Association analysis of HSP70A1A haplotypes with heat tolerance in Chinese Holstein cattle

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Abstract Single-nucleotide polymorphisms (SNPs) in the coding and untranslated regions of *heat shock 70 kDa protein 1A* (*HSP70A1A*), an inducible molecular chaperone that is responsible for cellular protection against heat stress, have been reported as being associated with heat tolerance. A fragment of the *HSP70A1A* gene was amplified in Chinese Holstein cattle and eight novel mutations were found. We performed comprehensive linkage disequilibrium (LD) and haplotype analyses of the eight SNPs of the *HSP70A1A* gene and examined their involvement in heat resistance in 600 Chinese Holstein cattle. Our results revealed the presence of significant differences between individuals carrying haplotype 1 and those without haplotype 1 for most of the heat-tolerance traits. Haplotype 1 increased the risk of heat stress; however, association analysis of its combination with haplotype 2 showed the lowest rectal temperature and red blood cell K⁺ level, moderate respiratory rate, and the

highest red blood cell NKA level, suggesting a heterozygote advantage in the penetration of the phenotype. Protein expression levels in white blood cells among haplotype combinations further confirmed the hypothesis that heterozygotes for haplotypes 1 and 2 are more sensitive to heat stress. We presume that these mutations may be useful in the future as molecular genetic markers to assist selection for heat tolerance in cattle.

Keywords HSP70 · Heat stress · Haplotypes · Cattle · Association analysis

Introduction

Chinese Holstein is a heat-sensitive breed of cattle that originated in Europe. High environmental temperature exerts a negative influence on the performance of Chinese Holstein cattle, especially in South China. In summer, the high temperature and humidity can produce moderate heat stress, resulting in decreased dry-matter intake, immunity, milk secretion, and reproduction performance (Wolfenson et al. 2000; Berman 2011). Previous studies have focused on the alleviation of heat stress through physical monitoring of the environment (Collier et al. 2006). However, little is known about the molecular mechanisms underlying heat resistance in dairy cows.

Heat shock proteins (HSPs) have been implicated in thermotolerance and the ability of heat-induced denatured proteins to recover to their native state (Maloyan et al. 1999). The HSP70 protein, which is encoded by the *HSP70A1A* gene, is a widespread protein of the HSP family and an important intracellular molecular chaperone. The regulation of HSP70 production is critical to inhibit the apoptosis of cells (Mosser et al. 2000). Although stress-

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induced synthesis of HSP70 proteins represents a generalized molecular mechanism displayed by almost all cells, individual animals differ in their capacity to manage stress. In Chinese Holstein cows, a genetic polymorphism in the coding region of the *HSP70A1A* gene was associated with resistance to mastitis (Cheng et al. 2009). In human peripheral mononuclear cells, polymorphisms in the coding region of the *HSP70A1A* gene were associated with an increased ability to respond to heat stress (Singh et al. 2006). Moreover, it has been reported that nucleotide changes occurring naturally in the flanking regions of the *HSP70A1A* gene might affect the inducibility, degree of expression, and/or stability of the HSP70 mRNA and contribute to susceptibility to certain diseases and stress tolerance. In humans, an *HSP70A1A* gene polymorphism located in the 5' untranslated region (UTR) was positively associated with coronary heart disease risk, which was mediated by the level of synthesis of the HSP70 protein (He et al. 2009). In pigs, an *HSP70.2* gene polymorphism located in the functional promoter and 3' UTR was associated with mRNA stability and stress response (Schwerin et al. 2001; Schwerin et al. 2002).

Although there are many previous studies on the function of the HSP70 protein, few reports have explored the association between *HSP70A1A* gene haplotypes and thermotolerance in Chinese Holstein cattle. Therefore, studies to verify the effects of *HSP70A1A* haplotypes on thermotolerance traits in Chinese dairy cows may uncover genetic tools that will improve tolerance to heat stress in cattle.

Materials and methods

Animals and DNA isolation

The 600 Chinese Holstein Cows used in this study came from four dairy farms located in Huanggang City, Hubei Province, China, which is situated at a latitude of 30°27'N and a longitude of 114°52'E in the middle reaches of the north shore of the Yangtze River in South China. Blood samples were collected using anticoagulant acid citrate dextrose and stored at -80 °C. DNA was extracted from 1 mL of frozen/thawed blood and diluted with distilled water to a final concentration of 50 ng/μL.

