Detection of functional polymorphisms in the *hsp70* gene and association with cold stress response in Inner-Mongolia Sanhe cattle

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Received: 21 October 2018 / Revised: 18 January 2019 / Accepted: 22 January 2019 / Published online: 5 March 2019
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Abstract

The genetic mechanisms underlying the cattle resilience to severe cold temperatures are still unknown. In this study, we observed that four blood biochemical parameters were significantly altered, i.e., blood adrenocorticotrophic hormone (ACTH), triiodothyronine (T3), thyroxine (T4), and potassium (K⁺) after expose to −32 °C for 3 h. This was observed using 105 healthy Sanhe heifers with similar weight (398.17 ± 34.06 kg) and age (19.30 ± 4.91 months). A total of 20 single nucleotide polymorphisms (SNPs) were identified in 5'-flanking region of the *hsp70* gene in Sanhe cattle, while only 10 SNPs were segregating when comparing genetic variations between Sanhe cattle and 285 Chinese Holstein samples. Statistically significant associations between the genomic markers SNP-42[−], SNP-105⁺, SNP-181⁺, and SNP-205⁺ with blood T3 and between SNP-105⁺ and blood T4 were observed by applying the general linear model procedure and Bonferroni *t* test. Furthermore, we demonstrated that the T alleles of SNP-42[−] and SNP-205⁺ in the GC box and Kozak sequence of the *hsp70* gene, respectively, significantly decreased the green fluorescent proteins activity in vitro GFP reporter assays. These findings suggest that these two SNPs are causative polymorphisms involved in the regulation of *hsp70* promoter activity and might contribute to the observed association between the *hsp70* gene and T3 and T4 levels in Sanhe cattle. Thus, *hsp70* gene is a promising candidate gene to be validated in independent cattle populations and functional studies related to cold stress resilience in cattle.

Keywords Inner-Mongolia Sanhe cattle · Severe cold stress · Climate resilience · *hsp70* gene · Functional polymorphisms

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s12192-019-00973-5>) contains supplementary material, which is available to authorized users.

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Introduction

Over the past decades, the evidences of climate change have increased substantially, which will largely affect animal welfare, fertility, health and production efficiency, and consequently, food availability (e.g., Rojas-Downing et al. 2017). The characterization of climate resilient breeds and further investigation of the genetic mechanisms that confer a more efficient response to temperature stress is of utmost value in an era of challenging environmental conditions. The majority of dairy cattle breeds is highly sensitive to temperatures out of the range from -5 to 25 °C (Hahn 1999). However, under rain or snow fall, the effects of cold stress are intensified due to the greater increase in heat loss as consequence of evaporation (Schutz et al. 2010). Animals under severe cold stress conditions will alter metabolizable energy intake and heat production, which will consequently result in a reduction in production efficiency, reproduction, metabolic disorders, and animal welfare (Angrecka et al. 2015; Broucek et al. 1991; Sasaki et al. 2016). Despite the importance of breeding cold-resilient animals, there is still a lack of studies investigating the biological mechanisms of response to severe cold stress in cattle, as well as the identification of functional polymorphisms in candidate genes and genetic association analyses with cold stress variables.

Inner-Mongolia Sanhe is a dual-purpose (milk and meat) cattle breed originated in the Inner Mongolia Autonomous Region of China, which has severe winter conditions in approximately 200 days per year. The Sanhe breed is well known for its high resilience to harsh environments, especially extremely low temperatures (e.g., below 30 °C), and elevated disease resistance and production efficiency under harsh winter conditions and grazing systems.

Though exotic breeds such as Holstein and Simmental have been imported into China, the Sanhe breed still remains as a major breed due to its high milk and meat production efficiency and resilience to extremely low temperatures, as observed in Northern China (Wu et al. 2012). Li et al. (2015) reported statistically significant changes in biochemical parameters and gene expression in response to severe cold stress in Sanhe cattle. After cold exposure, triiodothyronine (T3), thyroxine (T4), and adrenocorticotrophic hormone (ACTH) in blood were found to be significantly altered ($P < 0.01$) (Li et al. 2015). Temperature regulation under cold stress conditions is a complex physiological process, where the autonomic nervous system and neuroendocrine system control the main regulation activities, resulting in concentration changes of blood biochemical parameters. In addition, Hu et al. (2018) observed significant changes in blood biochemical parameters in Chinese Holstein cows after exposure to cold weather conditions. The authors reported blood biochemical parameters that can be used as potential indicators of individual response to cold stress in cattle.

