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LETTER

An Association Study of *CASQ1* Gene Polymorphisms and Heat Stroke

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Abstract Although molecular mechanisms of heat stroke under physiological and pathological conditions have not yet been elucidated, a novel disease-associated gene encoding a calcium-binding protein, *calsequestrin-1* (*CASQ1*), was suggested relevant based on results from a transgenic murine model. Here, we show the association between single nucleotide polymorphisms (SNPs) of *CASQ1* and physiological parameters for heat stroke from a study involving 150 patients. Pooled DNA from heat stroke patients were subjected to sequencing and 3 SNPs were identified. Genotypes were assigned for all patients according to g. 175A > G, one SNP which leads to a nonsynonymous substitution (N59D) in the first exon of human *CASQ1* gene. We analyzed the genotypic data with a linear model based on significance scores between SNP (175A > G) and heat stroke parameters. As a result, we found a significant association between SNP A175G and heat stroke ($P < 0.05$). Further bioinformatics analysis of the 1-Mb flanking sequence revealed the presence of two genes that encode DDB1 and CUL4 associated factor 8 (DCAF8), and peroxisomal biogenesis factor 19 (PEX19), respectively, which might be functionally related to *CASQ1*. Our results showed that the blood calcium of patients with allele D increased significantly, compared to patients with allele N ($P < 0.05$), which may result from the decreased calcium in muscle, suggesting that N59D in CASQ1 might account for the dysfunction of CASQ1 in calcium regulation during heat stroke.

Introduction

Heat stroke is a life-threatening illness commonly found in tropical areas and during hot seasons elsewhere. It is characterized by an elevated core body temperature above 40 °C, which induces multi-organ failures (such as circulatory shock, central nervous system dysfunction, acute renal and liver failures, *etc.*) and sometimes followed with heat cytotoxicity, coagulopathies and systemic inflammatory response syndrome [1]. Heat stroke is experienced primarily by

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immunocompromised individuals, such as the very young and the elderly [2]. The inability to properly predict, diagnose and treat long-term sequelae of heat stroke is a serious concern of modern medicine, which reflects our limited understanding of its pathophysiological mechanisms mediating tissue injuries. Recent epidemiological studies of short- and long-term heat stroke outcomes indicate that multiple organ dysfunctional syndrome continues to manifest in patients following clinical treatment and increases mortality during the ensuing months and years of recovery [3,4]. Therefore, application of novel technologies, including radiotelemetric, genomic and proteomic analyses, in heat stroke research is of essence in advancing our knowledge on its pathophysiology. The association between heat stroke and *calsequestrin 1* (*CASQ1*) gene was reported in a recent study, which represented a significant step forward [5]. CASQ1 can modulate skeletal muscle contraction by regulating Ca^{2+} release in sarcoplasmic reticulum [6,7]. Muscle is known to act as the most important organ that produces heat for the body and is considered as heat production pivot [5]. Previous studies have assumed that varied abilities to tolerate torrid and humid environments among people are attributed to the differential capacities of their muscles to modulate production and release of heat [8–10]. Therefore, CASQ1, which regulates the skeletal muscle movement, could underlie the different resistance of people to heat stroke.

There are two *calsequestrin* genes in humans, *CASQ1* and *CASQ2*, residing on the two different arms of chromosome 1. Both CASQ1 and CASQ2 are Ca^{2+}-binding proteins with high capacity. CASQ1 isoform present in the skeletal muscles binds around 80 Ca^{2+} ions, whereas CASQ2 isoform present in the cardiac muscle binds approximately 60 Ca^{2+} ions. However, these two isoforms function differentially, CASQ1 regulates the Ca^{2+} homeostasis while CASQ2 does not [11]. Several single nucleotide polymorphisms (SNPs) have been found in the CASQ1 coding sequences including a rare nonsynonymous SNP in exon 11 (A348V), albeit lack of disease association [12]. Recently, it has been indicated that heat stroke survivors have a significant elevation in the 30-year mortality rate, compared to individuals who have never experienced heat stroke [13,14]. In this study, we aim to discover sequence variations in *CASQ1* gene and carry out an association study on patients who suffered from heat stroke.

Results

Discovery of *CASQ1* sequence variation and genotyping

DNA was extracted from the peripheral blood of 150 patients who were diagnosed with heat stroke. Then, PCR amplification was performed with primers designed to cover different portions of *CASQ1* genomic sequence and the resulting PCR products were sequenced. Sequencing analysis indicated that three SNPs were identified, including one nonsynonymous SNP in exon 1(g. 175A > G), one intronic SNP in intron 2 (g. 2968C > T) and one synonymous SNP in exon 3 (g. 3015C > T) (**Table 1** and **Figure 1**). Database search indicated that the intronic SNP was reported in the NCBI dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP) as rs3747623, but without any functional annotation. On the other hand, the two exonic SNPs were not published before. In particular, the newly-identified SNP in exon 1, g. 175A > G, leads to the alteration of amino acid residue at codon 59 from asparagine to aspartic acid (N59D).