Measurement of heat-resistance traits

Sodium chloride was added in a 1:2 (w/v) ratio and the tube was shaken lightly and then centrifuged at 4 °C, 7,000 rpm for 8 min. The latter step was repeated twice; an erythrocyte-gained moiety was used to determine K⁺ and another to determine NKA levels (Wang et al. 2011). The rectal temperature and respiratory rate of cows were measured between 1200 and 1400 hours during the middle 10 days of

August 2012 (the average temperature was 33.5 °C and the temperature–humidity index was 85.7, during which time the cows were regarded as being under heat stress). Milk samples were taken and milk yield recorded once a month for each cow in the course of routine controlled milking over the whole lactation. Milk yield decline=(corrected milk in March–corrected milk in August)/corrected milk in March.

Identification and genotyping of *HSP70A1A* variants

In an effort to discover SNPs in a cost-effective manner, SNP discovery was implemented by sequencing pooled PCR products, which were amplified from genomic DNAs from 60 Chinese Holstein cows. PCR primers (PF: 5'-CATCCTTATTACCAACTTGCGTG-3'; PR: 5'-CGGACAAGAAGAAGGTGCTG-3') were designed for the partial amplification of the exons and 3' flanking region of the *HSP70A1A* gene based on the genome sequence fragments from the assembly of chromosome 23 reported in GenBank (accession no. AC_000180.1). PCR was carried out in a 50 μL mixture containing 200 ng template DNA, 200 nm each primer, 2.5 mm MgCl₂, 200 nm each dNTP, and 0.25 U Taq DNA polymerase with 1 μL PCR buffer supplied by the manufacturer (Takara, Tokyo, Japan). The PCR conditions were: initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 40 s, annealing at 59 °C for 40 s, elongation at 72 °C for 40 s, and final elongation at 72 °C for 10 min. The PCR products were purified with the MinElute PCR purification kit (QIAGEN, Madison, WI), and SNPs in the *HSP70A1A* bovine genomic

Fig. 1 SNPs detected in the *HSP70* gene, as shown by DNA antisense strand (a) and sequencing chromatograms (b) in Chinese Holstein cows. The STOP codon is boxed. Detection primers are underlined. The SNPs are indicated in red color

DNA were identified by direct sequencing on an ABI PRISM 377 sequencer using the BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (PE Applied Biosystems, Foster City, CA). Eight SNPs were found distributed over the entire gene (five in the 3' flanking region at positions 2066, 2040, 2027, 1990, and 1980; three in the CDS at 1923, 1761, and 1737; Fig. 1). All eight SNPs were genotyped in all study individuals. All SNPs could be tested using direct sequencing of PCR products by the same primers.

Western blotting

White blood cells collected from fresh blood of the indicated population during high temperature were solubilized in 100 μ L of sodium dodecyl sulfate (SDS) sample buffer, boiled for 5 min, and ultrasonically homogenized for 3 min. Equal amounts of total protein were separated by electrophoresis on 10 or 12 % SDS–polyacrylamide gels and then transferred onto polyvinylidene difluoride membranes. The membranes were blocked in TBS-T with 3 % BSA for 2–3 h. Next, the membrane was incubated with rabbit anti-HSP70 polyclonal antibody (CST, Beverly, MA) at 4 °C overnight. This HSP70 antibody detects endogenous levels of total HSP70 protein and does not cross-react with other HSPs. After washing the membrane, the proteins were detected using peroxidase-conjugated immunopure goat anti-rabbit IgG (H+L) (Millipore, Bedford, MA).

Statistical analyses

Genotype and allele frequencies were estimated by gene counting. A Hardy–Weinberg equilibrium (HWE) test was performed for each SNP to determine deviations of observed genotypic distributions from the expected distribution. We used the Haploview software [version 2.1.1] to determine the LD structure of the SNPs located within the *HSP70A1A* gene and to test for HWE.