Xu et al. (2017) identified 193 differentially expressed genes in peripheral blood samples of Sanhe cattle exposed to severe cold stress (-32 °C for 3 h). Out of the 193 candidate genes, the expression of *hsp70* (heat-shock protein 70) significantly increased after cold exposure ($P < 0.05$) (Xu et al. 2017). In addition, Kumar et al. (2015) investigated the expression pattern of the *hsp70* gene in both cattle and buffalo and reported an increased expression of the *hsp* family genes during the winter season. According to the authors, variations in the expression pattern of the *hsp* family genes during different seasons are likely to be associated with improved adaptability to tropical climate conditions. The differential expression of the *hsp70* gene during the winter season suggests that these genes might play important roles in the cold stress response in cattle.

Most genes of the HSP family have crucial cytoprotective effects and are involved in many regulatory pathways related to cell stress response (Feder and Hofmann 1999). *Hsp70* is one of the most conserved, sensitive, and abundant gene related to stress response among the HSP family genes (Kiang and Tsokos 1998). There are almost no introns in the *hsp70* gene to ensure that they can rapidly produce mature mRNA to meet the need of a large quantity of *hsp70* expression (Muller et al. 1992). There are rich binding sites for various transcription factors in the 5'-flanking region of *hsp70* gene which directly influence the *hsp70* expression (Behl et al. 2014; Schwerin et al. 2001; Wu et al. 1986). In humans, there are evidences of genetic variations in the 5'-flanking region of the *hsp70* gene associated with the risk of diseases or longevity (He et al. 2009; Singh et al. 2010). These genetic variations modulate the *hsp70* expression through translation or post-transcriptional regulation during stress periods (He et al. 2009; Singh et al. 2010). Additionally, genetic polymorphisms in the 5'-flanking region of *hsp70* gene have been associated with heat stress response in both pigs (Huang et al. 2002; Schwerin et al. 2001) and cattle (Deb et al. 2013; Li et al. 2011; Xiong et al. 2013). However, to our best knowledge, there are no reports of the genetic mechanisms of the *hsp70* gene underlying cold stress response in cattle.

Considering that the Sanhe breed is well known to be more resilient to extreme cold conditions compared to other worldwide cattle breeds and due to the fact that the *hsp70* gene plays an important role in thermotolerance mechanisms in livestock species (Xu et al. 2017), the objectives of this study were as follows: (1) to investigate severe cold stress-induced changes in concentrations of six blood biochemical parameters in Sanhe cattle, (2) to characterize genomic variations in the 5'-flanking region of the *hsp70* gene, (3) to perform association analyses between polymorphisms in the *hsp70* gene and cold stress response variables, and (4) to explore its biological associations with cold stress response mechanisms in cattle.

Material and methods

Animals and genomic information

The use of the animals was in agreement with the Committee on Ethics of Animal Experimentation from the Beijing Jiaotong University, Beijing, China. Two groups of experimental animals were involved in the current study. The first group included 105 healthy Sanhe heifers with similar weight (398.17 ± 34.06 kg) and age (19.30 ± 4.91 months), which were randomly selected from the Xiertala Cattle Breeding Farm population in Inner Mongolia, China. The animals were semi-housed in the same cowshed and fed a total mixed ration (TMR). In order to induce severe cold stress, the cattle were transferred outdoors and exposed to a temperature of -32 °C for 3 h followed by cowshed housing at 5 °C for 15 h (Fig. 1). The experimental procedure was conducted in accordance with the management of a local commercial farm in adverse winter conditions (Wu et al. 2012). Blood samples (10 mL) were collected from each animal before and after the cold exposure, and centrifuged at $\times 1400g$ for 10 min. Finally, the upper serum was collected for detecting the changes in blood biochemical parameters in response to cold stress. In addition, 5 mL blood samples with anticoagulant (ethylene diamine tetraacetic acid) were collected and used for the isolation of genome DNA for subsequent analyses.

The second group consisted of 285 healthy lactating Chinese Holstein cattle (average weight, 600.35 ± 43.82 kg; average age, 49.08 ± 20.20 months) from the Sanyuan Lvhe Dairy Cattle Centre located in Beijing, China. This group was only used to collect 5 mL blood samples for genome isolation and comparison of genetic variations the group mentioned previously.

Genome DNA was isolated from blood samples with standard procedure of TIANamp blood DNA kit (TIANGEN, Beijing, China).

Detection of blood biochemical parameters

Six blood biochemical parameters were measured as potential indicators of cold stress response. The laboratorial analyses were performed at the Beijing Huaying Biotechnology Research Institute (Beijing, China). The method of radioimmunoassay (Li et al. 2015) was used to detect ACTH, cortisone (CORT), glutathione peroxidase (GSH-Px), triiodothyronine (T3), and thyroxine (T4), and erythrocyte cells were used to measure potassium (K^+) concentration.

PCR amplification and sequencing

Primers for amplifying the 5'-flanking region of the *hsp70* gene were designed with Primer version 3.0 (PREMIER Biosoft, CA, USA) based on the DNA sequence of AY149618.1 (GenBank Accession No.). The primer sequences are: forward: 5'-GTCGTGTAGCCCTTAATTCTA-3', and reverse: 5'-ACGCAGGAGTAGGTGGTG-3'.

The polymerase chain reaction (PCR) analyses were performed in a total volume mixture of 50 μ L consisting of 0.2 μ g genomic DNA, 0.4 μ M each of primers, 0.8 mM dNTP mixture (0.2 mM each), $10 \times$ PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$), and 1.25 units rTaq DNA polymerase (Takara, Kusatsu, Japan). The thermal cycle profile was 94 °C for 5 min, followed by 30 cycles at 94 °C for 45 s, 58 °C for 30 s and 72 °C for 1 min, and extension at 72 °C for 5 min. The purification of the 390 PCR products from the two groups of animals was carried out using a Gel Extraction Kit (Omega Bio-Tek, GA, USA). The targeted

Fig. 1 Sanhe breed animals during severe cold stress challenge

fragments were then sequenced in both directions using BigDye® Terminator v3.1 Cycle Sequencing Kit (ThermoFisher, CA, USA).

Bioinformatic analyses

Single nucleotide polymorphism discovery was accomplished by multiple sequence alignments in the 105 (Sanhe) and 285 (Holstein) DNA samples for the *hsp70* gene promoter and the corresponding flanking regions. SNP discovery, homology searches, comparison among sequences, and multiple alignments were performed using DNAMAN version 6.0 (Lynnon Biosoft, USA) and Chromas 2.0 software (Technelysium, Australia), whereas the putative transcription factor binding sites were identified using the Cister (<https://zlab.bu.edu/~mfrith/cister.shtml>), Softberry (<http://www.softberry.com/cgi-bin/programs/promoter/tssg.pl>), and ProScan (<https://www.bimas.cit.nih.gov/cgi-bin/molbio/proscan>) software.

Association analyses

Association analyses between individual polymorphisms and the four biochemical parameters that significantly changed after cold stress (ACTH, T3, T4, and K^+) were estimated using the GLM procedure of SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). The statistical model can be described as follows: $Y_{ijk} = \mu + G_i + b_j \times B_j + e_{ijk}$, where Y_{ijk} is the difference in biochemical parameters between individual measurements before and after the severe cold temperature exposures; μ is the overall mean; G_i is the effect of the i th genotype (AA, AB, BB); B_j is the effect of the initial (before cold challenge) biochemical parameters concentration, and e_{ijk} is the vector of residual effects. Least square means were tested using the Bonferroni t test. The significance threshold used here was $P < 0.05$.

Plasmid construction and mutagenesis

The target 5'-flanking region was synthesized based on the sequence of Sanhe cattle *hsp70* gene available at the National Center for Biotechnology Information (NCBI, GenBank accession no. MH153782). The oligonucleotides spanned the DNA sequence from -399 to +208 bp of the *hsp70* gene (relative to the transcription start site, as shown in Fig. 2). The synthesized DNA fragment was sub-cloned into pEGFP-N1 expression vector (Clontech, Mountain View, CA, USA), using *AseI* and *BamHI* as cloning sites. The yielded construct was confirmed to contain SNP-42⁻C and SNP-205⁺C through sequencing and thus designated p42C-205C. SNP allele combinations for the polymorphisms SNP-42⁻ and SNP-205⁺ were then generated with the QuikChange II XL Site-Directed Mutagenesis Kit (StrataGene, La Jolla, CA), and designated p42T-205C and

p42C-205T, separately (Fig. 3a). All three plasmid constructs were verified by automated DNA sequencing in both directions (5'–3' and 3'–5').

Cell culture and transient expression

Cells of a human renal epithelial cell line (293T cells) were purchased from the American Tissue Cell Culture Inc. (Manassas, VA, USA), and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) at 37 °C in 5% CO₂. Cells were transfected with 5'-flanking region of *hsp70* constructs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol in six-well cell culture plates (Corning, NY, USA). After 48 h of transfection, cells were collected and analyzed. The mean fluorescence intensity of green fluorescent proteins (GFP) were measured by flow cytometry on a FACSCalibur cell analyzer (BD Biosciences, CA, USA) using a 488-nm laser. The BD FACSaria cell sorter (BD Biosciences, CA, USA) was used for the data analyses. Fluorescence was visualized with an Axiophot microscope (Carl Zeiss Microscopy GmbH, Göttingen, Germany) coupled to an AxioCam HR digital camera. Each transfection was repeated six times in triplicate, and results were averaged.