Asparagine 59 is located in the alpha helix 1 of *CASQ1* [15] and substitution with the acidic amino acid residue aspartic acid could have functional implications. To explore this possibility, we then performed genotyping for all the patients. To do this, we designed a PCR-RFLP assay using the restriction enzyme BtsCI to digest PCR products before gel electrophoresis (**Figure 2**). The three genotypes, NN, ND and DD, were classified according to band patterns of three fragment groups, 605 bp, 605 + 481 + 224 bp and 481 + 224 bp, respectively.

Association analysis between genotypes and heat stroke parameters

Physiological indices were commonly used clinically for heat stroke diagnosis and served as a sign for heat stroke [16]. We thus evaluated the biochemical data to see whether there is any correlation with CASQ1 genotypes. The patients were divided into three groups according to genotypes. Analysis of critical blood physiological indices indicated all indices except platelet counts tended to increase in genotypes containing D allele; however, there were no significant differences between patients with NN genotype and ND genotype (**Table 2**). Instead, platelet count was significantly lower in the DD group than that in the NN group, whereas an opposite trend is observed for blood calcium. In addition, both leukocyte and creatinine were significantly high in DD groups, compared to both NN and ND groups ($P < 0.05$). No significant alteration in urea nitrogen was observed.

Next, we used a general linear model to assess the significance of the association between genotypes and demographic data such as age, gender, smoking, drinking, family history of heat stroke and weight index. Our results showed that age, gender, smoking and drinking did not differ significantly between the genotypes ($P > 0.05$). However, significantly more patients with genotype DD had a family history of heat stroke, compared to those with genotypes NN or ND ($P < 0.05$) (**Table 3**). Furthermore, recurrence of heat stroke was significantly high in patients with S allele. These data suggest that there is no significant correlation between heat stroke and age, gender, smoking or drinking, whereas family history of heat stroke may indicate a higher risk of heat stroke

Table 1 SNPs identified in *CASQ1* from heat stroke patients

Nucleotide variation	Reference sequence	Variant sequence	Location	dbSNP ID	Chr position	Amino acid variation
g. 175A > G	5′…TACAAG**AAT**GT…3′	5′…TACAAG**GAT**GT…3′	Exon 1	–	160190926	N59D
g. 2968C > T	5′…ACTACC**C**CACCC…3′	5′…ACTACC**T**CACCC…3′	Intron 2	rs3747623	160193719	–
g. 3015C > T	5′…GAC**AGC**ATGTAT…3′	5′…GAC**AGT**ATGTAT…3′	Exon 3	–	160193766	–

Note: SNPs are highlighted in bold and the codon where the SNP is located is underlined. The sequences of PCR products were aligned with the reference sequence NC_000001.11 for human chromosome 1.

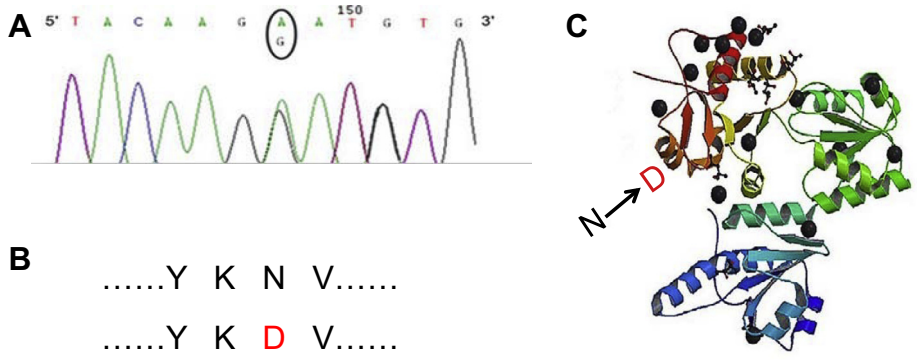

Figure 1 The newly-identified nonsynonymous SNP in *CASQ1*
A. Chromatograph of the SNP g175A > G in exon 1. Note the double peaks for A and G at position 148 (open circle). Sequence is aligned against the NCBI Reference Sequence NC_000001.11 for human chromosome 1, GRCh38 Primary Assembly. **B.** The nonsynonymous SNP 175A > G in *CASQ1* leads to amino acid substitution from asparagine (N) to aspartic acid (D, in red) at position 59 (N59D). **C.** The location of the altered amino acid residue in the CASQ1 protein structure (PDB ID:P31415) [15] (ribbon diagram). The arrow indicates the position of the sequence variation and the solid circles are calcium binding sites.