Haplotype construction and LD analysis

Haplotype analyses and graphical representation of the linkage disequilibrium (LD) structure as measured by coefficient D' and r^2 using expectation–maximization algorithm were performed with the HAPLOVIEW software (ver 3.32) (Barrett et al. 2005). Haplotypes were obtained for each animal using the PHASE computer program (ver 2.1), and the haplotype frequencies were estimated via permutation methods (Stephens et al. 2001). In large samples, $D'=1$ indicates complete LD, i.e., no evidence for recombination between the SNP pairs—such SNPs are good surrogates for each other; $D'=0$ indicates no LD. “Strong” LD was defined as having a pairwise $D'>0.85$. Haplotype-block structure was examined using the criteria described by Gabriel et al.

(Gabriel et al. 2002), which use the 90 % confidence bounds of D' to define sites of historical recombination between SNPs. Patterns of LD were visualized using Haploview.

Association analysis

The association analyses between *HSP70* haplotype combinations and heat-resistance traits was performed using GLM process of SAS 8.0 and the following liner model: $Y_{ijklm} = \mu + G_i + Y_{sj} + H_k + P_l + S_m + e_{ijklm}$, where Y_{ijklm} =the heat-resistance capability value; μ =the overall mean; G_i =the effect of genotype; Y_{sj} =the effect of seasons; $j=1$ for April to September and 2 for October to next March; H_k =the effect of different farms; $k=1, 2, 3$, or 4; P_l =the effect of paternal descent; S_m =the effect of maternal descent; and e_{ijklm} =random error.

Differences in baseline characteristics between the different genotype groups of the *Hsp70* gene were compared as haplotype-dose effect. Haplotype-dose effect was defined as an increase or decrease in value per copy of the haplotype. The association analyses between different copy numbers of certain haplotypes and traits were implemented using GLM process of SAS 8.0 as mentioned above.

Results

Identification of eight polymorphisms in the bovine *HSP70A1A* gene and analysis of their characteristics

We identified eight polymorphisms in the bovine *HSP70A1A* gene using genomic DNA from 60 Holstein cows (Fig. 1). Five polymorphisms were located in the 3' flanking region (HSP70a_1–HSP70a_5). Three polymorphisms were found in the *HSP70A1A* coding region (HSP70b_1–HSP70b_3). HSP70a_1 and HSP70a_5 were identified in the 3' flanking region and resulted in a T to C change at positions 2066 and 1980 of the *HSP70A1A* gene, respectively. HSP70a_2 and HSP70a_3 were also located in the 3' flanking region and resulted in an A to G change at positions 2040 and 2027 of the *HSP70A1A* gene, respectively. HSP70a_4 was identified in the 3' flanking region and resulted in a G to T change at position 1990 of the *HSP70A1A* gene. Regarding the other three polymorphisms, which all led to silent mutations, HSP70b_1 and HSP70b_3 were detected in the CDS and resulted in an A to G change at positions 1923 and 1737, whereas HSP70b_2 resulted in a C to A change at position 1761 of the *HSP70A1A* gene.

Analyses of haplotypes and LD among the eight SNPs identified

The genotyping of all eight SNPs was successfully implemented using direct sequencing in 600 Holstein cows

in primary studies. The minor allele frequency (MAF) at each marker ranged between 27 and 36 %. Table 1 shows allele frequencies and Hardy–Weinberg test P values. All eight SNPs, with the exception of HSP70a_4, were in Hardy–Weinberg equilibrium ($P=0.0004$) for the combined data. All eight SNPs genotyped exhibited heterozygosity. The corresponding estimated D' values are depicted using a color scheme in Fig. 2. All of the SNPs were identified as an LD block. “Strong” LD was observed in 39 % (11 of 28) SNP pairs (dark shading). Among these, we identified two distinct sets of “family of SNPs” that mutually exhibited strong LD with one another within the family. These were: I = {HSP70a_1, HSP70a_2, HSP70b_1, HSP70b_2, HSP70b_3} and II = {HSP70a_3, HSP70a_5}. However, we observed that 17 SNP pairs showed evidence of recombination (gray shading). Interestingly, five out of the 17 SNP pairs that showed evidence of recombination (HSP70a_2 and HSP70a_3, HSP70a_3 and HSP70a_4, HSP70a_4 and HSP70a_5, HSP70a_2, and HSP70b_1) in the 3' flanking region were neighboring SNPs. The lower-than-expected degree of LD in the cases, even between closely spaced SNPs, might be caused by gene conversion.

