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A novel SNP of the *ATP1A1* gene is associated with heat tolerance traits in dairy cows

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Abstract PCR low ionic strength single-strand conformation polymorphism (PCR-LIS-SSCP) and DNA sequencing methods were used to analyze the polymorphisms within the coding region of bovine *ATP1A1* gene. A novel C/A mutation was identified at the nucleotide position 2789 of the *ATP1A1* mRNA, but it was silent with respect to the amino acid sequence of the protein. The LIS-SSCP banding pattern could be divided into three kinds of genotypes, named *CC*, *CA*, and *AA*. Also, the association of the novel SNP of *ATP1A1* gene with heat tolerance traits was studied, we found that the individuals with genotype *CC* showed significantly higher heat resistance than those of genotype *CA* ($P < 0.05$). Further, the mRNA levels of *ATP1A1* gene in lymphocytes of peripheral blood in dairy cows among various temperature groups and genotypes were analyzed by using real-time RT-PCR. Results showed that the expression of *ATP1A1* mRNA was highest in heat-stressed cows with *CC* genotype among the three genotypes ($P < 0.01$), and the *ATP1A1* mRNA level at temperature 32.5°C was higher than that at optimal temperature 12.5°C in dairy cows ($P < 0.01$). Simultaneity, the plasma potassium (K^+, mmol/l) and sodium (Na^+, mmol/l) declined when the temperature dropped ($P < 0.05$). Our findings implied that the novel SNP here could be a potential genetic marker for anti-heat stress trait in dairy cow breeding.

Keywords Dairy cow · *ATP1A1* · SNP · Cow · Heat stress · Gene expression

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Introduction

As subtropical area, one of the problems encountering the dairy industry is the hot and humid climate during summer (May–September) in southern China. A temperature range of −0.5–20°C is known as the "thermoneutral range" for dairy cow [1], increasing milk yield increases sensitivity of cattle to thermal stress and reduces the "threshold temperature" at which milk losses occur [2]. At high temperatures the animal has to consume energy to maintain its body temperature [3]. According to documents, heat stress has detrimental effects on milk production in dairy cows [4, 5]. It was suggested that selection for milk production and heat tolerance simultaneously is possible [6], and heat tolerance can be improved in a few generations for the high-yielding breed [7]. It was also reported that temperature-humidity index (THI) can be used to account for the effect of heat stress on milk production [8]. Therefore, heat tolerance may be act as a quantitative trait in dairy cows. Bernabucci et al. [9] demonstrated that high temperature can produce moderate heat stress and cause oxidative stress in transition dairy cows. Calamari et al. [10] revealed weak negative effects of heat stress on some plasma markers of oxidative status in mid-lactating cows. As we know, addition of solutes ($NaHCO_3$ and KCl) benefited heat-stressed cows in terms of milk yield, regulation of acid-base balance, and lowering body temperatures [11, 12], which may be related to the deficiency of the major monovalent ions (sodium and potassium) under heat load [13, 14]. Other studies showed that heat stress influenced the level of plasma Na^+ and K^+ in Holstein cows [15, 16]. Above studies seem to imply that variational ion concentration was correlative with oxidative stress occurring after heat stress. Since Na^+, K^+-ATPase is especially sensitive to oxidative stress [17] and its function is maintaining the

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electrochemical gradient of Na^+ and K^+ ions across the cytomembrane, which provides energy for the membrane transport of metabolites, nutrients, and ions [18]. Therefore, the bovine Na^+, K^+-ATPase gene represents a plausible candidate for heat tolerance traits.

The bovine Na^+, K^+-ATPase gene is composed of principal catalytic α subunit, sugar-rich auxiliary β subunit, and an associated γ subunit. The catalystic α subunit, an 110 kDa trans-membrane protein, carries the binding sites for sodium, potassium, ATP and the specific inhibitor ouabain [19]. The α1 isoform of the enzyme predominant in red blood cells and nerve tissue is encoded by the *ATP1A1* gene, which spans about 20.9 kb and includes 21 exons. Up to now, only one single nucleotide polymorphism (SNP) was found in bovine *ATP1A1* gene [20]. It is well known that SNPs contribute to conducting association analysis and evaluating them as genetic markers of selection for economic character [21, 22]. There are also evidences of a possible effect of heat shock on DNA synthesis and gene expression [23]. However, few studies on expression of the *ATP1A1* gene in the dairy cows undergoing heat stress were reported.

In this study, we analyzed polymorphisms within the coding region of bovine *ATP1A1* gene by using PCR low ionic strength single-stranded conformation polymorphism (PCR-LIS-SSCP) and determined its possible association with heat tolerance traits in dairy cows. Further, the mRNA level of *ATP1A1* gene in the lymphocytes of the peripheral blood in dairy cows among various temperature groups and genotypes, as well as the plasma potassium (K^+, mmol/l) and sodium (Na^+, mmol/l) were also analyzed.

Materials and methods

Animals, DNA isolation, plasma potassium, and sodium

A total of 160 Holstein healthy cows were randomly selected from Weigang farm in Nanjing, Jiangsu Province, China, homogenous for stage of lactation and parity, and under the same management and nutritional regimen. The blood samples were collected with anticoagulant acid citrate dextrose (ACD) at different daily-mean-temperature (32.5°C, high temperature in August; 12.5°C, optimum temperature in November).

Genomic DNA was extracted from the blood samples and detected by 0.8% agarose gel electrophoresis. The content of DNA was estimated by ultraviolet spectrophotometer, and the genome DNA was diluted to a final concentration of 50 ng /μl, stored at 4°C. Plasma was removed from 3 ml fresh blood (40 samples in each group with different temperature) through centrifuged at 900×*g* for 15 min, and stored at −20°C within 2 h of collection, the concentrations of plasma potassium (K^+, mmol/l) and sodium (Na^+, mmol/l) were measured by Olympus AU2700 biochemical auto-analyzer.

Heat tolerance traits measurement

Heat Tolerance Coefficient (HTC) and Respiratory Rate (RR) were measured in 160 Holstein cows. HTC was determined according to the Iberia heat tolerance test [24] with the following equation: HTC = 100-10 (ART –38.3), where ART = average rectal temperature before and after 3 h exposure to solar radiation for three consecutive days and 38.3°C is the average normal rectal temperature of cow. HTC was calculated for each cow to assess its heat adaptability. Average RR was determined by monitoring three times per cow between 13:00 and 16:00 during the same days. Records of daily milk yields at 32.5°C in summer and 305 daily milk yields from DHI documents were also collected for statistical analysis.

Polymorphism detection

The primers of four fragments covering exons 16, 17, 18, and 19 were designed by Oligo 6.12 based on the genomic sequence of bovine *ATP1A1* gene (GenBank accession No. NC_007301.3), including P16 (forward 5′-AGT GCT GCG TGA AAC CTG-3′, reverse 5′-GTG ATG TGT GGA ATG TGT GC-3′), P17 (forward 5′-ACA AAC AAA AGG GTC ACA ACA T-3′, reverse 5′-CTT ACC CTA GAT CCT GGC TCA T-3′), P18 (forward 5′-CCC GCA GAT GAT TTG TAC CAG-3′, reverse 5′-GCA CAA GGC AGA CGG ATG AG-3′), and P19 (forward 5′-TCA TCC GTC TGC CTT GTG-3′, reverse 5′-AGG TGC TGG GCT TTT TAT TTAG-3′). The PCR reactions were carried out in a total of 20 μl volume of containing 50 ng genomic DNA, 1× buffer (Tris–HCl 100 mmol/l, pH 8.3;KCl 500 mmol/l), 0.25 μmol/l primers, 2.0 mmol/l $MgCl_2$, 0.25 mmol/l dNTPs, and 0.5 U Taq DNA polymerase (TaKaRa). Amplification was performed in a PTC-200 PCR machine (MJ Research). The thermal cycling consisted of an initial denaturation step at 94°C for 5 min, followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 56–60°C for 30 s and extension at 72°C for 1 min. The reaction was terminated by a final extension at 72°C for 8 min. PCR products were detected by electrophoresis on 1.5% agarose gel stained with ethidium bromide. Subsequently, two microliters of the PCR products was added to 10 μl of LIS loading solution (10% sucrose, 0.01% bromophenol blue, and 0.01% xylenecyanol FF). The mixture was incubated at 98°C for 5 min and then cooled on ice, loaded onto a 12% polyacrylamide gel (acrylamide/bisacrylamide 29:1, v/v). Electrophoresis was carried out using 1× TBE (45 mM