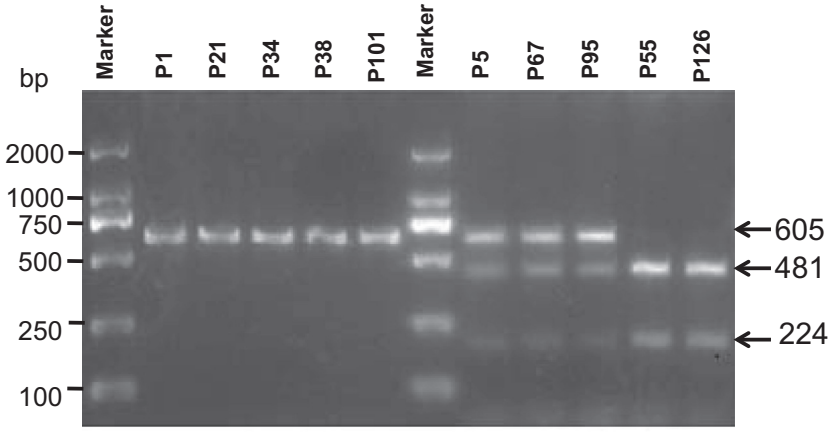

Figure 2 *CASQ1* genotyping using PCR-RFLP
CASQ1 PCR products were digested using BstU I and separated on 1% agarose gel. Lanes M: DL 2000 DNA Marker. Lanes are labeled with patient No. on top to indicate the source of DNA.

occurrence for DD genotype. In addition, patients who carry an allele D would be more prone to heat stroke recurrence.

Functional analysis of related genes found in the mutation-residing locus

Linkage disequilibrium (LD) analysis is often served as a post-genome-wide association study (GWAS) method in genetic research, and LD generally triggers the reduction of polymorphism in locus around the causative SNP, in selective theory, which is called as a selective sweep [17]. To explore the linkage disequilibrium effect between different genes and to identify the functional genes physically around the mutation-residing locus, we used BioMart (http://www.biomart.org/) and DAVID (http://david.abcc.ncifcrf.gov/), two common bioinformatic software tools, for functional analysis to evaluate genes in the 1-Mb flanking sequences of the mutation-residing locus with 0.5 Mb on each side. BioMart helps to find homologous genes in the target region, and DAVID could cluster all the genes into pathways or systematic biological functions. The first two genes standing out are those encoding DDB1 and CUL4 associated factor 8 (*DCAF8*) and peroxisomal biogenesis factor 19 (*PEX19*). As far as we know, the functional association between *CASQ1* and *DCAF8* or *PEX19* has not been reported.

Discussion

Heat stroke usually occurs in tropic regions or during hot climate and heat is often attributed as the causative factor [18,19]. Additionally, genetic susceptibility has also been reported for heat stroke [20,21], indicating the involvement of genetic background in heat stroke. Based on high throughput data as well as a limited number of association studies, recent studies have indicated *CASQ1* as a candidate gene associated with heat stroke [22–25]. In this study, we discovered a novel *CASQ1* SNP, which causes a nonsynonymous amino acid substitution N59D.

Our association analysis on demographic and clinical data indicated that family history is significantly associated with heat stroke occurrence for patients with DD genotype, *i.e.*, patients whose family or relatives have previously suffered

Table 2 Blood physiological indices of heat stroke patients

	Genotype NN (n = 35)	Genotype ND (n = 72)	Genotype DD (n = 43)
Leukocyte (count/μL)	$10.6 \times 10^3 \pm 0.10 \times 10^3$	$11.1 \times 10^3 \pm 0.25 \times 10^3$	$13.3 \times 10^3 \pm 0.22 \times 10^{3*,\#}$
Platelet (count/μL)	$9.6 \times 10^3 \pm 0.67 \times 10^3$	$9.3 \times 10^3 \pm 0.50 \times 10^3$	$7.7 \times 10^3 \pm 0.61 \times 10^{3*}$
Creatinine (mM)	39.12 ± 3.20	37.17 ± 9.20	$57.79 \pm 5.60^{*,\#}$
Ca^{2+} (mM)	6.11 ± 1.06	7.52 ± 0.98	$8.04 \pm 0.88^{*}$
Urea nitrogen (mM)	0.80 ± 0.31	0.82 ± 0.21	0.89 ± 0.30

Note: The data were analyzed by SAS 8.0 ANOVA one-way analysis and indicated as mean ± SD. Significant difference was indicated with * (compared to NN) or # (compared to ND) ($P < 0.05$).