Haplotype association analysis

Three major haplotypes accounted for 91.8 % of the alleles: haplotype 1, –TAATTACG– (35.8 %); haplotype 2, –CGAGTGAA– (29.7 %), and haplotype 3, –TAGGCACG– (26.3 %). So, the six haplotype combinations 11 (TAATTACG/TAATTACG), 12 (TAATTACG/CGAGTGAA), 13 (TAATTACG/TAGGCACG), 22 (CGAGTGAA/CGAGTGAA), 23 (CGAGTGAA/TAGGCACG), and 33 (TAGGCACG/TAGGCACG) accounted for the major genotypes of the *HSPA1A* gene, and we named the corresponding genotypes as genotype 11, 12, 13, 22, 23, or 33. The results of association analyses between haplotype combinations and the heat-tolerance traits are shown in Table 2. There were significant differences ($P<0.05$) between haplotype combinations regarding some of the heat-tolerance traits analyzed. There was a significant difference between individuals carrying

Fig. 2 Linkage disequilibrium (LD) patterns of the *HSP70* gene in a Chinese Holstein population. LD was estimated based on the pairwise LD coefficient, D' . The color scheme is dark gray for “strong” LD and light gray for “evidence of historical recombination”. Patterns of LD were visualized using Haploview

haplotype 1 and those without haplotype 1 for all traits, with the exception of decreased rate of milk yield. The presence of haplotype 1 favored red blood cell K^+ levels with a very high significance ($P<0.0001$). Haplotype 3 showed no significant associations with any of the heat-tolerance traits. Haplotype 2 was only associated with rectal temperature and red blood cell K^+ levels. In contrast to what was observed for haplotype 1, cows with haplotype 2 had a tendency for decreased rectal temperature and red blood cell K^+ levels during the hot days of summer; i.e., to some extent, cows with haplotype 2 had more thermal resistance than did cows with haplotype 1. However, cows with genotype 12 had much lower respiratory rate, rectal temperature, and red blood cell K^+ levels, but higher red blood cell NKA levels, than did cows with genotype 22, suggesting a heterozygote advantage in the penetration of the phenotype.

Table 1 Characteristics of the SNPs located in the 3' flanking and coding regions of the *HSP70* gene and their MAF

No.	Name	Region	Position	Alleles	MAF	HW P
1	HSP70a_1	3' Flanking region	2066	T>C	0.345	0.3698
2	HSP70a_2	3' Flanking region	2040	A>G	0.302	0.3892
3	HSP70a_3	3' Flanking region	2027	A>G	0.289	0.9853
4	HSP70a_4	3' Flanking region	1990	G>T	0.358	0.0004
5	HSP70a_5	3' Flanking region	1980	T>C	0.267	0.7565
6	HSP70b_1	Coding region	1923	A>G	0.345	0.3698
7	HSP70b_2	Coding region	1761	C>A	0.353	0.4462
8	HSP70b_3	Coding region	1737	G>A	0.345	0.3698

MAF minor allele frequency, A>B implies that B is the minor allele; HW P Hardy–Weinberg test P value

Table 2 Associations between *HSP70* haplotype combinations and copy numbers of specific haplotype and heat-tolerance traits, respectively

Trait	LSM (SE) of haplotype combination							LSM (SE) of copies of haplotype 1-TAATTACG-				
	11, n=120	12, n=100	13, n=72	22, n=44	23, n=144	33, n=34	P value	0, n=222	1, n=172	2, n=120	P value	P value
RT	40.25±0.51a	39.89±0.74b	40.16±0.65ab	39.98±0.98ab	39.95±0.56b	40.07±0.41ab	0.035	40.00±0.36b	40.03±0.58b	40.25±0.51a	0.004	
RR	91.18±6.14a	88.48±7.68bc	86.40±5.55c	89.73±4.91ab	90.14±9.08ab	89.15±4.87abc	0.043	89.67±4.81a	87.44±5.48b	91.18±6.14a	0.0002	
K ⁺	628.24±72.65a	491.45±58.15d	583.15±49.13b	534.59±78.93c	545.37±36.74c	558.67±58.92bc	<0.0001	546.21±35.83b	537.3±45.72b	628.24±72.65a	<0.0001	
NKA	9.84±4.31b	13.01±5.43a	11.16±4.93ab	12.25±3.85a	10.25±4.13b	12.08±	0.003	11.53±3.58a	12.09±4.75a	9.84±	0.005	
MYD	28.45±9.84	27.39±10.65	26.17±9.99	25.69±12.26	31.94±11.67	29.48±3.72ab	0.954	29.04±9.73	26.78±9.62	28.45±9.84	0.262	