Results

The effect of cold stress on blood biochemical parameters in Sanhe cattle

To identify and define severe cold stress-induced changes in concentrations of blood biochemical parameters, 105 Sanhe cattle individuals were exposed to a temperature of -32 °C for 3 h. Four out of the six blood biochemical compounds analyzed here, that are usually used to indicate animal response to cold stress, showed statistically significant changes ($P < 0.01$ for ACTH, T3, and T4, and $P < 0.05$ for K^+) after the cold stress challenge. No statistically significant differences were observed in CORT concentration and the levels of GSH-PX activity before and after the cold stress challenge (Table 1).

Sequence of 5'-flanking region of the *hsp70* gene of Sanhe cattle

Based on the DNA sequence of Holstein–Friesian (AY149618.1), 624 bp 5'-flanking region of the *hsp70* gene was sequenced and analyzed in Sanhe cattle. Figure 2 shows the sequence that contains 399 bp of the 5'-flanking region and 225 bp of the first exon, 208 bp of 5'-untranslated region (5'-UTR), and 17 bp of coding sequence. The comparison with

Fig. 2 Nucleotide sequences of 5'-flanking region of the *hsp70* gene in Sanhe cattle. Gray shadings indicate deletions in Hereford sequence (AC_000180.1). Underlined sequences represent elements (HSE heat-shock element, C/E CCAAT enhancer). Boxed sequences indicate the transcription start site. Capital and bold letters indicate the variations within Sanhe cattle. Italics indicate nucleotides (ATG) that code for the first amino acid

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-399 CCTTAATTCT ACTTTAAACC ACCAAACTAA TTTGAGCCCC GAGATCCTCT CACCGCCCTA
-339 CAATTAATTA CAAGCCCAGG GCTGATCCTT CCAGTCGACT CCAAACACTT TGGTGCGCTG
-279 GTCGCCAGGA AACCAGAGAC AGAGTGGGTG GACCTTCCCA GCCCTCTCTC CCCTCTCCTT
-219 AGGACTCCTG TTTCTCTCAG CGAATCCTAG AAGAGTCTGG AGAGTTCTGG GAGGAGAGGC
          C/E      HSE                                GC box
-159 ATCCAGGGCG CTGATTGGTT CCAGAAAGCC AGGGGGCAGG ACTTGAGGCG AAACCCCTGG
          CCAAT box                                GCC box      HSE      -42
-99  AATATTCCCG ACCTGGCAGC CCCACTGAGC TCGTCAATTG GCTGACGAGG GAAAAGGTGG
          CCAAT box                                GC box
-39  GGCTTGATGA AGAATTATAA ACACAGAGCC GCCTGAGGAG AAACAGCAGC CTGGAGAGAGG
          TATA box                                +1
+22  CTGATAAAAC TTGCGGCTTA GTCCGTGAGA GCAGCTTCCG CAGACCCGCT ATCTCCAAGG
+82  ACCGCCCCGA GGAGCACCAG CGCGTTCAGT TTTCGGGTTC CGAAAAGCCC GAGCTTCTCG
+142 TCGCAGATCC TCTTCACCGA TTTCAGGTTT GAAGCTTATT TCGGAGCCGG AAAAGCAGGG
          +205
          T
+202 CACCGGCATG GCGAAAAACA CAGC
          Kozak

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the corresponding sequence from other Chinese cattle breeds (Chinese Holstein KY366444.1, Chinese Simmental KY366445.1, and Xinjiang Brown KY366446.1) showed a similarity rate greater than 98.00%. Approximately, the same level of homology was also found by the following comparison to the *hsp70* gene in other cattle breeds: 94.29% with Hereford (AC_000180.1), 97.36% with Hariana (EU872051.1), and 97.84% with Holstein-Friesian (AY149618.1). As shown in Table 2, out of the six bovine

sequences mentioned above, one deletion of AGGC between -44 and -45 bp appeared in each bovine sequence with the exception of Holstein-Friesian (AY149618.1), while two other large deletions (-399 to -202 bp and 101 to 131 bp) were observed only in the Hereford sequences (AC_000180.1). This is most likely due to incomplete assembly of certain genomic regions. Regarding to other species, the homology was considerably lower: 65.60% with Human (NM_005346.5), and 51.63% with Mus (NM_010478.2).

Fig. 3 GFP reporter expression analysis of SNP-42- and SNP-205+ polymorphisms. **a** Illustration of three plasmids constructed for promoter activity analysis of the *hsp70* gene. **b** Statistical analysis of flow cytometry data for the three plasmids. Comparisons of p42C-205C, p42T-205C, and p42C-205T showed lower fluorescence intensity of GFP, by 21.38% and 13.90%, respectively. Data is given as means $\pm$ SD (* P < 0.05; n = 5/group). **c** Representative fluorescence images for GFP by microscope

Table 1 Changes in blood biochemical parameters in Sanhe cattle exposed to severe cold stress

Parameter	Before cold stress*	After cold stress*	<i>P</i> value
T4, ng/mL	70.83 ± 22.40	75.77 ± 18.76	0.000087
T3, ng/mL	1.19 ± 0.21	1.34 ± 0.20	0.00016
ACTH, pg/mL	22.72 ± 5.40	25.60 ± 5.40	0.00052
K ⁺ , mmol/L	17.00 ± 2.02	16.60 ± 2.01	0.044
CORT, ng/mL	274.74 ± 26.01	269.78 ± 27.49	0.091
GSH-PX, U/mL	821.24 ± 34.78	811.27 ± 62.13	0.13

*Mean ± SD. A paired *t* test was used to analyze the changes in blood biochemical parameters after challenging animals to severe cold stress

Based on the integrated overlapping hits of the bioinformatic packages used here and in previous studies, various potentially important cis-acting elements were identified in the proximal sequence of 5'-flanking region, which revealed the presence of consensus sequences for the crucial regulators of the *hsp70* gene expression (Fig. 2). In particular, two CCAAT enhancers (C/E), two heat-shock elements (HSE), two GC box, one CCAAT box, one GCC box, and one TATA were found in this region. At the same time, a consensus Kozak sequence for translation initiation factor (TIF) located in the region from 203 to 212 bp downstream of transcription start site was identified.

SNP identification and genotyping

In comparison to the sequence available for *Bos taurus* (AY149618.1), no insertion was observed in the nucleotide sequence of the 5'-flanking region of the *hsp70* gene obtained in the Sanhe breed. In all samples, 20 SNP alleles were observed with varying degrees of occurrence; four of the observed changes were in the promoter region, and 16 were in the 5'-UTR. Allele and genotype frequencies of each variant, as well as respective genomic regions, are shown in Table 3 and Fig. 2. In addition, 9 out of the 20 SNPs that

were identified had their rs IDs in the NCBI SNP Database (<https://www.ncbi.nlm.nih.gov/snp/>), whereas the other 11 SNPs with a minor allele frequency (MAF) of 0.05 or greater were reported for the first time in the current study. Furthermore, by analyzing the same fragment in 285 Chinese Holstein, we identified 10 SNPs, all of which were common to those identified in Sanhe cattle, while the other 10 SNPs were specific to Sanhe cattle. These findings confirmed that the level of genetic variations in 5'-flanking region of the *hsp70* gene detected within Sanhe cattle is different from the corresponding variability found in other cattle groups, especially Holstein. Finally, the MAF of 16 out of 20 polymorphisms in Sanhe cattle was greater than 0.10 and the transition A/G at position + 102 bp in 5'-UTR showed the highest MAF (0.44). Furthermore, two SNPs at position - 42 and + 205 bp were located within the potential transcription and translation factor binding sites, respectively.

Association analyses between polymorphisms and changes in blood biochemical parameters after exposure to severe cold stress

We performed the association analyses between 16 individual SNPs with a MAF greater than 0.10 (Table 3) and the four blood biochemical parameters (ACTH, T3, T4, and K⁺) that significantly differed before and after cold stress challenge in Sanhe individuals (Table 1). As shown in Table 4, one SNP in the *hsp70* gene promoter region and three SNPs in the 5'-UTR were found to be significantly correlated with the difference in two blood biochemical parameters induced by severe cold stress in Sanhe cattle ($P < 0.05$). We observed significant associations of SNP-42⁻, SNP-105⁺, SNP-181⁺, and SNP-205⁺ with T3 ($P < 0.001$ –0.02), and of SNP-105⁺ with T4 ($P = 0.008$). None of the other 12 SNPs in the 5'-flanking region of the *hsp70* gene were significantly associated with any of the other four blood biochemical parameters (data are given in Online Resource 1).

Table 2 Deletions in multi-base difference among bovines

Sequence genome	Relative to the transcription starting site of Sanhe cattle		
	- 399 to - 202	- 45 to - 44	101~131
Sanhe (MH153782)	+	–	+
Xinjiang Brown (KY366446.1)	+	–	+
Chinese Simmental (KY366445.1)	+	–	+
Chinese Holstein (KY366444.1)	+	–	+
Holstein-Friesian (AY149618.1)	+	+	+
Hereford (AC_000180.1)	–	–	–
Hariana (EU872051.1)	+	+	+

+ existing, – missing

Table 3 Frequencies of variations in the 5'-flanking region of the *hsp70* gene in Sanhe cattle and Chinese Holstein cattle

Sites*	Variation	ID	Allele frequency		Region
			Sanhe	Chinese Holstein	
-261	A/T	Novel	0.89/0.11	0.81/0.19	Promoter
-135	C/-	Novel	0.83/0.17	0.72/0.28	Promoter
-42	C/T	Novel	0.76/0.24	NA	GC box
-11	C/T	Novel	0.84/0.16	NA	Promoter
+12	C/T	rs137078625	0.81/0.19	NA	5'-UTR
+21	G/A	rs378256122	0.96/0.04	NA	5'-UTR
+22	C/T	Novel	0.86/0.14	0.95/0.05	5'-UTR
+34	A/G	rs267240780	0.75/0.25	NA	5'-UTR
+45	C/T	rs211506802	0.83/0.17	0.80/0.20	5'-UTR
+52	G/A	rs267240778	0.79/0.21	NA	5'-UTR
+72	G/A	rs471604061	0.96/0.04	0.93/0.07	5'-UTR
+94	G/A	rs438646103	0.58/0.42	0.81/0.19	5'-UTR
+102	A/C	rs478612967	0.56/0.44	0.58/0.42	5'-UTR
+105	G/T	Novel	0.87/0.13	0.65/0.35	5'-UTR
+109	A/G	Novel	0.83/0.17	NA	5'-UTR
+111	T/C	Novel	0.95/0.05	NA	5'-UTR
+115	C/T	Novel	0.80/0.20	NA	5'-UTR
+131	C/G	Novel	0.95/0.05	0.94/0.06	5'-UTR
+181	C/T	rs473916108	0.67/0.33	0.59/0.41	5'-UTR
+205	C/T	Novel	0.80/0.20	NA	Kozak

*Relative to the transcription start site

NA not applicable

Biological effect of the polymorphisms SNP-42⁻ and SNP-205⁺ on the promoter activity of the *hsp70* gene

We used Cister, Softberry, and ProScan packages to perform the bioinformatic analyses to identify potential binding sites for important factors in the 5'-flanking region of the *hsp70* gene, and observed 11 potential binding sites for transcription or translation factor, in which SNP-42⁻ was located in a GC

box element for stimulatory protein 1 (Sp1), and SNP-205⁺ in Kozak for TIF. To define the functional significance of SNP-42⁻ (C/T) and SNP-205⁺ (C/T) on the *hsp70* promoter activity, we constructed three plasmids (Fig. 3a) for the possible allele combinations (i.e., p42C-205C, p42T-205C, and p42C-205T) by site-directed mutagenesis from a synthesized fragment based on the sequence of the 5'-flanking region of the *hsp70* gene in Sanhe cattle at NCBI (GenBank accession no. MH153782), and then transfected them into 293T cells.

Table 4 Association results between SNPs in 5'-flanking region of the *hsp70* gene and difference of biochemical parameters during cold stress in Sanhe cattle

SNP	Genotype	Number	T3 (ng/mL)		T4 (ng/mL)	
			LS means $\pm$ SE	P-value*	LS means $\pm$ SE	P-value*
-42	CC	67	0.39 $\pm$ 0.02 ^a	0.020	4.14 $\pm$ 1.26 ^a	0.40
	CT	21	0.14 $\pm$ 0.04 ^{ab}		4.60 $\pm$ 2.24 ^a	
	TT	14	0.11 $\pm$ 0.05 ^b		4.06 $\pm$ 2.77 ^a	
+1-05	GG	76	0.17 $\pm$ 0.02 ^a	0.020	6.60 $\pm$ 1.14 ^a	0.0080
	GT	26	0.07 $\pm$ 0.02 ^b		0.39 $\pm$ 1.09 ^b	
	TT	0	NA		NA	
+1-81	CC	55	0.09 $\pm$ 0.03 ^a	0.0090	3.83 $\pm$ 1.40 ^a	0.43
	CT	25	0.19 $\pm$ 0.04 ^b		5.62 $\pm$ 2.06 ^a	
	TT	20	0.23 $\pm$ 0.04 ^b		4.28 $\pm$ 2.33 ^a	
+2-05	CC	71	0.15 $\pm$ 0.04 ^a	0.010	4.76 $\pm$ 1.23 ^a	0.84
	CT	19	0.16 $\pm$ 0.02 ^a		5.15 $\pm$ 2.39 ^a	
	TT	11	0.06 $\pm$ 0.02 ^b		4.55 $\pm$ 3.13 ^a	

*P-value < 0.05 means significant difference among the genotypes. LS: least square; SE: standard error. NA: not applicable. Bonferroni t-test was used to compare the changes in T3 and T4 between different genotypes. Different letters in the same row mean significant difference between the genotypes ($P < 0.05$).

Following the transient transfection, we measured the effect of the two variants on the *hsp70* gene promoter activity by GFP reporter assay in which the mean fluorescence intensity was measured by flow cytometry. As presented in Fig. 3b, c, constructs p42T-205C and p42C-205T showed significantly less GFP activity than p42C-205C (i.e., 21.38% and 13.90%, respectively; $P < 0.05$). These results indicate that both alleles of SNP-42⁻ and SNP-205⁺ have impact on the *hsp70* gene promoter activity. Furthermore, the effect of SNP-42⁻ in the GC box is more notable than SNP-205⁺ according to the differences observed among the three constructs, of which the T allele caused lower expression of GFP compared to the C allele. This finding indicates that these two SNPs are functional polymorphisms contributing at least partly to the observed association of the gene with cold stress response in Sanhe cattle.

Discussion

Sanhe cattle is an important dual-purpose breed that originated as a result of the long-term selection in the grasslands of Northeastern Inner Mongolia (China) where the monthly average temperature is usually below 0 °C, and the animals are raised in a semi-extensive production system during severe winter conditions (Wu et al. 2012). Sanhe cattle are more cold-resilient compared to other cattle breeds likely due to the capability to maintain stable body temperature in extremely low temperatures, by reducing heat losses and increasing heat load (Nienaber and Hahn 2007). Also, Sanhe cattle are well known for its disease resistance, hardiness, and survival under low-input production systems and severe winter conditions. For instance, both Wang et al. (2007) and Yuan et al. (2012) have reported significantly lower estimates of somatic cell count in Sanhe cattle compared to Chinese Holstein, indicating that Sanhe cattle are more resistant to mastitis compared to Chinese Holstein. The effective cold resilience and economic importance of Sanhe cattle to the Chinese livestock production systems highlight the value of this population as an interesting animal model and important genetic resource for studying cold stress at the physiological and molecular levels, and increasing food production in harsh environmental conditions.

We previously observed that the concentrations of blood ACTH, T3, and T4 were significantly upregulated in Sanhe cattle after severe cold exposure (Li et al. 2015). These findings were confirmed in the present study using an independent sample set. Given these consistent results, we confirmed that blood ACTH, T3, and T4 can be used as indicators for measuring cold stress response in Sanhe cattle. Hormones are known to be pivotal regulators in response to temperature stress (Nazifi et al. 2003). Under cold stress conditions, the physiological responses are regulated by the hypothalamic–

pituitary–adrenal (HPA) and hypothalamic–pituitary–thyroid (HPT) axis, eventually inducing the concentration changes of CORT, T3, and T4 in circulation of blood to maintain the body temperature stable and then adapt to changes in the external environment, whereas ACTH is synthesized and secreted by anterior pituitary gland in stress responses and acts on the adrenal gland to regulate CORT. Similarly to our findings, it has been reported that T3 and T4 significantly increased concentration levels in fat-tailed sheep (Nazifi et al. 1999) and rats (Park et al. 2017), after exposure to cold temperatures, and ACTH in both Wistar Kyoto and Sprague Dawley rats (Pardon et al. 2003). Future studies should investigate whether these blood biochemical parameters will have the same change patterns in other cattle breeds in response to cold stress.

Differently gene expression due to cold stress has been well documented in both animals (Shore et al. 2013) and plants (Sharma and Nayyar 2014). We previously reported, in a transcriptome study, various differentially expressed genes in Sanhe cattle, including *hsp70*, after exposure to extremely low temperatures (Xu et al. 2017). Due to the role of HSP in the protection of cells against stress effects, we hypothesized that *hsp70* is likely an important candidate gene for differential cold stress response. Thus, in the current study, we unraveled the genetic variability in the *hsp70* gene 5'-flanking region and performed genetic associations and identification of functional polymorphisms located in the *hsp70* gene with cold stress response in Sanhe cattle. We firstly obtained the consensus sequence of the *hsp70* gene 5'-flanking region in Sanhe cattle and submitted to the NCBI GenBank (GenBank accession no. MH153782). A similarity level greater than 93% in sequences between Sanhe cattle and other six cattle breeds shown by sequence comparison suggested a high conservation of the 5'-flanking region in the *hsp70* gene, as observed by Sodhi et al. (2013). Additionally, important regulation elements, particularly a potential translation initiation factor binding site known as Kozak, were found in this region (Fig. 2). Earlier studies reported that the promoter of the *hsp70* gene contained those core domains in pigs (Schwerin et al. 2001) as well as in Zebu cattle (*Bos indicus*) (Behl et al. 2014). In this study, we identified 11 out of 20 SNPs which were reported for the first time in the 5'-flanking region of the *hsp70* gene by direct sequencing of Sanhe animals. In order to explore whether these 20 genetic variations were specific for Sanhe cattle, we sequenced the same fragment in 285 Chinese Holstein samples and found that 10 out of the 20 SNPs were segregating (Table 3). The higher polymorphism number observed in the Sanhe breed may be a result of the biological mechanisms related to cold stress response, which improves the cold adaptation of this breed compared to other cattle breeds. Four out of the 16 SNPs with MAF greater than 0.10 located in the *hsp70* gene promoter or 5'-UTR region were found to be significantly ($P < 0.05$) associated with the