Tris–borate/1 mM EDTA) at 160 V for 12–15 h. LIS-SSCP gel were detected by silver staining. The bands representing different LIS-SSCP patterns were purified with Agarose Gel DNA Purification Kit (TaKaRa), then cloned into the PMD19-T Vector (TaKaRa) and sequenced by Shanghai Invitrogen Biotechnology.

Real-time RT-PCR

Total RNA was extracted from 5–10 × 10^6 dissociated lymphocytes with RNAiso reagent (TaKaRa). Five samples were included in each genotype group and 10 samples in each group of different temperature. The quality of isolated RNA was verified by electrophoresis in 1% agarose gel (Sigma). The reverse transcript reaction was conducted in a 20 μl reaction system according to the manufacturer's protocol (TaKaRa). Real-time PCR was performed with the ABI 7300 machine and Software SDS v.1.4. The reaction mixture contained 10 μl 2 × SYBR premix Ex Taq (TaKaRa), 50 × Rox Dye 0.4μL, 1μL cDNA (three repeats for each sample), 10 pmol of each primer, and ddH_2O up to 20 μl. The following primers were used to amplify the fragment encompassing exons 20 and 21 of *ATP1A1* (GenBank accession No. NM_001076798): 5′- CAG CAG GGG ATG AAG AAC AAG -3′ (forward) and 5′- GGA AGG CAC AGA ACC ACC A -3′ (reverse). The internal reference standard was 18S rRNA (GenBank accession No. DQ222453.1), which forward and revere primer sequences were 5′- TTG ACG GAA GGG CAC CA -3′ and 5′- CCC ACG GAA TCG AGA AAG AG -3′. The thermal profile was as follows: initial denaturation (95°C for 10 s), 40 cycles with denaturation (95°C for 5 s), annealing (60°C for 31 s). Subsequently, a melting step was performed consisting of denaturation (95°C for 15 s), annealing (60°C for 1 min), slow heating with a rate of 0.1°C for 1 s up to 95°C, with continuous fluorescence measurement, and finally cooling to 35°C. The relative mRNA level of *ATP1A1* was determined by the $2^{-\Delta\Delta CT}$ method [25]. Then, the PCR products were cloned and sequenced in both directions. Specificity of real-time PCR products was verified by aligning the sequenced PCR products and the corresponding published coding of bovine *ATP1A1* mRNA sequences.

Statistical analysis

Software POPGENE 1.31 was used to calculate the frequencies of both alleles and genotypes and test their accordance with the Hardy-Weinberg equilibrium. Student's *t*-test or one-way ANOVA as appropriate was used to test the differences at different temperature groups or in different genotypes by SPSS 13.0 software. Population genetic indexes, such as He (gene heterozygosity), Ho (gene homozygosity), Ne (effective allele numbers), and PIC (polymorphism information content) were calculated according to Nei's methods [26], respectively. The formulas were as follows:

$$H_O = \sum_{i=1}^{n} p_i^2 \quad He = 1 - \sum_{i=1}^{n} p_i^2 \quad Ne = \frac{1}{\sum_{i=1}^{n} p_i^2} PIC$$
$$= 1 - \sum_{i=1}^{n} p_i^2 - \sum_{i=1}^{m-1} \sum_{j=i+1}^{m} 2p_i^2 p_j^2$$

The association between the polymorphism of the *ATP1A1* gene and heat tolerance traits was analyzed using the general linear models procedure of SPSS 13.0. The following model was used: $Y_{ijk} = \mu + A_i + G_j + e_{ijk}$,where: Y_{ijk} was the observation of the trait; μ is overall mean; A_i was the fixed effect of *i*th age; G_j was the fixed effect of *i*th genotype; e_{ijk} was random error. Significance level of differences between the means for each group was considered at $P < 0.05$ and $P < 0.01$.