Trait	LSM (SE) of copies of haplotype 2-CGAGTGAA-				LSM (SE) of copies of haplotype 3-TAGGCACG-			
	0, n=226	1, n=244	2, n=44	P value	0, n=264	1, n=216	2, n=34	P value
RT	40.16±0.38a	39.92±0.55b	39.98±0.98ab	0.003	40.04±0.42	40.06±0.48	40.07±0.41	0.926
RR	88.91±4.62	89.31±7.43	89.73±4.91	0.799	89.80±4.76	88.27±5.36	89.15±4.87	0.066
K ⁺	590.02±47.63a	518.41±33.98b	534.59±78.93b	<0.0001	551.43±55.53	564.26±33.86	558.67±58.92	0.120
NKA	11.03±3.54	11.63±3.95	12.25±3.85	0.266	11.70±3.66	10.71±3.98	12.08±3.72	0.094
MYD	28.03±9.43	29.67±10.37	25.69±12.26	0.174	27.18±9.63	29.06±9.74	29.48±9.87	0.275

Letters a, b, and/or c differed significantly ($P<0.05$) from each other

Trait: 0, 1, 2 represents different copy numbers of certain haplotypes

RT rectal temperature, RR respiratory rate, K⁺ red blood cell K⁺ level, NKA red blood cell Na⁺/K⁺-ATPase level, MYD milk yield decline, LSM (SE) least squares means and their standard errors

Polymorphisms and HSP70 expression

To investigate the possible functional significance of the SNPs of the *HSPA1A* gene, HSP70 protein levels were compared between haplotype combinations in white blood cells during high temperature (Fig. 3a). Under heat-stress status, the levels of expression of the HSP70 protein in cows with genotype 12 were remarkably higher than those observed in cows carrying other genotypes (Fig. 3b). As for the other five distinct combined genotypes, the expression of genotypes 11, 13, and 33 was significantly lower than that of genotypes 22 and 23; however, no differences were found between genotypes 11 and 13 nor between genotypes 22 and 23, suggesting that the reduction in expression was due to the presence of haplotype 1. This result further confirmed the suggestion that heterozygotes for haplotypes 1 and 2 may be more sensitive to heat stress and that the occurrence of heat stress upregulated the synthesis of the HSP70 protein to protect the individual from heat stress.

Discussion

In cattle, several promoter and 3' UTR variants have been identified in *HSP70A1A* (Adamowicz et al. 2005; Basirico et al. 2011; Brown et al. 2010; Grosz et al. 1994; Li et al. 2011; Rosenkrans et al. 2010). However, to date, only a few

variants have been found in the coding and 3' flanking regions of *HSP70A1A*, which may also affect inducibility, degree of expression, and/or stability of the HSP70 mRNA and may contribute to different individual stress tolerances. In our study, the bovine *HSP70A1A* 3' flanking region is different from the 3' UTR, which is transcribed from upstream of the bovine *HSP70A1A* gene. In addition, five novel SNPs located in the 3' flanking region of the inducible bovine *HSP70A1A* gene were identified in a Chinese Holstein population. These data are not consistent with the information of the *HSPA1A* SNP GeneView report published in NCBI (geneID 281825). These comparison data indicated that the allele frequency of a specific SNP is not identical in different populations of the same breed. There is increasing evidence that the influence of noncoding regulatory variants on complex traits may be beyond the effects of coding-region variants (Glazier et al. 2002). To date, a good number of noncoding DNA variants have been identified in candidate genes that affect production traits in livestock. There is evidence that conserved elements located in 3' flanking regions control transcriptional gene regulation. Beck et al. (1991) have identified an enhancer element located in the 3' flanking region of the human erythropoietin gene that controls transcriptional response to hypoxia. Trujillo et al. (2006) have also found that the human growth hormone gene contains a silencer embedded within an Alu repeat located in its 3' flanking region. Moreover, three novel SNPs identified in the coding region of the bovine *HSP70A1A* gene were not reported by Li et al. (2011) in a Chinese Holstein population. In addition, the three SNPs were all silent mutations that did not change the amino acid sequence. However, the silent mutations might also affect the protein expression level through a synonymous codon usage bias or transcription differences. Duan et al. (2003) have reported that silent mutations in the human dopamine receptor D2 (*DRD2*) gene affect its mRNA stability and the synthesis of the receptor (Duan et al. 2003). Parmley et al. (2006) have found that silent mutations affect the stability of the secondary structure of mRNA in mammals (Parmley et al. 2006).