Table 3 Demographics of heat stroke patients

	Genotype NN (n = 35)	Genotype ND (n = 72)	Genotype DD (n = 43)	P value
Age	35.1 ± 8.6	33.0 ± 9.2	36.2 ± 7.9	0.8613
Male (%)	46.6	40.3	40.1	0.5827
Smoking (%)	35.7	34.2	38.2	0.8926
Drinking (%)	40.0	39.5	43.5	0.9667
Family history of heat stroke (%)	19.6	21.2	39.5	0.0166^{*}
Body mass index	23.2 ± 1.3	23.3 ± 1.3	23.2 ± 1.1	0.6914
Recurrence of heat stroke (%/3 years)	2.80	10.72	11.63	0.0296^{*}

Note: The data were indicated as least square mean ± SD for age and body mass index. P values were calculated using general linear model analysis. Significant difference was indicated with * ($P < 0.05$).

from heat stroke are more likely to possess DD genotype ($P < 0.05$). Moreover, the heat stroke recurrence in the patients with allele D is also remarkably higher than those with allele N (NN) ($P < 0.01$), suggesting that allele D increases the possibility of heat stroke recurrence. However, a few recent reports argued that *CASQ1* is associated with diabetes mellitus but not heat stroke [12], when investigating elderly people in North America, who are in an extreme pathological condition and more vulnerable to heat stroke [26–28], which might complicate data interpretation. In contrast, patients in our current study were relatively young (below 40 years old on average) and they did not have other major diseases. In addition, patients recruited in their and our studies were of different ethnic backgrounds, thus we could not rule out the possible effect of ethnicity. A larger cohort involving more heat stroke patients, followed by functional studies of the CASQ1 protein with N59D, would be essential to address the discrepancy.

Blood physiological indices, which are usually considered as initial indicators for the health status of patients, have been used to group patients for heat stroke studies on CASQ1 mutations. Altered blood physiological indices, especially increase in leukocyte count and decrease in platelet count, were observed in people with heat stroke in relation to healthy controls. Therefore, alterations in these blood physiological indices often serve as the early response of heat stroke [16,29,30]. Similar observations were also revealed in our study. Furthermore, our results indicated that significantly higher leukocyte count and lower platelet count were found in the blood from patients with genotype DD. In addition, no significant alteration in amount of blood urea nitrogen was detected among different patients, whereas creatinine in patients with DD phenotype was significantly increased. Blood urea nitrogen and creatinine are critical indices of renal function in the human [31]. However, it is possible that the increase of creatinine in blood could result from increased metabolic rate in muscle, since patients with heat stroke may endure a severe physical activity. Interestingly, significantly higher amount of Ca^{2+} was found in the blood from patients with genotype DD as well. Bouchama et al previously reported that patients with heat stroke had increased amount of Ca^{2+} [28]. Since CASQ1 is known as the major Ca^{2+}-binding protein in the skeletal muscle, we speculate that N59D might reduce the calcium binding ability of CASQ1 in skeletal muscle at high temperature, which may lead to increased calcium in blood when patients suffered from heat stroke. Further investigation would be necessary to test this hypothesis.

Our clinical practice indicated that the recurrence rate of heat stroke was 6% on average for patients. Moreover, the frequency and severity of recurrence in patients also differed; some patients suffered from recurrence often and showed worsened immunity with recurrence. Our study indicated that patients carrying allele D had a higher recurrence rate than the average, suggesting that they were more vulnerable to heat stroke. Further investigation of differential recurrence among patients could provide some insights into the mechanisms underlying heat stroke.

Finally, in the region flanking the nonsynonymous SNP, we found two genes *DCAF8* and *PEX19* that might be related to the pathogenesis of heat stroke. DCAF8 and PEX19 are known to mediate the body repairing system and work as the important component in the binding of substrates to the Cul4-Ddb1 E3 ligase macromolecular complex and ubiquitination [32–35]. DDB1 is a large subunit of the heterodimeric DNA DDB complex. It was reported that defective activity of this complex causes repair defect in patients with xeroderma pigmentosum (XP), an autosomal recessive disorder characterized by photosensitivity and early onset of carcinomas [15,36]. Additionally, PEX19 is necessary for early peroxisomal

biogenesis and plays important roles in many biological pathways such as ATP-binding cassette (ABC) family protein-mediated transport and organism-specific biosystem [37]. The ubiquitin–proteasome pathway (UPP) is a common mechanism to degrade endogenous proteins. Furthermore, ubiquitination is indispensible in regulating biological functions of a vast number of proteins [35]. Further investigation in the functional interaction between CASQ1 and DCAF8, PEX19 would be required to test this possibility.

In summary, we identified a new nonsynonymous SNP (N59D) in CASQ1 from heat stroke patients. Analyses of clinical data indicated that there were significant differences between NN and DD genotypes in recurrence of heat stroke, familiar history of occurrence and some blood physiological indices in heat stroke patients. