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mRNA Expression of Glutathione S-Transferase Pi (GSTP1) under Heat Stress and Association of Genotypes with Heat Tolerance Ability in Holstein

[1]Jia Yao, [1]Kun Liao, [2]Qiu-Ling Li, [2]Chang-Fa Wang,
[2]Hong-Mei Wang, [1]Song-Jia Lai and [2]Ji-Feng Zhong
[1]College of Animal Science and Technology, Sichuan Agricultural University,
Xinkang Road, 625014 Ya'an, Sichuan, China
[2]Dairy Cattle Research Center, Shandong Academy of Agricultural Science,
250100 Jinan, Shandong, China

Abstract: High ambient temperature has largely reduced the milk production due to the weak heat tolerance ability of Holstein. One potential mechanism for the destructive effect on organism is that the heat stress stimulated excessive cellular toxicants. The glutathione S-transferase Pi (GSTP1) has been proposed to play an important role to inactivate toxic metabolites in human malignant tumors. In this study, researchers evaluated the effect of heat stress on GSTP1 mRNA expression in Holstein using semi-quantitative RT-PCR method. With the liver tissue exception, the *GSTP1* gene showed relatively high expression level in heart, spleen and kidney tissues in cool ambient temperature. After heat stress treatment, the GSTP1 mRNA expression increased significantly in all studied tissues. We sequenced the 3705 bp fragment containing complete sequence of *GSTP1* gene among 15 cows and detected 31 variations. The nonsynonymous variation of G18C (p.M6I) was further scanned in 106 Holsteins using RFLP method to analyze the association of genotypes with heat tolerance ability. However, we did not detect statistical difference of heat tolerance ability among genotypes. To the knowledge, this is the first report to study GSTP1 mRNA expression under heat stress, SNPs distribution and association of genotypes with heat tolerance ability in Holstein. The significantly elevated expression of GSTP1 would suggest the positive role to resist heat stress, especially in liver tissue.

Key words: GSTP1, mRNA expression, SNPs, heat stress, Holstein, tissue

INTRODUCTION

The milk production of Holstein is very susceptible to high ambient temperature due to the weak heat tolerance ability which has strongly restricted the development of dairy industry in tropical regions and caused considerable economic loss (St-Pierre *et al*., 2003). The continuous genetic selection for higher production performances will inevitably result in greater susceptibility to heat stress. Although, many reports have been conducted to study the effect of heat stress on milk production, reproduction performance and immune system function in Holstein, the detailed molecular mechanisms has remained unknown (West, 2003; Argov *et al*., 2005; Aguilar *et al*., 2010). One alternative mechanism for the destructive effect on organism is that the heat stress directly and indirectly stimulates excessive intra/inter-cellular toxicants such as Malondialdehyde (MDA) and Reactive Oxygen Species (ROS) (Zuo *et al*., 2000; Yang and Lv, 2006). These intermediates can destroy cell membrane integrity by oxidating polyunsaturated fatty acids and further disturb biological metabolic function (Ikeda *et al*., 1999; Valko *et al*., 2004; Mene-Saffrane *et al*., 2009). Ravagnolo and Misztal estimated the genetic parameters in Holstein and found that the heat tolerance ability is partially genetically controlled (Ravagnolo and Misztal, 2000). However, the underlying functional gene(s) and the corresponding mechanism have largely remained unknown until now. The Glutathione S-Transferase Pi (GSTP1) belonging to glutathione S-transferase superfamily has been proposed to consistently express in a wide range of tissues in human including cornea, ciliary epithelium, prostate, placenta, breast, esophagus and lung (Ishioka *et al*., 1991; Hernando *et al*., 1992).

The best known physiological role of Glutathione S-Transferases (GSTs) is the detoxification by catalyzing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione (Perally *et al*., 2008; Chronopoulou and Labrou, 2009). Among them, the

Corresponding Author: Song-Jia Lai, Laboratory of Animal Genetic and Breeding, College of Animal Science and Technology, Sichuan Agriculture University, 46 Xingkang Road, 625014 Ya'an, Sichuan Province, China

expression level and Single Nucleotide Polymorphisms (SNPs) of *GSTP1* gene have been wildly reported to associate with the susceptibility to human tumors such as prostate cancer (Millar *et al*., 1999), acute lymphoblastic leukemia (Stanulla *et al*., 2000), endometrial cancer (Chan *et al*., 2005) and breast cancer (Oliveira *et al*., 2010). Under these biological processes, GSTP1 would play the central role in the detoxification of ROS and other toxic metabolites induced by carcinogenesis (Millar *et al*., 1999; Fryer *et al*., 2000; Yeh *et al*., 2010). In bovine, the GSTP1 shows >85% homology of amino acid sequence with human (Hernando *et al*., 1992). So, researchers tentatively proposed that GSTP1 would play positive response under heat stress to alleviate the destructive effect on organism in Holstein by inactivating excessive metabolic toxicants which has not been reported to the knowledge.

During last decades, some physiological and biochemical indexes have been explored to evaluate the ability of heat tolerance. Among them, the Rectal Temperature (RT) and Potassium Content in Erythrocytes (PCE) were wildly adopted as heat tolerance indexes in Holstein. The lower RT and PCE values were always associated with better ability to resist heat stress (Olson *et al*., 2003; Su *et al*., 2006; Chen *et al*., 2007). In this study, we studied the effect of heat stress on mRNA expression of *GSTP1* gene in Holstein. The SNPs distribution and their association of genotypes with heat tolerance ability evaluated by RT and PCE values were further analyzed. To systematically deduce this subject will be significant to improve the heat tolerance ability by genetic selection in Holstein.

MATERIALS AND METHODS

Animals: Total 112 healthy Holsteins at the 2nd or 3rd lactations were randomly collected in this study. They were treated with same feed nutrition supply and management schedule in experimental farm at Shandong Academy of Agricultural Science, Jinan, China. Among them, 6 cows with similar body weight and body condition score were further selected to study the effect of heat stress on *GSTP1* gene mRNA expression while the remaining 106 individuals were further adopted to analyze the association of SNP in *GSTP1* gene with heat tolerance ability.

Experimental design: The Temperature Humidity Index (THI) was employed to evaluate the heat stress degree (Bohmanova *et al*., 2007). This experiment began at early April, 2007 which was designed as cool ambient temperature (average THI was ~60). Before test day, researchers measured dry bulb and wet bulb temperatures 3 times every day (8:00, 14:00 and 20:00) and calculated the average THI for 3 days (Liu and Liang, 2006; Bohmanova *et al*., 2007). After this, the 6 cows were randomly divided into control group (continually kept in cool ambient temperature) and experimental group (treated with heavy heat stress). The 3 cows in experimental group were kept in artificial climate chamber for 24 h which was designed for THI of about 85 (corresponding to dry bulb temperature of 32±1°C and relative humidity of almost 75%). Researchers evaluated *GSTP1* gene mRNA expression between control group and experimental group by using the semi-quantitative RT-PCR method.

The individual heat tolerance ability was assessed based on the Rectal Temperature (RT) and Potassium Content in Erythrocytes (PCE). Researchers measured the RT value twice daily (8:00 and 14:00) and PCE value from jugular vein blood for the 106 cows at test day in the cool ambient temperature. The RT measurement was further repeated when the mean THI was up to 76.5, almost in early August of the same year. The average RT values both in cool and hot ambient temperatures were finally adopted. The SNP distribution and frequencies of *GSTP1* gene were determined by sequencing and Restriction Fragment Length Polymorphism (RFLP) methods.