Because the eight SNPs located in the coding and 3' flanking region of the *HSP70A1A* gene at adjacent positions were detected in a 426-bp fragment, we performed comprehensive LD and haplotype analyses of these SNPs and examined possible interactions of the genotypes with the level of heat stress. In the present study, LD analysis showed a strong association between tightly linked variants. Thus, three major haplotypes accounted for 91.8 % of the alleles. Association results revealed the presence of significant genotypes with probable consequences on heat tolerance. We suggest that genotype 12 confers better heat tolerance and higher HSP70 protein levels in response to high temperature compared with any of the other genotypes. Interestingly, six

Fig. 3 Effects of heat stress on heat shock protein 70 (HSP70) expression levels in bovine white blood cells. Cells with genotypes 11, 12, 13, 22, 23, and 33 were analyzed for protein level of HSP70 and quantified by western blotting under heat-stress status (a). Relative levels of HSP70 protein were calculated by imaging densitometer with reference protein (β -actin) in each sample (b). Bars represent the mean $\pm$ SE ($n=3$). * $P<0.05$, the differences in the levels of expression were significant by t test

pairs of alleles of genotype 12 were heterozygous. These findings indicate that potential overdominance of the *HSP70A1A* gene may play a role in mediating resistivity to heat stress. Heterozygote advantage at a locus (often called overdominance) is one form of balancing selection. Balancing selection favors guarding resistance proteins (Van der Hoorn et al. 2002). Indeed, heterozygote advantage has been used to explain the polymorphisms in the major histocompatibility complex, as heterozygotes presented two sets of epitopes to T cells and were able to respond more efficiently to pathogens (Carrington et al. 1999; Jeffery et al. 2000; Thursz et al. 1997). Heat shock proteins are known to protect cells from several stressors. Basirico et al. (2011) have also found that cows heterozygous at g1128G/T in the *HSP70A1A* 5' UTR had higher cell viability and an increase in the levels of the *HSP70A1A* gene and HSP70 protein after HS compared with cows homozygous for guanine or homozygous for thymine. Furthermore, the population genetics of balancing selection shows that in addition to maintaining diversity at the selected sites themselves, it increases diversity at closely linked neutral sites. This concept provides an explanation for the high number of polymorphisms found here in the short sequence of the *HSP70A1A* gene analyzed. Moreover, we cannot formally exclude the possibility that the associations observed result from distinct sequence variants in LD with the heat-resistant traits because a high level of genetic variation may be closely related to the gene migration flow, which is attributed to long-term adaptation to the environment. Some loci known to be involved in defense processes have high sequence polymorphism. One such locus in *Arabidopsis thaliana* is estimated to have nucleotide diversity above 4 % for synonymous sites (and even for nonsynonymous ones) (Rose et al. 2004).

Here, we identified eight SNPs in the *HSP70A1A* 3' flanking and CDS regions of a Holstein population from South China. The high sequence polymorphism observed may be a reflection of the fact that a self-protection mechanism present in Holstein cattle from South China under heat stress may bring self-regulation into full play to adapt to the hot environment. It is probably the case that haplotypes 1 and 2, with different structures, act together to exert stronger phenotypic effects. However, further studies of the underlying molecular mechanisms are needed to determine whether heterozygote advantage does exist for the *HSP70A1A* polymorphisms and how these polymorphisms alter HSP70 gene or protein expression. The mutation sites ascertained in this study may be useful in the future as molecular genetic markers to assist selection for heat tolerance in cattle.

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