welfare issues, and food security challenges (IPCC, 2018). Dairy cows are essential for food production, but high-producing cows are more susceptible to heat stress (Tao et al., 2018), highlighting the need for the development of mitigation strategies such as genetic selection (Mauger et al., 2015; Min et al., 2017). For instance, genetic selection for improved heat tolerance in dairy cows has become a reality in the Australian (www.holstein.com.au/services/genetics/genomic-testing) and Italian (www.holsteininternational.com/en/story/new-heat-tolerance-index-in-italy) dairy industries since 2017 and 2022, respectively. In addition, when performing genetic selection based on the additive genetic effect for heat tolerance, there is a great value in understanding the biological and genomic mechanisms influencing heat stress (HS) response in the species of interest.

Heat shock proteins (HSP) act as molecular chaperones that are preferentially transcribed in response to HS (Abbas et al., 2020; Fang et al., 2021). HSP90 is one of the most studied genes of the HSPs family and has been associated with HS response in cattle.
There are 2 main cytoplasmic HSP90 isoforms, including the inducible form (HSP90α) and the constitutive form (HSP90β), which resulted from gene duplication (Chen et al., 2006). HSP90α is an intracellular molecular chaperone that functions as a homodimer and aids in the proper folding of specific target proteins through an ATPase activity that is modulated by co-chaperones. Its secretion is regulated by the C-terminal EEVD motif via interacting with proteins containing tetratricopeptide repeat domains (Wang et al., 2009).

Apoptosis and autophagy are 2 important processes of the cellular stress response and reflect a spectrum of cellular activities that varies based on the severity of the stress source (McCormick et al., 2021). During HS, cell apoptosis is caused by the disruption of dynamic mitochondrial networks (Chen et al., 2020). Another indelible phenomenon is cell autophagy, which can result from elevated levels of reactive oxygen species (ROS) during HS (Kassis et al., 2021). A recent study reported that the HSP90α gene increased chemoresistance in osteosarcoma cells by inhibiting apoptosis and inducing autophagy through the PI3K/Akt/mTOR pathway (Xiao et al., 2018). Moreover, the HSP90α gene induced autophagy in early avibirnavirus infection through the Akt/mTOR pathway, thus highlighting the role of HSP90α in autophagy (Hu et al., 2015). However, little is known about the functional role and significance of HSP90α in heat-induced cell apoptosis and autophagy.

HSP90α is encoded by the heat shock protein 90 α family class A member 1 gene (HSP90AA1), which is located on the Bos taurus autosome 21 and spans nearly 8,137 bp comprising 11 exons, out of which the first exon is not translated. Several studies have reported the upregulation of the HSP90AA1 gene in cattle under HS conditions (Zeng et al., 2014; Khan et al., 2020; Fang et al., 2021). When Sahiwal (Bos taurus indicus) was compared with Frieswal (Bos taurus taurus × Bos taurus taurus), experimental cells in Sahiwal exhibited a greater HSP90AA1 mRNA expression (Deb et al., 2014), indicating that genetic factors may affect the expression of HSP90AA1 and regulation of HS response. Moreover, polymorphic variations in the HSP90AA1 gene were found to be significantly associated with thermoregulatory traits in Deoni (Shergojry et al., 2012), Sahiwal (Kumar et al., 2015), and Karan Fries (Kumar et al., 2016) cattle populations. Single nucleotide polymorphisms (SNP) have been identified in functional regions (e.g., promoter, 3’ untranslated region – UTR) of the HSP90AA1 gene in Holstein cattle (Badri et al., 2018). However, the molecular mechanism and the role of HSP90AA1 and its SNP in heat stress-induced cell autophagy and apoptosis are still unclear in Holstein cows.

Therefore, the primary objectives of this study were to: 1) investigate the cellular and molecular functions of the HSP90AA1 gene in heat-induced cell apoptosis and autophagy using the Madin–Darby bovine kidney cells as a cellular model; 2) identify SNP associated with HS response in dairy cows; and 3) investigate the underlying molecular regulatory mechanism of the identified functional SNP.

MATERIALS AND METHODS

Animals and Sample Collection

This study was approved by the Animal Care and Use Committee of Beijing Jiaotong University (Protocol number: SS-QX-2014–06). A total of 1,655 healthy Holstein cows owned by the Beijing Sunlon Livestock Development Co., Ltd. (Beijing, China) were selected for blood sample collection via a dorsal vein in the tail using ethylene diamine tetraacetic acid (EDTA) as an anticoagulant. These cows were from 9 farms located in the Beijing region, China. The number of individuals born before 2010, in 2010, 2011, 2012, 2013, 2014, 2015 were 107, 121, 194, 287, 316, 452, and 178, respectively. Their parities ranged from 1 to 8, with a mean of 1.56 and a median of 1.00. Their lactation stage ranged from 4 to 575 d in milk (DIM), with a mean of 143.35 and a median of 123.00. DNA samples were further extracted for SNP screening in the HSP90AA1 gene according to the manufacturer’s instructions (DE-05121, Chengdu Foregene Biotechnology Co., Ltd., Chengdu, Sichuan, China) from August to September, 2019.

SNP Screening

According to the reference gene sequence of bovine HSP90AA1 (ENSBTAT000000008225.3), specific primers were used to amplify its exons and 2,000 bp of upstream and downstream genomic regions (Supplemental Table S1: https://doi.org/10.6084/m9.figshare.24534808; Hu, 2023a). Polymerase chain reaction (PCR) was performed using pool DNA containing equivalent genomic DNA from 20 unrelated Holstein cows based on their pedigree information. In summary, they originated from the same farm and were all born in 2011. At the time of blood sampling, they were in their second lactation and had DIM ranging from 63 to 314 d. The amplification system and reaction conditions were in accordance with our previous study (Hu et al., 2019). The PCR products were sequenced both in forward and reverse directions. Candidate SNPs were detected by visualization of the sequencing peaks and
alignment analyses using the DNAMAN 5.2.2 software (Lynnon Biosoft, St-Louis, QC, Canada). Subsequently, 1,655 cows were genotyped using competitive allel-specific PCR (KASP).

**Cell Culture**

The Madin–Darby bovine kidney (MDBK) and human embryonic kidney (HEK) 293T cell lines were cultured in Dulbecco’s modified eagle medium (DMEM, Gibco Invitrogen, La Jolla, CA, USA) supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 1% (vol/vol) penicillin–streptomycin (Gibco Invitrogen, La Jolla, CA, USA). Given the limitations associated with the MDBK cell line (derived from kidney tissue) as a systemic cow model, we also isolated the peripheral blood mononuclear cells (PBMC) to evaluate the usefulness of MDBK cells. The PBMC were isolated from blood samples of experimental cows within 4 h of collection by using the Ficoll-Paque density gradient centrifugation and cultured in the Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco Invitrogen, La Jolla, CA, USA) containing 10% (vol/vol) FBS and 1% (vol/vol) penicillin-streptomycin, as previously described (Fang et al., 2021).

**Heat Stress Treatment**

Cells were exposed to 42°C for 1 h to mimic an acute heat treatment (HS) and 37°C for 1 h as the thermo-neutral group (TN), with the parameters of temperature and duration defined based on our previous study (Fang et al., 2021). A humidified atmosphere consisting of 95% air and 5% CO2 was used. Specifically, the 1-h exposure time was chosen to ensure the reproducibility and control of the experiment. Longer exposure times could result in cell death or irreversible damage, making the interpretation of the results more difficult. In addition, the exposure temperature of 42°C for simulating HS conditions is also commonly used in the literature (e.g., Kishore et al., 2014; Kim et al., 2020).

**17-allylamino-17-demethoxygeldanamycin (17-AAG) Treatment**

The 17-allylamino-17-demethoxygeldanamycin (17-AAG) is an HSP90 inhibitor that blocks ATP binding to HSP90, resulting in the destabilization of HSP90-client protein complexes. In this study, 17-AAG (CS-Npharm, Chicago, IL, USA) was dissolved in dimethyl sulfoxide (DMSO, 14.1 M, Invitrogen, La Jolla, CA, USA) and was used to inhibit the HSP90 expression in heat stress-induced MDBK cells. The experimental group was treated with a final concentration of 5 μM of 17-AAG in media for 48 h based on our preliminary experiments and literature (e.g., Siegelin et al., 2009; Yun et al., 2015). The same volume of DMSO used to dissolve 17-AAG in the experimental group was applied to the control group. Further, 2 groups of cells were subjected to the HS treatment as described above. In addition, the increased concentrations of 17-AAG (0 μM, 5 μM, and 10 μM) were added to the MDBK cells, respectively, and then the cells were subjected to HS as described above after 48 h of incubation.

**Transmission Electron Microscope Investigation**

After the MDBK cells were subjected to HS for 1 h, the cells from both the TN and HS groups were collected and mixed with a fixing solution containing of 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M sodium cacodylate buffer. Cells were further embedded in the resin, which served as a fixative to hold them securely in place for sectioning, and then sectioned at a thickness of 50 nm using the Leica microtome (EM UC7, Leica, Wetzlar, HE, Germany). The sections were stained with uranyl acetate for 20 min followed by lead citrate for 15 min. Finally, cell morphology was observed using a transmission electron microscope (JEM-1400, JEOL Ltd., Tokyo, Japan).

**Cell Viability Assay**

After temperature treatment for 1 h, the cell viabilities of MDBK and PBMC were assayed by the MTS test (Cell Titer96, Promega, Madison, WI, USA) according to the manufacturer’s protocol. In brief, a 20 μL working solution was added to each well of the 96-well plates and these plates were shielded with foil and incubated in 5% CO2 at 37°C for 4 h. The optical density was measured at 490 nm in a microplate reader (Multiskan FC 357, Thermo Scientific, Waltham, MA, USA).

**Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick-end Labeling (TUNEL) Assay**

Apoptosis in the MDBK cells was assessed by TUNEL staining according to the manufacturer’s instruction of the TUNEL Apoptosis Detection Kit (Yeasen Biotech Co., Ltd., Shanghai, China). Briefly, the slides were first incubated with 100 μL 1 × Equilibration Buffer for 30 min at room temperature, then incubated with Alexa Fluor 488–12-dUTP in TdT buffer for 1 h at 37°C in the dark, and followed by staining with 4’,6-diamidino-2-phenylindole (DAPI) solution (2 μg/mL, Invitrogen) at room temperature for 5 min. Green fluorescence targeting apoptotic cells was observed at
520 ± 20 nm and blue fluorescence targeting all cells was observed at 460 nm using a fluorescence microscope (TE2000-S, Nikon Corp., Tokyo, Japan). Three non-repeating micrographs for each well were randomly captured. The numbers of corresponding cells were counted using the Image J software (National Institutes of Health, Bethesda, MD, USA). The apoptosis rate was measured as: apoptosis rate (in %) = (green fluorescence targeting cells / blue fluorescence targeting cells) × 100.

**Bioinformatic Analyses**

The online software miRWalk 3.0 (http://mirwalk.umm.uni-heidelberg.de/) and miRanda (www.microrna.org/) were used to predict the candidate microRNAs (miRNA) binding to the 3′ UTR of bovine HSP90AA1 gene. The prediction of the secondary structure for miRNAs was performed using the RNAfold WebServer (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). Relative thermodynamic stability of miRNAs was predicted using the RNAhybrid software (https://bibiserv.cbi.tie.de/rnahybrid).

**Plasmid Construction**

According to the mRNA sequence of the HSP90AA1 gene in the Ensembl database (ENS-BTAT00000008225.3), the 691 bp genomic region in the 3′ UTR region was taken as the HSP90AA-3′ UTR-A sequence. Based on the mutation allele of rs109256957 (A > C), the 65th base was changed from A to C as HSP90AA1–3′ UTR-C. Moreover, 33 bp of the adjacent sequence near the binding region was modified as the HSP90AA1–3′ UTR-Mut sequence. Three nucleotide sequences were synthesized and ligated into the pCHECK2 vectors by HITRO BioTech (Beijing, China), which were digested with the same restriction enzymes.

**Cell Transfection and Luciferase Assay**

To validate whether bta-miR-1224 and bta-miR-2451 target the 3′ UTR of the HSP90AA1 gene, the mimics, inhibitors, and their negative controls (NC) of 2 miRNAs were synthesized by GenePharma (Suzhou, Jiangsu, China). Their sequences are shown in Table S2 (Supplementary Table S2; https://doi.org/10.6084/m9.figshare.24534871; Hu, 2023b). In this study, MDBK cells were plated in 24-well plates at 0.5 × 10⁵ cells/well. Transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) with 80% confluency, using a 1.5 to 1 transfection ratio. The MDBK cells were then incubated at 37°C and 5% CO₂ for 48 h before the cells were harvested for further experiments.

The co-transfections of the luciferase reporter plasmid containing HSP90AA-3′ UTR-A, HSP90AA1–3′ UTR-C, or HSP90AA1–3′ UTR-Mut with bta-miR-1224 mimic and ta-mir-1224 mimic NC were performed according to similar procedures mentioned previously to determine the effect of SNP rs109256957 on the binding affinity of bta-miR-1224. The luciferase activity was measured using a Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA). For each sample, renilla luciferase activities were normalized to firefly luciferase after correction for blank control.

**Quantitative Real-time PCR (qRT-PCR)**

Total RNA was isolated using the Trizol reagent from the Invitrogen reagent (Invitrogen, Carlsbad, CA, USA). Then, the RNA was reverse-transcribed into cDNA using the PrimeScript RT reagent Kit with a gDNA Eraser according to the manufacturer’s instruction (Takara Bio Inc., Kusatsu, Shiga, Japan). The qRT-PCR was performed on the SLAN-96P real-time system (Shanghai Hongshi Biotechnology Co., Ltd., Shanghai, China) using the GoTaq® qPCR Master Mix (Promega, Madison, WI, USA). Relative expression levels were calculated using the 2−ΔΔCt method (Fang et al., 2021). The expression level of the target genes was normalized with the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each treatment group had at least 3 biological replicates. The primers used in this study are listed in Supplementary Table S3 (https://doi.org/10.6084/m9.figshare.24534916; Hu, 2023c).

**Western Blotting**

Cells were lysed using the Radio Immunoprecipitation Assay Buffer (Beyotime Biotechnology Co., Shanghai, China), and protein concentration was measured by a Bicinchoninic Acid (BCA) Protein Assay Kit (Beyotime Biotechnology Co., Shanghai, China). A total of 20 μg protein extracts were separated on 12% SDS-PAGE gels (Genscript, Biotechnology, Piscataway, NJ, USA) and blotted onto nitrocellulose membranes. The nitrocellulose membranes were blocked with 5% nonfat dry milk for 1 h at room temperature, and then they were incubated overnight with the primary antibodies of HSP90α (1:500; Abcam Technology, Cambridge, UK) and LC3B (1:1000; Novus Biologicals, Littleton, CO, USA), and rabbit polyclonal antibodies of p62 (1:1000; ProteinTech Group Inc., Chicago, IL, USA), Cleaved caspase-3 (1:1000; Beijing Bios Biotechnology Co., Ltd., Beijing, China), Bcl-2 (1:500; ProteinTech Group Inc., Chicago, IL, USA), and GAPDH (1:1000; Bioworld Technology Co., Ltd., China). The membranes were then incubated with the secondary antibodies conjugated with horseradish peroxidase (1:10000; BioRad, Hercules, CA, USA) for 1 h. After washing, the blots were visualized using the ECL Plus Western Blotting Detection Reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and exposed to X-ray film (Eastman Kodak, Rochester, NY, USA).
(1:1000; ProteinTech Group Inc., Chicago, IL, USA) at 4°C, followed by incubation with an HRP-conjugated secondary antibody (1:2000; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) for 1 h at room temperature. The images were performed by the Flour Chem chemiluminescent imaging system (ProtainSimple, San Francisco, CA, USA). The band density was analyzed using the Image J software (National Institutes of Health, Bethesda, MD, USA). GAPDH was used as a housekeeping protein for the assay.

Data Analyses

**Estimation of breeding values for heat tolerance traits.** Estimated breeding values (EBVs; additive genetic effect) for 3 physiological indicators, including rectal temperature (RT), respiration score (RS), and drooling score (DS) were calculated after adjusting for known environmental effects such as lactation stage, age at calving, parity, and temperature-humidity index. More information about the models, data collection scheme, and farm management has been described in detail by Luo et al., 2021.

**Association analyses.** The extent of linkage disequilibrium between the identified SNPs was estimated using the Haploview 4.2 software (Broad Institute of MIT and Harvard, Cambridge, MA, USA). The association analyses between SNPs or haplotype combinations and EBVs of the DS, RS, and RT were performed using the GLM procedure of SAS 9.2 software (SAS Institute Inc., Cary, NC, USA) with the following model:

\[ Y_{ij} = \mu + G_i + e_{ij}, \]

where \( Y_{ij} \) is EBV of RT, RS, or DS; \( \mu \) is the overall mean; \( G_i \) is the effect of genotype or haplotype combination for animal \( i \); and \( e_{ij} \) is the random residual. The least squares means were tested using the Bonferroni t-test. The significance threshold was \( P < 0.05 \).

Statistical analyses.

Each experiment in this study was performed in triplicate, and all experimental data were expressed as the mean ± standard deviation (Mean ± SD). The SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analyses, and the mean comparison between the 2 groups was performed using the student’s t-test. While this study involved the assessment of multiple independent variables, including HS and 17-AAG treatments, the comparison among multiple groups was conducted through one-way ANOVA (ANOVA), followed by post hoc analyses using the Bonferroni t-test.

The purpose of using one-way ANOVA was to observe and assess the variations between the 4 corresponding groups. Differences between the 2 groups were considered significant if \( P < 0.05 \) and highly significant if \( P < 0.01 \).

**RESULTS**

Heat Stress Induced Cell Apoptosis, Cell Autophagy, and Upregulated Expression of the HSP90AA1 Gene in MDBK Cells

We first used transmission electron microscopy to assess the effects of HS on the MDBK cells, which has been used as a useful in vitro model for a variety of applications in laboratories. As shown in Figure 1A, HS exposure (42°C) for 1 h resulted in significant changes in cells’ morphology. Compared with the TN group (37°C), heat exposure increased membrane rupture, mitochondrial swelling, chromatin condensation, and apoptotic process, which showed the apoptosis of MDBK cells significantly increased due to acute HS exposure. Enhanced autophagosomes-structures also appeared in the presence of acute HS, indicating the presence of autophagy as a cellular response to acute HS exposure. Figure 1B shows that cell proliferation of MDBK was significantly attenuated by HS based on the results of the cell viability assay with 95.71 ± 4.94% in TN versus 88.72 ± 7.45% in HS (\( P < 0.01 \)). Finally, the expression pattern of the HSP90AA1 gene was evaluated at both transcriptional and translational levels in heat-induced responses. In comparison with the TN group, the HSP90AA1 mRNA level was significantly upregulated by acute HS (Figure 1C; 0.61 ± 0.13 versus 0.90 ± 0.07; \( P < 0.01 \)), as well as the protein (HSP90α is coded by HSP90AA1 gene) level (Figure 1D; 0.67 ± 0.01 versus 1.00 ± 0.13; \( P < 0.01 \)). Moreover, HS also significantly decreased cell viability and increased HSP90AA1 expression in PBMC (Supplementary Figure S1; https://doi.org/10.6084/m9.figshare.24534946; Hu, 2023f), which is consistent with what we found when using MDBK cells.