PCE measurement: After 10 mL vein bloods were carefully collected at test day in the cool ambient temperature in the 106 cows, we isolated erythrocytes at a relative centrifugal force of 3,000 g for 20 min. The erythrocytes were treated with deionized water and further digested overnight by mixed acid (the volume ratio of HNO_3 and $HClO_4$ was 5:1). After the pretreatment, the PCE was measured in AA6300 atomic absorption spectrophotometer (Shimadzu Co., Japan) and was presented as milligram per unit erythrocyte volume (mg L^{-1}).

Total RNA extraction and cDNA synthesis: After the 6 cows from control group and experimental group were slaughtered by using electrical stunning, we immediately isolated the tissues of heart, liver, spleen and kidney. Total RNA was prepared for each tissue sample (100 mg) using 2 mL TRIzol reagent (Invitrogen Life Technologies, Shanghai, China) according to manufacturer's instructions. RNA integrity and concentration were subsequently tested. The total RNA treated with DNase was then converted to cDNA using the first-strand cDNA Reverse Transcription Kit (TaKaRa Bio Inc., Dalian, China).

Semi-quantitative RT-PCR: One primer pairs in exon 3 for *GSTP1* gene amplification were designed according to

Table 1: The primers used to RT-PCR analysis and to amplify *GSTP1* gene

Purposes	Genes/ fragments	Primers (5'→3')	Locations	Annealing temperatures (°C)
RT-PCR	GSTP1	AACTACGAGGCGGGCAAGG; CTGGTCGCCCACGATGAA	404-517	60.5
	β-actin	CATCCGCAAGGACCTCTAC; ATGCCAATCTCATCTCGTTTT	956-1295	60.5
To amplify	Primer 1	CTCCAAACGGTCCACG; GGGTAGCCTATCCCTTCG	-408-1146	63.5
GSTP1 gene	Primer 2	AATGGAGGCGTGTGGAGGTT; CCACCCAGAACCAGAAGCAGC	1017-1667	60.5
	Primer 3	ACGGTGTAGAGGACCTTCGCT; TGTTTCCCATTGCCGTTGAT	1486-2784	62.5
	Primer 4	ACCTGCTGGACCTGCTTCG; AGGCAGATTCTTTCCTGTTTGAG	2619-3285	62.0
	Primer 5	GCCAGGAGGATGATACCCAG; TGTTTCCCATTGCCGTTGAT	2290-2784	61.0
	Primer 6	CCTGCTGGACCTGCTTCGGA; CTCCCACATATGAGGCAGA	2620-3297	63.0

mRNA sequence of cattle (GenBank acc. no. NM_177516). Another primer pairs were also used to amplify *β-actin* gene (Table 1). The 2 primer pairs were approved to be noncompetitive. Preliminary experiments were conducted to determine the appropriate number of PCR cycles to assure that the amplification was in the exponential range and could be linearly quantified; the 24-30 PCR cycles were tested. Semi-quantitative RT-PCR was conducted in the same tube to compare the relative expression ratio of *GSTP1* gene to *β-actin* gene. PCR amplifications were carried out in 50 μL reaction mixture containing 0.5 μL of each primer (10 pM), 10 μL cDNA, 2.5 unit LA Taq DNA Polymerase and 25 μL 2×GC buffer I (TaKaRa Bio Inc., Dalian, China) followed by 4 min at 94°C, 26 cycles of 30 sec at 94°C, 30 sec at 60.5°C, 30 sec at 72°C, an additional 5 min extension step. The RT-PCR products were stained by GoldView on 1.2% agarose gel. The signal intensity of each PCR product was scored by using quality one 4.6.2 software to calculate the ratio of GSTP1 to β-actin mRNA expression. This protocol was performed in the 6 cows from both control group and experimental group.