Results

Identification of polymorphisms

The four fragments with the expected length of 295, 301, 318, and 246 bp were successfully amplified (Fig. 1a). By separated in a polyacrylamide gel electrophoresis, the PCR products of exon17 were shown polymorphic and three kinds of band patterns were observed, named *AA*, *CC*, and *CA* (Fig. 1b). By comparing the sequences of two types of homozygotes, we found a substitution of C/A at the nucleotide position 2,789 of the *ATP1A1* mRNA (GenBank: NM_001076798), which did not induce any change in amino acids of bovine *ATP1A1* gene (Fig. 1c). The novel SNP was deposited in dbSNP of GenBank (ss159831435).

The allele frequencies were 0.86 for *C* and 0.14 for *A*. The value of He, Ho, Ne, and PIC in the population was 0.24, 0.76, 1.32, and 0.21, respectively. The frequencies of genotypes in the population was in accordance with the Hardy-Weinberg equilibrium ($P > 0.05$).

Association between bovine heat tolerance traits and the polymorphism of ATP1A1 gene

With association analysis, significant associations of *ATP1A1* polymorphism with HTC, RR, daily milk yield in summer and 305 daily milk yields were revealed ($P < 0.05$). Because of a small number of animals with genotype *AA*, we excluded this group from statistical analysis. Cows with genotype *CC* showed a higher HTC (+7.22), daily milk yield in summer (+9.83), and 305 daily milk yields

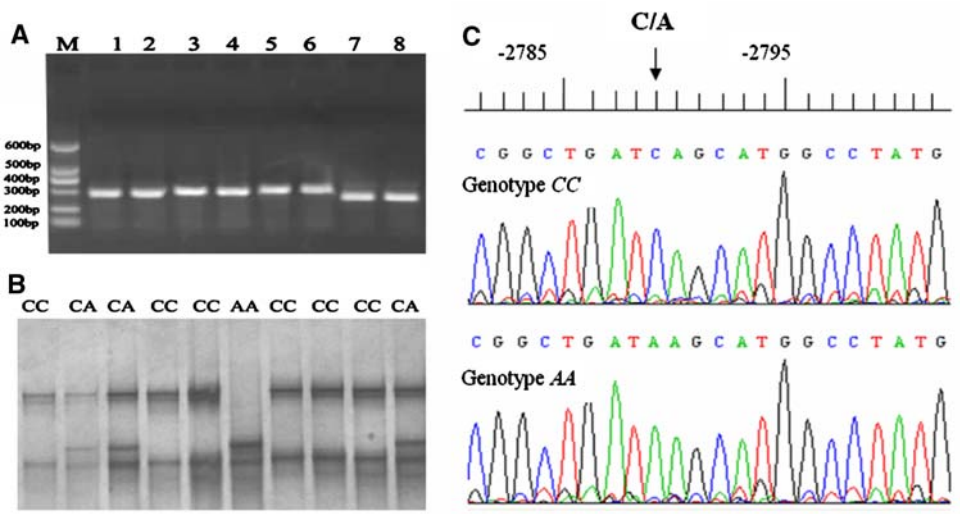

Fig. 1 The electrophoresis result of PCR products, LIS-SSCP patterns and sequences of two homozygotes. **a**The PCR results of four pairs of primers: Marker; P16:1, 2; P17:3, 4; P18:5, 6 and P19:7, 8. **b** LIS-SSCP results of the PCR products P17, corresponding to *CC*, *CA*, and *AA* genotypes. **c** Sequences of two types of homozygotes. Sequence I occurs in the *CC* genotype, II in *AA*

(+417.35) but lower RR (-8.85) than that with genotype *CA* ($P < 0.05$, Table 1) (on-line supplementary data). Such results indicated that the cows with *CC* genotype showed higher heat tolerance than those with genotype *CA*.

The mRNA level of ATP1A1 in lymphocytes of peripheral blood and plasma concentrations of Na^+, K^+

The mRNA levels of *ATP1A1* in lymphocytes of peripheral blood among various temperature groups and genotypes were investigated by using real-time RT-PCR. We found that the mRNA level of *ATP1A1* was higher in genotype *CC* (0.87 ± 0.13) than that in genotypes *CA* (0.39 ± 0.03) or *AA* (0.20 ± 0.02) ($P < 0.01$, Fig. 2a). On the other hand, the mRNA level of *ATP1A1* at 32.5°C (1.38 ± 0.21) was shown higher than that at 12.5°C (0.55 ± 0.12) ($P < 0.01$, Fig. 2b).

At the same time, we found that the concentrations of the plasma potassium (K^+, mmol/l) and sodium (Na^+, mmol/l) increased with heat stress in dairy cows ($P < 0.05$, Fig. 2c, d). Results indicated that the plasma potassium (K^+, mmol/l) and sodium (Na^+, mmol/l) were changed by temperature.

Discussion

Previous literatures showed that some potential candidate genes for heat tolerance traits in dairy cows [27, 28]. Among various candidates, Na^+, K^+-ATPase seems to be interesting and it could be necessary to fulfill different physiological roles. In human, Na^+, K^+-ATPase was relevant to diabetes[19, 29] and plays an important role in the pathogenesis of many cardiovascular diseases [30]. It was reported that the association of human *ATP1A1* ($P < 0.000005$) with hypertension by SNP haplotype analysis [31]. Polymorphism of intron 1 in the human *ATP1A1* gene could influence the phenotype (red blood cell Na^+, K^+-ATPase activity) in case of C-peptide deficiency [32]. Some extent influence on physiological functions could be induced by SNPs occurring within the Na^+, K^+-ATPase gene or at least it can be adopted as DNA marker of dairy cattle genome. Take that into account, in marker-assisted selection of dairy cow, we selected four fragments of the *ATP1A1* gene for screening of SNPs, a novel SNP was identified (GenBank accession No. ss159831435) in the exon17. According to the classification of PIC (PIC value < 0.25, low polymorphism; $0.25 <$ PIC value < 0.5, intermediate polymorphism; and PIC value > 0.5, high polymorphism), the Holstein breed are low genetic polymorphism, which reflected that there was low genetic diversity within bovine *ATP1A1* gene in the analyzed population.

In present study, considering that all experimental dairy cows were descendants of 18 unrelated proven bulls. Anyway, we did not observe any preferential sire in the genotype groups, so the sire effect was not included in the statistical model for association analysis. HTC, RR, as physical factors were considered to be indicators for evaluating the heat tolerance of animals [33, 34], and improving the daily milk in summer and 305 daily milk yields were the purpose of marker-assisted selection of anti-heat stress dairy cow. Statistical results indicated that the heat-stressed cows with genotype *CC* showed highest mRNA level of *ATP1A1* gene. Meanwhile, the association analysis results also proved that the genotype *CC* was related to heat tolerance and allele *C* might be the beneficial allele, while the genotype *CA* was associated with heat stress susceptibility. Compared to a random population, the observed allele frequencies (0.86 for *C* and 0.14 for *A*) may be underestimated for allele *C* and overestimated for allele *A*. It could be concluded that bovine *ATP1A1* gene may

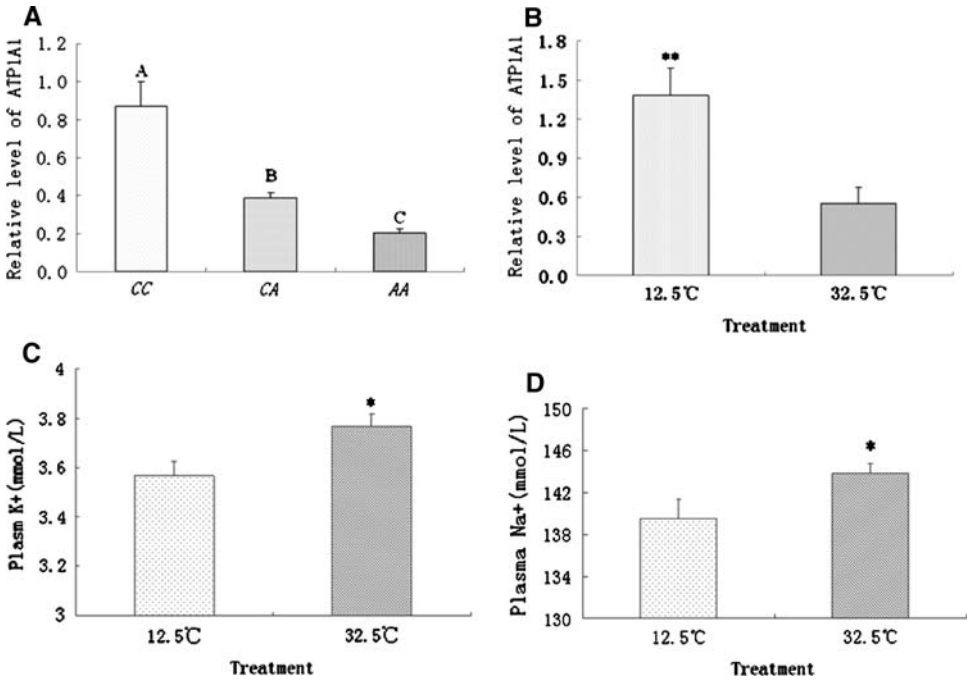

Fig. 2 ATP1A1 mRNA expression in lymphocytes in peripheral blood and the concentrations of plasma K^+ and Na^+ in different groups. **a** Relative level of *ATP1A1* expression in different genotypic heat-stressed cows. **b** Relative level of *ATP1A1* expression at different daily-mean temperatures. **c** Plasma potassium (K^+, mmol/l) at different daily-mean-temperatures. **d** Plasma sodium (Na^+, mmol/l) at different daily-mean-temperatures. ** $P < 0.01$, * $P < 0.05$, and *bars* represent standard error

play an important role in heat shock response. Our results implied that increases the frequency of allele *C* of the *ATP1A1* gene might attribute to breed high-yielding cows with heat tolerance by artificial selecting a few generations, which is agree with the previous study that possibility could be existed in selection for milk production and heat tolerance simultaneously [6].

The Na^+, K^+-ATPase gene expression has been studied in human and rat. Previous studies showed that significantly linear positive correlation between Na^+, K^+-ATPase activity and mRNA level of α1 isoforms [29, 35]. In the present study, we found that the mRNA level of *ATP1A1* at 32.5°C was higher than that at 12.5°C, implying dairy cows may need higher Na^+, K^+-ATPase activity subjected to prolonged and continuous periods of high heat and humidity. In addition, there was numerous studies in the past few decades have clearly shown that synonymous mutations although do not produce altered coding sequences, but might affect directly or indirectly the production traits of cattle [36–39] through alteration of mRNA stability, modulation of the efficiency of translation of mRNA, and consequently influence of encoded protein structure and property [40], which is similar to our finding that the novel synonymous SNP in the exon17 of *ATP1A1* changed its mRNA expression. The heat-stressed cows with *CC* genotypes showed a higher efficient transcription and a stronger ability to adapt heat stress environment. We also noticed that heat stress decreased the plasma potassium (K^+, mmol/l) and sodium (Na^+, mmol/l) in the dairy cows undergoing prolonged high temperature ($P < 0.05$). It is possible that a declination in Na^+ and K^+ can attribute to increased urinary Na^+ and K^+ excretion during respiratory alkalosis in heat stress environment [41]. Our finding is in agreement with El-Nouty et al. [15], but different from the other findings [16], which might be caused by the different stages of heat-stress in experimental dairy cows.

In conclusion, our results showed that the *ATP1A1* gene possibly affected heat tolerance traits in dairy cows, the novel SNP here could be a potential useful genetic marker to assisted selection for anti-heat stress traits in dairy cattle breeding programs. Therefore, further work will be necessary to use the SNP for marker-assisted selection in larger population.

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