**The Role of the HSP90AA1 Gene in Heat-induced Cell Apoptosis and Autophagy**

To investigate whether the HSP90AA1 gene plays an important role in heat stress-induced cell apoptosis and autophagy, we evaluated the knockdown efficiency of 17-AAG for HSP90α expression. As 17-AAG is an HSP90 inhibitor that may block ATP binding to HSP90 and destabilize HSP90-client protein complexes, as shown in Figure 2A, 48 h of 17-AAG treatment (0.37 ± 0.03) significantly inhibited the expression of the...
HSP90α protein in comparison with the control group (0.19 ± 0.08; \( P < 0.05 \)). When exposed to acute HS (42°C for 1 h), HSP90α was significantly upregulated in both control and 17-AAG groups (HS-Control, 1.10 ± 0.06; HS-17-AAG, 0.79 ± 0.04) more than that in TN groups (TN-Control, 0.44 ± 0.06; TN-17-AAG, 0.30 ± 0.01; 37°C), but the expression of HSP90α was still significantly inhibited in the 17-AAG group when compared with the control group (\( P < 0.01 \); Figure 2B).

To further understand the function of the bovine HSP90AA1 gene in heat stress-induced cell apoptosis, we further examined the role of HSP90AA1 gene on apoptosis. As shown in Figure 2C, the treatment with 17-AAG could increase the apoptosis rate of MDBK under TN state (TN-17-AAG, 15.70 ± 2.90; TN-Control, 4.10 ± 1.00; \( P < 0.01 \)). When exposed to HS, the apoptosis rates of MDBK cells were significantly higher in HS-Control (40.80 ± 3.60) or HS-17-AAG (55.50 ± 4.50) than those in TN-Control or TN-17-AAG (\( P < 0.01 \)), and the highest apoptosis rate was observed in the HS-17-AAG group, which was treated with both HS and 17-AAG. The Western blot analyses further corroborated these results. As shown in Figure 2D, the heat-treated cells exhibited higher levels of Caspase-3 (HS-Control, 1.49 ± 0.23 versus TN-Control, 0.58 ± 0.08; HS-17-AAG, 2.14 ± 0.04 versus TN-17-AAG, 1.01 ± 0.11) and Bax but lower levels of Bcl-2 than TN cells, so the ratio of Bax/Bcl-2 was elevated in the HS groups.

**Figure 1.** The effects of acute heat exposure on cell morphology, cell viability, and HSP90AA1 expression in MDBK cells. (A) Transmission electron microscopy analysis. (B) Cell viability assay. (C) The mRNA expression of the HSP90AA1 gene. (D) The protein expression of the HSP90AA1 gene. MDBK: Madin-Darby bovine kidney; TN: Thermoneutral group (37°C for 1 h); HS: Heat stress group (42°C for 1 h); Values are means ± standard deviation; *\( P < 0.05 \), **\( P < 0.01 \), and NS is \( P > 0.05 \).
Holstein Cows

Association Analyses of HSP90AA1 Genetic Variants challenged with acute HS treatment. apoptosis while induced cell autophagy when cells were... 0.01). In summary, the HSP90AA1 gene suppressed heat-induced cell apoptosis in MDBK cells (Figures 2B-D).

Heat-treated cells showed increased autophagic structures compared with untreated cells, as shown in Figure 1A. To further validate autophagic induction in heat-stressed MDBK cells, we measured the protein levels of autophagy substrate p62 and the conversion of LC3-I to LC3-II known as the hallmarks of autophagy induction. The expression of p62 was significantly decreased (HS-Control, 0.30 ± 0.03 versus TN-Control, 0.80 ± 0.04; HS-17-AAG, 0.51 ± 0.03 versus TN-17-AAG, 0.76 ± 0.06) whereas the conversion of LC3-I to LC3-II was significantly increased (HS-Control, 0.96 ± 0.03 versus TN-Control, 0.21 ± 0.01; HS-17-AAG, 0.76 ± 0.03 versus TN-17-AAG, 0.20 ± 0.02) in MDBK cells after heat treatment (Figure 2E; P < 0.01), suggesting that the autophagy level was higher in heat-stressed cells. Accordingly, a significant difference in their expressions was found in 17-AAG treated cells compared with the control group under acute HS (HS-17-AAG versus HS-Control). Our data demonstrate that HSP90AA1 has a facilitative role in the regulation of heat-induced cell autophagy. Furthermore, the inhibitory intensity for the HSP90α (11.44 ± 0.50; 8.16 ± 0.66; 3.71 ± 0.69) was increased by the elevated 17-AAG content (0 μM, 5 μM, and 10 μM) in dose-dependent fashion (Figure 2F; P < 0.01). Notably, pretreatment with higher 17-AAG enhanced apoptosis but inhibited autophagy in MDBK cells in response to acute HS (Figures 2F-H), which further confirmed the changes in the expression of HSP90α. All these results indicate that the expression of the HSP90AA1 gene significantly inhibited cell apoptosis while induced cell autophagy when cells were challenged with acute HS treatment.

Association Analyses of HSP90AA1 Genetic Variants with Physiological Indicators of Heat Stress in Holstein Cows

A total of 13 SNPs were identified by Sanger sequencing on the entire coding region, 5′- and 3′- flanking regions of the bovine HSP90AA1 gene in 20 unrelated Holstein cows (Supplementary Table S4; https://doi.org/10.6084/m9.figshare.24534925; Hu, 2023d). Among them, 5 SNPs (rs109014822, rs209516308, rs135053858, rs109256957, and rs110026192; Figure 3A) were genotyped in 1,655 individuals. Further analyses showed that their allele frequencies were high and all of them were in accordance with Hardy-Weinberg equilibrium (Table 1; P > 0.001). The association analyses between them and 3 physiological indicators (RT, RS, and DS) of HS in Holstein cows are presented in Table 1. Five SNPs were significantly associated with RT (P < 0.05), in which 3 SNPs (rs209516308, rs109256957, and rs110026192) showed significant correlation with RS (P < 0.05). However, no SNP was found to be significantly associated with DS (P > 0.05). Figures 3B-F show the results of the Bonferroni t-test where both RT_EBV and RS_EBV had significant differences among genotypes of rs109256957 (P < 0.05) located in the 3′ UTR region. The CC and AC genotypes were associated with lower RT_EBV. Similar results were observed for rs110026192, which also belongs to the 3′ UTR variant. The values of least squares means (LSM) ± standard error (SE) are presented in Supplementary Table S5 (https://doi.org/10.6084/m9.figshare.24534931; Hu, 2023e).

One block with strong linkage disequilibrium was found based on current genomic data (Figure 3G) and formed 4 haplotypes, including H1 (CACAT, 56.5%), H2 (TGTCC, 28.6%), H3 (TACAT, 18.8%), and H4 (CGCAT, 1.7%). Consequently, 5 haplotype combinations (H1H1, H1H2, H1H3, H2H2, and H2H3) with a frequency greater than 5% were selected for further analyses. There was a significant association between haplotype combinations and RT_EBV (P < 0.05), as well as RS_EBV (P < 0.05) as shown in Table 2. The Bonferroni t-test showed that cows with H1H1 had significantly lower values for RT_EBV and RS_EBV when compared with H2H2 (P < 0.05). Furthermore, a significant difference between H1H2 and H2H2 was also observed for RS_EBV (P < 0.05), and H1H2 individuals had a lower RS_EBV. Notably, we found that H1H1 was homozygous with lower values of physiological indicators in each SNP, while H2H2 was a combination of homozygous with higher values. The H1H2 was the combination of their heterozygotes.

Bta-miR-1224 Directly Targets Binding the 3′ UTR of the HSP90AA1 Gene

miRNAs are non-coding RNAs of approximately 22 nucleotides in length that have a significant impact on biological processes by degrading targeted mRNAs and repressing translation. As the binding sites of miRNAs are usually on the 3′ UTR region of the target genes, the hypothesis was that the SNP in HSP90AA1 gene 3′ UTR region caused changes in binding activity, which caused different genotypes of the HSP90AA1 gene to
Figure 2. The role of the HSP90AA1 gene in cell apoptosis and autophagy in response to acute heat exposure in MDBK cells. (A) The impact of 5 μM 17-AAG on HSP90α expression. (B) The impact of 5 μM 17-AAG on HSP90α expression under TN and HS conditions. (C) TUNEL assay. Cells stained with Tunel indicated apoptosis, and all cells were counterstained with DAPI. (D) The expression pattern of apoptosis-related genes. (E) The expression pattern of autophagy-related genes. (F) The impact of 5 μM and 10 μM 17-AAG on HSP90α expression under the HS condition. (G) The expression pattern of apoptosis-related genes with increased 17-AAG concentrations under the HS condition. (H) The expression pattern of autophagy-related with increased 17-AAG concentrations under the HS condition. MDBK: Madin-Darby bovine kidney; TN: Thermoneutral group (37°C for 1 h); HS: Heat stress group (42°C for 1 h); TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; DAPI: 4',6-diamidino-2-phenylindole; Values are mean ± standard deviation; *P < 0.05, **P < 0.01, and NS is P > 0.05; Different letters indicate a significant difference between groups based on the Bonferroni t-test (lowercase letter, P < 0.05; uppercase letter, P < 0.01).
Figure 3. Single nucleotide polymorphisms (SNPs) in the bovine HSP90AA1 gene associated with physiological indicators of heat stress (HS) in Holstein cows. (A) Diagrammatic representation of the detected SNPs of the HSP90AA1 gene. (B) Multiple comparisons of different genotypes in SNP1 rs109014822. (C) Multiple comparisons of different genotypes in SNP2 rs209516308. (D) Multiple comparisons of different genotypes in SNP3 rs135053858. (E) Multiple comparisons of different genotypes in SNP4 rs109256957. (F) Multiple comparisons of different genotypes in SNP5 rs110026192. (G) The linkage disequilibrium analysis of 5 SNPs in the HSP90AA1 gene. RT: rectal temperature; RS: respiration rate score; DS: drooling score; EBV: estimated breeding values. Values are least squares means (LSE) ± standard error (SE); Different letters indicate a significant difference between genotypes based on the Bonferroni t-test (lowercase letter, $P < 0.05$; uppercase letter, $P < 0.01$).
be associated with heat stress response in cattle. The results above indicate that 2 SNPs (rs109256957 and rs110026192) in the 3′ UTR region are associated with physiological indicators of heat stress in Holstein cows. So, we next predicted putative miRNAs that may target the 3′ UTR region of the HSP90AA1 gene using miRWalk and miRanda. Two putative miRNAs named bta-miR-1224 and bta-miR-2451 target binding the 3′ UTR region of the HSP90AA1 gene, and rs109256957 was located within miRNA target sites and potentially resulted in the creation of miRNA target sites while rs110026192 did not locate in or near the miRNA binding sites. Therefore, we next investigated the molecular mechanism involved in the modulation of rs109256957 on the HSP90AA1 gene expression.

We first verified whether bta-miR-1224 and bta-miR-2451 may regulate the expression of the HSP90AA1 gene. Figure 4A showed the binding sites in the HSP90AA1 gene for bta-miR-1224 and highlighted the similarity among different species. Based on the binding character, 4 specific sequences (mimic negative control; mimic; inhibitor negative control; inhibitor) were synthesized to test whether bta-miR-1224 targets the HSP90AA1 gene. Compared with the mimic negative control group (bta-miR-1224 mimics NC, 1.01 ± 0.09), the mRNA expression of the HSP90AA1 gene in MDBK was significantly reduced by the bta-miR-1224 mimic (0.70 ± 0.16; Figure 4B; \( P < 0.01 \)), a corresponding decrease was also found in the translation level (bta-miR-1224 mimics NC, 0.18 ± 0.01 versus bta-miR-1224 mimic, 0.13 ± 0.01; Figure 4C; \( P < 0.01 \)). Using an exogenous bta-miR-1224 inhibitor, the mRNA (1.37 ± 0.19) and protein (0.32 ± 0.01) levels of the HSP90AA1 gene were significantly increased in comparison to that in the inhibitor negative control group (mRNA, 1.00 ± 0.16; protein, 0.19 ± 0.02; Figures 4B-C; \( P < 0.01 \)). Base pairing between bta-miR-2451 and the 3′ UTR of the HSP90AA1 gene is shown in Figure 4D. MDBK cells were transfected with mimic negative control, mimics, inhibitor negative control, and inhibitor of bta-miR-2451. However, no difference of HSP90AA1 expression was observed among the 4 groups at both transcriptional (bta-miR-2451 mimics NC, 1.00 ± 0.08; bta-miR-2451 mimic, 0.85 ± 0.15; bta-miR-2451 inhibitor NC, 0.72 ± 0.34; bta-miR-2451 inhibitor, 0.84 ± 0.19; Figure 4E; \( P > 0.05 \)) and translational levels (bta-miR-2451 mimics NC, 0.43 ± 0.03; bta-miR-2451 mimic, 0.41 ± 0.04; bta-miR-2451 inhibitor NC, 0.45 ± 0.05; bta-miR-2451 inhibitor, 0.38 ± 0.01; Figure 4F; \( P > 0.05 \)). Overall, these findings suggest that bta-miR-1224 can target HSP90AA1 mRNAs for degradation and further inhibit translation.

### Table 1. Allele frequency and P-value of single nucleotide polymorphisms in the HSP90AA1 gene associated with physiological indicators of heat stress in Holstein cows.

<table>
<thead>
<tr>
<th>SNP</th>
<th>rs ID</th>
<th>Position</th>
<th>Call rate (%)</th>
<th>MAF</th>
<th>HW</th>
<th>RT_EBV</th>
<th>RS_EBV</th>
<th>DS_EBV</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>rs109014822</td>
<td>21:66944159</td>
<td>95.01</td>
<td>0.41</td>
<td>0.61</td>
<td>0.028</td>
<td>0.398</td>
<td>0.881</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>rs209516308</td>
<td>21:66943771</td>
<td>98.18</td>
<td>0.31</td>
<td>0.43</td>
<td>0.002</td>
<td>0.008</td>
<td>0.253</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>rs135653858</td>
<td>21:66942278</td>
<td>98.22</td>
<td>0.30</td>
<td>0.97</td>
<td>0.047</td>
<td>0.066</td>
<td>0.409</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>rs109256957</td>
<td>21:66940241</td>
<td>96.54</td>
<td>0.30</td>
<td>1.00</td>
<td>0.031</td>
<td>0.013</td>
<td>0.150</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>rs110026192</td>
<td>21:66940204</td>
<td>97.13</td>
<td>0.30</td>
<td>0.53</td>
<td>0.034</td>
<td>0.048</td>
<td>0.343</td>
<td></td>
</tr>
</tbody>
</table>

Note: MAF: minor allele frequency; HW: Hardy-Weinberg test; \( P \)-value suggests whether SNPs and physiological indicators were significantly associated; \( P \)-value <0.05 shows a significant association; RT: rectal temperature; RS: respiration score; DS: drooling score; EBV: estimated breeding values.

### Table 2. P-values of association analysis between haplotype combinations and physiological indicators of heat stress in Holstein cows.

<table>
<thead>
<tr>
<th>Haplotype combinations</th>
<th>Number</th>
<th>RT_EBV</th>
<th>RS_EBV</th>
<th>DS_EBV</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1H1</td>
<td>437</td>
<td>0.006 ± 0.005a</td>
<td>0.003 ± 0.005b</td>
<td>−0.015 ± 0.004</td>
</tr>
<tr>
<td>H1H2</td>
<td>470</td>
<td>0.014 ± 0.005ab</td>
<td>0.006 ± 0.005b</td>
<td>−0.016 ± 0.004</td>
</tr>
<tr>
<td>H1H3</td>
<td>209</td>
<td>0.020 ± 0.008ab</td>
<td>0.010 ± 0.008ab</td>
<td>−0.007 ± 0.006</td>
</tr>
<tr>
<td>H2H1</td>
<td>118</td>
<td>0.043 ± 0.010a</td>
<td>0.042 ± 0.011a</td>
<td>0.001 ± 0.007</td>
</tr>
<tr>
<td>H2H3</td>
<td>95</td>
<td>0.010 ± 0.011ab</td>
<td>0.000 ± 0.012ab</td>
<td>−0.022 ± 0.008</td>
</tr>
</tbody>
</table>

\( P \)-value: 0.020 0.020 0.151

Note: \( P \)-value suggests whether haplotype combinations and physiological indicators were significantly associated; \( P \)-value <0.05 shows a significant association; Different letters in the same column indicate a significant difference between haplotype combinations (\( P < 0.05 \)) based on the Bonferroni \( t \)-test; RT: rectal temperature; RS: respiration score; DS: drooling score; EBV: estimated breeding values.
SNP rs109256957 Regulated the Expression of the HSP90AA1 Gene by bta-miR-1224

To investigate the mechanisms for the genetic association, we examined the influence of rs109256957 in the 3′ UTR on the mRNA folding and post-transcriptional regulation of the HSP90AA1 gene. As shown in Figure 5A, the allele A of rs109256957 contributed more strongly to mRNA stability with a marginal variation in the minimum free energy (MFE) than the allele C (ΔG = −22.32 kcal/mol), indicating that the A allele helps to protect the mRNA from being degraded. Further, we evaluated whether the rs109256957 could influence the binding between bta-miR-1224 and the 3′ UTR of the HSP90AA1 gene. The bioinformatic analysis showed that there was a difference in MFE for the preferential binding between the A and C alleles (Figure 5B; ΔG = −2.90), suggesting that the C allele had a more stable binding. To validate these findings, we constructed 3 full-length 3′ UTRs of the HSP90AA1 gene, containing either the G or the A allele or the mutated version, and cloned them into the psiCHECK-2 vector (Figure 5C). Their constructs were then co-transfected into HEK-293T cells with bta-miR-1224 mimic or bta-miR-1224 mimic negative control. As shown in Figures 5D and 5E, the luciferase activity was significantly lower in the bta-miR-1224 mimic group (HSP90AA1–3′UTR-A, 0.99 ± 0.02; HSP90AA1–3′UTR-C, 0.67 ± 0.04) in both A and C alleles than in bta-miR-1224 mimic negative control (HSP90AA1–3′UTR-A, 0.92 ± 0.03; HSP90AA1–3′UTR-C, 0.88 ± 0.05), suggesting the specificity of decreased luciferase activity by bta-miR-1224. Moreover, the C allele demonstrated significantly lower luciferase activity compared with the A allele in the presence of the β-miR-1224 mimic (Figure 5F), which is consistent with the prediction. These results indicate that rs109256957 is a key functional polymorphism affecting the target binding between bta-miR-1224 and 3′ UTR of the HSP90AA1 gene.

DISCUSSION

Dairy cows are susceptible to HS when raised in environments with high temperatures and humidity. Also, HS can disrupt the normal physiology of dairy cows, leading to a decrease in production (Abbas et al., 2020; Tao et al., 2020), reproductive performance (Turk et al., 2015), and immune function (Bagath et al., 2019), ultimately resulting in major economic losses and welfare issues to the dairy industry. Therefore, genetic selection and breeding of heat tolerant cows are of great importance. A better understanding of the molecular regulatory mechanisms of HS and the identification of key genes will provide fundamental insights for the development of more effective breeding strategies. Our previous study identified HSP90AA1 as a key gene associated with HS responses in dairy cows (Fang et al., 2021). However, the cellular roles of the HSP90AA1 gene and its genetic variation in the responses to HS in dairy cows have not been well documented. This study aimed to reveal the mechanism of the HSP90AA1 gene involved in the HS responses of dairy cows, as well as to identify genetic markers that have the potentials to be used in the selection of more heat tolerant dairy cows (Badri et al., 2018), and to conduct in-depth analyses on the functional implications and mechanisms responsible for genetic markers.

As an important member of the HSP family, HSP90 protects cells by binding to abnormal proteins produced in the cells, helping them restore their correct conformation, or transporting them to lysosomes for degradation (Biebl and Buchner, 2019). HSP90 is also involved in important processes such as transmembrane transport and signal transduction (Biebl and Buchner, 2019). Numerous studies have shown that it is massively induced and expressed in heat-exposed dairy cows (Liu et al., 2020), goats (Hooper et al., 2018), pigs (Cervantes et al., 2016), and chickens (Cedraz et al., 2017). In the present study, HSP90AA1 gene expression was significantly upregulated in MDBK after exposure to 42°C for 1 h, which is consistent with previous findings. The same pattern was observed in PBMC. These results indicate that the response of cells to HS involves the activation of the HSP90AA1 gene. In the current study, the parameters such as temperature and duration were used to mimic an acute HS, typically characterized by short periods of heat exposure. The use of these specific parameters based on our previous study (Fang et al., 2021), which allows for a focused investigation of the immediate cellular responses to HS and ensures the reproducibility and control of the experiment. Our experimental design may oversimplify the complexity of real-world stress scenarios, where variables such as temperature fluctuations and duration can vary, but this study provides valuable insights into HS responses. Given the full range of responses to different types of HS, further research is essential to gain a full understanding of how organisms or cells cope with HS.

Apoptosis is a form of programmed cell death. And ROS induced by HS are responsible for the apoptosis of the cells (Ikeda et al., 1999; Reed, 2000). The proteins of the Bcl-2 family are key regulators of apoptosis, including both anti- and pro-apoptotic proteins (such as Bcl-2 and Bax), where the dynamic balance of these proteins is important for cell fate (Ikeda et al., 1999). In addition, caspases are a class of proteases. Activated caspases (e.g., Caspase-3) cleave critical cellular substrates, leading to the morphological changes associat-
Figure 4. The regulation of bta-miR-1224 and bta-miR-2451 on the expression of the HSP90AA1 gene. (A) Base pairing between bta-miR-1224 and the HSP90AA1 gene. (B) The effects of mimic and inhibitor of bta-miR-1224 on the mRNA expression of HSP90AA1 gene. (C) The effects of mimic and inhibitor of bta-miR-1224 on the protein expression of HSP90AA1 gene. (D) Base pairing between bta-miR-2451 and the HSP90AA1 gene. (E) The effects of mimic and inhibitor of bta-miR-2451 on the mRNA expression of HSP90AA1 gene. (F) The effects of mimic and inhibitor of bta-miR-2451 on the protein expression of HSP90AA1 gene. Values are mean ± standard deviation; *P < 0.05, **P < 0.01, and NS is P > 0.05.
ed with apoptosis (Boatright and Salvesen, 2003). Our results showed that the ratio of Bax/Bcl-2 and cleaved caspase-3 was increased in the heat-stress group compared with the control group, indicating a higher level of apoptosis in heat-stressed MDBK cells. Autophagy is another mechanism of programmed cell death that involves the degradation of cellular components in autophagosomes and autolysosomes (Tsujimoto and Shimizu, 2005). In contrast to apoptosis, autophagy allows the degradation of cellular components and pro-

Figure 5. The genetic variant rs109256957 affects the mRNA folding and regulation of the HSP90AA1 gene. (A) The secondary structures of HSP90AA1 mRNA for different alleles of rs109256957. (B) The minimum free energy (MFE) required for RNA hybridization between 3′ UTR and bta-miR-1224 for different alleles of rs109256957. (C) psiCHECK-2 reporters carrying the A-type or C-type or Mut-type of bovine HSP90AA1 3′ UTR. (D) Luciferase activities of psiCHECK-2 constructs carrying the bovine HSP90AA1 3′ UTR with either the A allele or Mut under co-transfection with bta-miR-1224 mimic or NC. (E) Luciferase activities of psiCHECK-2 constructs carrying the bovine HSP90AA1 3′ UTR with either the C allele or Mut under co-transfection with bta-miR-1224 mimic or NC. (F) The comparison of luciferase activities of psiCHECK-2 constructs carrying the bovine HSP90AA1 3′ UTR with A allele and C allele under co-transfection with bta-miR-1224 mimic. Values are mean ± standard deviation; * P < 0.05, ** P < 0.01, and NS is P > 0.05.
motes cell survival by providing energy (Tsujimoto and Shimizu, 2005). Moreover, HS induces autophagy in the pig oocyte during oocyte maturation, and autophagy is a potential mechanism by which the oocyte adapts to heat stress (Hale et al., 2021). Transmission electron microscopy was used to monitor the ultrastructure of autophagic structures in heat-stressed MDBK cells. A large number of autophagosomes were observed, along with mitochondrial swelling and chromatin condensation. We also detected the expression of autophagy markers based on immunoblot analysis, including p62, LC3-II, and LC3-I (Kuma et al., 2007; Su and Wang, 2011). In the present study, p62 decreased, while the LC3II/LC3I ratio increased in MDBC cells cultured at 42°C for 1 h, which is consistent with the morphological observations. Therefore, acute HS induced cell apoptosis and autophagy in MDBC cells. Given the complexity and multi-tissue involvement of HS, future studies on other cell types will enhance our understanding of the overall effects on the whole cow.

To investigate the relationships between heat stress-induced HSP90AA1 expression and cell apoptosis and autophagy, this study treated MDBC cells with the inhibitor 17-AAG to reduce HSP90AA1 expression at the protein level. The 17-AAG, a pharmacological inhibitor of HSP90 proteins, was first reported in 1998 (Schulte and Neckers, 1998). It inhibits the ATPase activity of HSP90 proteins, thereby promoting the degradation of HSP90 through the proteasome mechanism (Usmani et al., 2009). Our results showed that treatment with 17-AAG decreased the levels of HSP90 proteins in both HS and HS conditions. Accordingly, the inhibition of HSP90 proteins by 17-AAG increased the expression of cleaved caspase-3, the Bax/Bcl-2 ratio, and p62, while reducing the LC3-II/LC3-I ratio, especially in HS groups. The TUNEL assay for the detection of cell apoptosis validated the above results. Subsequent experiments with different doses of 17-AAG inhibitors further highlighted the relationships between HSP90 and cell apoptosis and autophagy. In response to HS, the upregulated expression of the HSP90AA1 gene can inhibit cell apoptosis and promote cell autophagy. Increasing the expression level of the HSP90AA1 gene can lead to the dissociation of phosphorylated JNK/P38, which can result in decreased apoptosis (Xiao et al., 2018). In addition, the HSP90AA1 gene is involved in autophagy through regulation of the PI3K/Akt/mTOR pathway, in which HSP90AA1 expression can induce the dephosphorylation of AKT and then decreases the expression of phosphorylation of mTOR. HSPs act as molecular chaperones and help adapt to thermal stress and balance the intracellular environment, especially protein function (Archana et al., 2017). Therefore, the increased expression of HSP90AA1 under heat stress is a self-protective mechanism of cells that maintains cell survival by inhibiting apoptosis and increasing autophagy. It is thought that the HSP90AA1 gene may have other roles in heat stress, such as immune response (Liu et al., 2022), which will be the subject of further investigation.

Furthermore, SNPs are the most common type of genetic variation in livestock animals, with millions of SNPs identified throughout the cattle genome (Stothard et al., 2011). Several studies have shown that there are SNPs in the HSP90AA1 gene associated with HS responses in cattle (Kumar et al., 2015), sheep (Astuti et al., 2022), and chicken (Irivboje et al., 2020). In particular, the rs451760757 polymorphism in the promoter region and rs110026192 in the 3′ UTR region of the HSP90AA1 gene are associated with HS responses and contribute to the modulation of HSP90AA1 expression in Holstein cows (Badri et al., 2018). In this study, 13 SNPs were identified by Sanger sequencing using pooled DNA from 20 unrelated individuals, of which 5 SNPs were genotyped in 1,655 individuals. Further analyses showed that rs109256975, located within the 3′ UTR region, was significantly associated with both RT_EBV and RS_EBV. Further, individuals with the AA genotype had lower RT_EBV than that of the CC genotype, as well as a lower RS_EBV value. Moreover, there is strong LD among 5 SNPs and the H1H1 haplotype comprising AA genotype in rs109256975 had lower RT_EBV and RS_EBV than those of H2H2 comprising CC genotype in rs109256975. We hypothesized that this SNP is a functional mutation associated with HS responses in Holstein cows by affecting the expression of the HSP90AA1 gene.

Generally, miRNAs are considered post-transcriptional gene expression regulators that regulate gene expression by hybridizing to target mRNAs and regulating their translation or stability (Filipowicz et al., 2008). In most cases, miRNAs interact with the 3′ UTR of target mRNAs, leading to translational repression or mRNA degradation (OBrien et al., 2018). Two miRNAs (bta-miR-1224 and bta-miR-2451) were predicted to target the 3′ UTR region of the HSP90AA1 gene containing rs109256975. However, only bta-miR-1224 specifically targeted the 3′ UTR region and reduced HSP90AA1 gene expression. Previous studies have shown that bta-miR-1224 is involved in the regulation of spermatogenesis in cattleyak (Xu et al., 2020) and foot-and-mouth disease virus infection in cattle (Stenfeldt et al., 2017). Our results suggest that bta-miR-1224 may be related to the response to heat stress by regulating the expression of the HSP90AA1 gene. There was a significant difference in the binding activity between the allele A and the allele C in SNP rs109256975 in the presence of the bta-miR-1224 targeting on the 3′ UTR region of
HSP90AA1 mRNA. Compared with the allele C, bta-miR-1224 significantly increased the luciferase expression in the presence of the allele A, indicating that the allele A had less binding activity and then induced the high expression of the HSP90AA1 gene. Previous studies have reported that polymorphisms in the 3′ UTR region, located within or near the miRNA binding site, are associated with human diseases by interfering with miRNA function and leading to differential gene expression levels (Wang et al., 2016; Zhang et al., 2022). Similarly, genetic variation in the 3′ UTR of the HSF1 gene is associated with thermal tolerance traits in Holstein cows through disruption of miRNA target binding (Li et al., 2011). It should be noted that SNPs affect the structural folds of the mRNAs and the accessibility of various RNA-binding elements, including miRNAs (Shen et al., 1999). Our prediction results suggest that the A allele will also have a more stable mRNA stability with a higher expression of the HSP90AA1 gene than the C allele. Therefore, the HSP90AA1 expression is higher in the presence of the A allele. mRNAs with the A allele are more stable and less able to bind miRNAs. These findings are supported by evidence of the expression of HSP90AA1 under a variety of environmental conditions. In summary, rs109256957 in the 3′ UTR region of HSP90AA1 gene is significantly associated with HS responses in Holstein cows by altering the HSP90AA1 expression based on different levels of binding activity with bta-miR-1224. Together with the biological functions of the HSP90AA1 gene under acute HS conditions, we suggest that individuals with AA genotype are more heat-tolerant (lower RT and RS) because they activate more HSP90AA1 gene expression to inhibit apoptosis and increase autophagy, thereby better coping with HS (Figure 6).

CONCLUSIONS

The HSP90AA1 gene is a key regulator of cell apoptosis and autophagy and plays an important functional role in the response to HS in cattle. Our results showed that heat stress-induced HSP90AA1 expression maintains cell survival by inhibiting cell apoptosis and increasing cell autophagy. Five genetic variations within the HSP90AA1 gene were significantly associated with HS responses in Holstein cows. Among them, rs109256957, located in the 3′ UTR region, was identified as a functional variant and affected HSP90AA1 expression by altering its binding activity with bta-miR-1224.

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SUPPLEMENTARY MATERIAL Supplementary Table S1: Primers for amplification of the HSP90AA1 gene. https://doi.org/10.6084/m9.figshare.24534808. Supplementary Table S2: Sequences of the mimics, in-

REFERENCES


Hu, L. 2022e. Table S5. figshare. Table. https://doi.org/10.6084/m9.figshare.24534931.

Hu, L. 2022f. Figure S1. figshare. Figure. https://doi.org/10.6084/m9.figshare.24534946.


Kumar, R., I. D. Gupta, A. Verma, N. Verma, and M. R. Vineeth. 2015. Im-
O'Brien, J., H. Hayder, Y. Zayed, and C. Peng. 2018. Overview of mi-
Prof. Geogr. 67:121–131. https:
Kuma, A., M. Matsui, and N. Mizushima. 2007. LC3, an autophago-
Tsujimoto, Y., and S. Shimizu. 2005. Another way to die: autopha-
Mauger, G., Y. Baumson, T. Nennich, and E. Salathé. 2015. Im-
O'Brien, J., H. Hayder, Y. Zayed, and C. Peng. 2018. Overview of mi-
Schulte, T. W., and L. M. Neckers. 1998. The benzoquinone ansa-
Siegelin, M. D., A. Habel, and T. Gaenser. 2009. 17-AAG sensitized ma-
Stenfeldt, C., J. Arzt, G. Smoliga, M. LaRocco, J. Gutooksa, and P. Lawrence. 2017. Proof-of-concept study: profile of circulating mi-
Su, H., and X. Wang. 2011. 362 Stages an interplay between the ubiqui-
Tsujiimoto, Y., and S. Shimizu. 2005. Another way to die: autophag-
Usmani, S. Z., R. Dona, and Z. Li. 2009. 17 AAG for HSP90 inhibi-
Wang, C., Y. Zhao, Y. Ming, S. Zhao, and Z. Guo. 2016. A poly-
tide polymorphism association study identifies significant prostate cancer risk-associated functional loci at Sp2 Al in Chinese popula-
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