***GSTP1* gene sequencing and SNPs identification:** Genomic DNA was extracted by using standard phenol/chloroform method. Total 6 primers were developed according to cattle genome sequence (GenBank acc. no. NW_001494541) to amplify the 5'-flanking region, exon/intron complete sequences and 3'-flanking region sequences among 15 randomly selected individuals (Table 1). PCR amplifications were performed in a 50 μL reaction mixture containing 100 ng of DNA, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 50 mM KCl, 10 pM of each primer and 2.5 unit LA Taq DNA Polymerase under the procedures of 35 cycles at 94°C for 45 sec at individual annealing temperature (Table 1) for 60 sec and at 72°C for 1 min. All PCR products were purified on spin columns and were directly sequenced for both strands with amplification primers using a Big Dye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI Prism 3100 DNA sequencer according to manufacturer's manual. After sequences were artificially checked, the variable sites were determined in Mutation Surveyor 2.28 software.

PCR-RFLP: In order to investigate, the allele frequencies, one nonsynonymous SNP (G18C in exon 1) was successfully developed for RFLP analysis using restriction endonuclease BstXI. The PCR reactions were performed in a volume of 25 μL reaction mixture containing 50 ng of DNA, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 50 mM KCl, 10 pM of each primer and 1 unit of Taq polymerase (S_{ABC}) which were carried out under the PCR program with 5 min denaturation at 94°C, 30 cycles for 1 min denaturation at 94°C, 1 min annealing at 63.5°C, 1 min extension at 72°C and a final extension for 7 min at 72°C. The PCR product was digested with restriction endonucleases BstXI and detected in a 1.5% agarose gel electrophoresis.

Statistical analysis: The effect of heat stress treatment on *GSTP1* gene mRNA expression in different tissues was statistically analyzed using t test. Least square means of RT and PCE values were estimated under general linear model with GSTP1 genotypes as fixed factor using SAS statistical software package (SAS Institute Inc., Cary, NC, USA). Considering the fact that all cows adopted in this study were from one breed, one farm, same seasons and similar ages (lactations), researchers only evaluated the effect of GSTP1 genotypes on heat tolerance ability.

RESULTS AND DISCUSSION

Effect of heat stress on GSTP1 mRNA expression: In cool ambient temperature (the average THI was ~60), the *GSTP1* gene showed relatively high expression level in heart, spleen and kidney tissues whereas we hardly detected mRNA expression in liver tissue (Fig. 1). After the simulated heat stress treatment, the GSTP1 mRNA expression significantly increased in all studied tissues ($p<0.05$ or $p<0.01$). Among the 4 tissues, heart tissue showed the highest expression level both in cool ambient temperature and after heat stress treatment.

SNPs identification in *GSTP1* gene: Researchers successfully obtained the 3705 bp fragment of *GSTP1* gene consisting of 408 bp of 5'-flanking region, the completesequence of 7 exons and 6 introns and 428 bp of

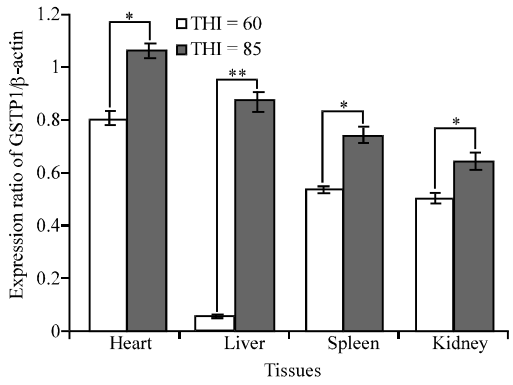

Fig. 1: Changes of mRNA expression ratio of *GSTP1* gene to *β-actin* gene under heat stress treatment in different tissues of Holsteins. Bars were calculated based on the density and width of electrophoresis bands using quality one 4.6.2 software. White and black showed the control group and experimental group. The significant difference were further marked by *and** on 0.05 and 0.01 levels, respectively

3'-flanking region. Total 31 variations were detected among the 15 sequenced individuals, consisting of three deletions, one insert, 24 transitions and 3 transversions (Table 2). Among them, 14 variations (45.2%) had already been reported in Hereford cattle. These variations were distributed in the region of -346th~3171st sites and showed relatively high mutation frequencies in 3rd-6th intron regionsand 7th extron. Total 2 fragment deletion events were detected at -346th and 3086th sites, respectively (Table 2).