differences in two blood biochemical parameters induced by severe cold stress in Sanhe cattle (Table 4): T3 with SNP-42⁻, SNP-105⁺, SNP-181⁺ and SNP-205⁺, and T4 with SNP-105⁺. For SNP-42⁻, there was only a significant difference in changes of T3 between CC and TT genotypes; and for SNP-181⁺, no significant difference in the changes of T3 was found between CT and TT genotypes; only those with CC genotypes had significantly less increase in T3 after cold stress. However, in the case of SNP-205⁺, only TT genotypes had significantly less increase in T3 when compared to CC and CT genotypes. These results indicate that allele effects among those SNPs vary in experimental individuals. SNP-42⁻ and SNP-205⁺ were located in GC box and Kozak elements; thus, we speculate that they may represent two functional polymorphisms affecting promoter activity. By GFP reporter assay containing different allele combinations of SNP-42⁻ and SNP-205⁺, we found that the plasmid constructs bearing SNP-42⁻T and SNP-205⁺T alleles produced significantly less GFP activity than those with C alleles (Fig. 3). We thus conclude that SNP-42⁻T and SNP-205⁺T are potential causative polymorphisms involved in the regulation of the *hsp70* promoter activity. Additionally, we observed that the changes in T3 among CC genotypes were higher than those in TT genotypes in both SNP-42⁻ and SNP-205⁺ (Table 4). It indicates that the C alleles of SNP-42⁻ and SNP-205⁺ may play a protective role against the cold stress response in Sanhe cattle. The GC box is upstream of the TATA box and approximately 110 bp upstream from the transcription initiation site, genetic variants in which can affect the expression of genes by altering the binding affinity to SP1 and enhancer activity, which finally causes the differences in phenotypic expression (Schwerin et al. 2001) (e.g., cold stress response). Various studies have shown evidences for that, such as the variants in the GC box of the *AGTRL1* and *PD-L1* genes which were associated with susceptibility to brain infarction (Hata et al. 2007) and clinicopathological features in gastric cancer (Tao et al. 2017), respectively. Whereas, the Kozak sequence occurs on eukaryotic mRNA and plays a major role in the initiation of the translation process (Kozak 1986). Polymorphisms in Kozak sequence in variant genes were also found to be associated with disease phenotypes in humans (Cheng et al. 2016). In this study, we observed that the SNP-42⁻ in GC box and SNP-205⁺ in Kozak affected the promoter activities of the *hsp70* gene. It could modulate the expression of *hsp70*, which in turn would contribute to the improved cold stress response in Inner-Mongolia Sanhe cattle.

Conclusion

Our findings suggest that SNP-42⁻ and SNP-205⁺ are causative polymorphisms involved in the regulation of *hsp70* promoter activity and might contribute to the observed

association between the *hsp70* gene and T3 and T4 levels in Sanhe cattle. Thus, *hsp70* gene is a promising candidate gene to be validated in independent cattle populations and functional studies related to cold stress resilience.

Author contributions Q Xu and YC Wang designed the experiment and supervised the project. LR Hu carried out the data analyses and wrote the initial manuscript of the paper. Y Ma, LL Liu, and L Kang conducted the lab work. LL Liu collected the samples and determined blood biochemical parameters. LF Brito helped to discuss the results and edit the manuscript. DS Wang, HJ Wu, and AR Liu provided the experimental animals and pedigree information.

Funding information This work is supported by the Research Fund for International Young Scientists by the National Natural Science Foundation of China (no. 31750110459), the National Natural Science Foundation of China Project (no. 31172191), the earmarked fund for Modern Agro-Industry Technology Research System (no. CARS-37), and the Program for Changjiang Scholar and Innovation Research Team in University (no. IRT1191).

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval The use of the animals was in agreement with the Committee on Ethics of Animal Experimentation from the Beijing Jiaotong University. The experiment was performed according to regulations and guidelines established by this committee.

Consent for publication Not applicable.

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