PCR-RFLP and association analysis: The nonsynonymous variation of G18C (p.M6I) occurred in exon 1 was successfully subjected to PCR-RFLP analysis by using restriction endonucleases BstXI. The PCR product was digested into 1123 and 431 bp fragments due to the transversion of G18C.

The frequencies of CC, CG and GG genotypes among 106 cows were 24.5% (26), 49.1% (52) and 26.4% (28), respectively (Table 3). The frequencies of C and G alleles were 49.1 and 50.9%. The effects of different genotypes on heat tolerance ability were evaluated (Table 3). There was no significant effect of genotypes on heat tolerance ability evaluated by both the PCE value (p = 0.6620) and the RT value (p = 0.5067). The highest and the lowest least square means were 677.44±57.01 mg L^{-1} (CG genotype) and 601.20±77.69 mg L^{-1} (GG genotype) for PCE value. Meanwhile, GG genotype had highest RT value (39.46±0.09°C) and CC genotype had the lowest RT value (39.32±0.10°C). Glutathione S-transferase superfamilies, consisting of 5 classes of alpha, pi, mu, theta and zeta in human have been identified to be involved in conjugation of electrophilic compounds to reduced glutathione (Mannervik *et al*., 1992; Ntais *et al*., 2005; Chronopoulou and Labrou, 2009). Among them, *GSTP1* gene (pi-class) polymorphism and expression analyses were wildly reported in various malignant diseases.

Wang *et al*. (2004) studied the expression of *GSTP1* gene in various prostatic disease types and suggested that GSTP1 may be associated with the genesis and progress of prostate carcinoma. Chan *et al*. (2005) found that the Ile105Val polymorphism of *GSTP1* gene was associated with an increased risk of endometrial cancer. The association of genotypes were also reported in

Table 2: Sequence variations of *GSTP1* gene in Holsteins

IDs	Locations	Types	GenBank accession nos.
1	-346-337 (5' end)	Del GGTGCACAGG	
2	-209 (5' end)	T>C	
3	-81 (5' end)	A>G	
4	18 (Exon 1)	G>C	
5	29 (Exon 1)	G>A	
6	210 (Intron 1)	A>G	
7	326 (Intron 2)	C>T	rs42188155
8	386 (Intron 2)	C>T	rs42188154
9	699 (Intron 3)	T>C	
10	708 (Intron 3)	Del C	
11	746 (Intron 3)	A>G	rs42188153
12	915 (Intron 4)	A>G	rs42188152
13	940 (Intron 4)	C>T	rs42188151
14	1229 (Intron 4)	A>G	rs17871923
15	1760 (Intron 5)	A>G	rs42188150
16	1793 (Intron 5)	T>C	rs42188149
17	1905 (Intron 5)	G>A	rs42188147
18	2332 (Exon 6)	A>G	
19	2453 (Intron 6)	G>A	
20	2508 (Intron 6)	G>A	
21	2567 (Intron 6)	G>A	rs42188146
22	2746 (Exon 7)	G>C	rs42188145
23	2796 (Exon 7)	T>C	rs42188144
24	2797 (Exon 7)	G>A	rs42188143
25	2809 (Exon 7)	G>T	rs42188142
26	2829 (Exon 7)	T>C	
27	2833-2834 (Exon 7)	Ins C	
28	3078-(3' end)	A>G	
29	3086-3092 (3' end)	Del AAAGTGA	
30	3166 (3' end)	T>C	
31	3171 (3' end)	G>A	

These variations already published in cattle were denoted by their GenBank ac-cession numbers and their IDs. are 1-31, respectivly

Table 3: Least square means of genotypes for PCE and RT values

		Traits[b]			
Sites	Genotypes	N (%)[a]	PCE (mg L^{-1})	RT (°C)	p-values
G18C	CC	26 (24.5)	609.05±80.62	39.32±0.10	0.6620 (PCE)
	CG	52 (49.1)	677.44±57.01	39.35±0.07	0.5067 (RT)
	GG	28 (26.4)	601.20±77.69	39.46±0.09	-
Total	-	106	-	-	-

[a]The genotypes which occurred in less than 10 individuals were excluded to calculate least square mean values; [b]All least square means for PCE (Potassium content in erythrocytes) and RT (Rectal temperatures) values had no statistical differences among different genotypes

prostate cancer (Millar *et al*., 1999), acute lymphoblastic leukemia (Stanulla *et al*., 2000) and breast cancer (Chan *et al*., 2005). The positive biological role of GSTP1 in these genesis processes is to detoxify ROS and other toxic metabolites induced by malignant pathological changes (Eaton and Bammler, 1999; Fryer *et al*., 2000; Chung *et al*., 2009). The Temperature Humidity Index (THI) was wildly adopted to evaluate the heat stress degree in Holstein (Bohmanova *et al*., 2007; Avendano-Reyes *et al*., 2010). When THI index exceeds 76 cow will be suffering from heavy heat stress and exhibited significantly decreased milk production (West, 2003). The destructive effect of heat stress on organism might take place via stimulating excessive cellular toxicants such as ROS and MDA (Zuo *et al*., 2000; Yang and Lv, 2006). So, researchers tentatively inferred that GSTP1 would play the protective role under heat stress by inactivating the excessive metabolic toxicants. In this study, we kept Holsteins in artificial climate chamber for 24 h which was designed the heavy heat stress condition and found that heat stress treatment significantly elevated the mRNA level of *GSTP1* gene in all studied tissues. Most interesting, the mRNA expression of GSTP1 in liver tissue was hardly detected in cool ambient temperature which was strikingly contrasted to that after heat stress treatment (Fig. 1).

One early report also suggested that *GSTP1* gene hardly expressed in normal liver tissue of rat while high expression appeared in pathological cells of liver cancer (Sato, 1989). In addition, high expression of *GSTP1* gene has been wildly reported in many malignant human tumors (Wang *et al*., 1997; Kolwijck *et al*., 2009). These results would suggest that the elevated expression of *GSTP1* gene especially in liver tissue might play the positive response to resist the destructive effect of heat stress in Holstein.

The RT was wildly acceptable to evaluate the ability for maintaining homeostasis in deep body temperature and the low RT value was associated with high resistance ability to heat stress (Olson *et al*., 2003). In addition, the PCE was proved to be a reliable and practical heat-tolerant index in Holstein and the high heat tolerance ability was proposed to be less than the threshold of 800 mg L^{-1} (Liu *et al*., 1997; Su *et al*., 2006; Chen *et al*., 2007). In this study, we combined the two indexes together to study the association of genotypes with heat tolerance ability in Holsteins on the SNP of G18C variation which occurred in exon 1 and resulted in amino acid mutation.

The lowest least square means of PCE and RT were presented in GG and CC genotypes, respectively. However, researchers did not detect statistical difference of heat tolerance ability among genotypes. In contrast, the significant associations of polymorphism in *GSTP1* gene with susceptibility to various tumors have been reported in human (Millar *et al*., 1999; Stanulla *et al*., 2000; Chan *et al*., 2005; Singh *et al*., 2008). More SNPs in *GSTP1* gene should be further investigated to deduce the association of genotypes with heat tolerance ability in Holstein.

CONCLUSION

To the knowledge, this is the first report to study GSTP1 mRNA expression under heat stress, SNPs distribution and association of genotypes with heat tolerance ability in Holstein. Although, researchers failed to detect the significant association of genotypes of GSTP1 with heat tolerance ability, the significantly elevated expression in all studied tissues under heat stress treatment especially in liver tissue also confirm the positive role of GSTP1 to resist the heat stress in Holstein.

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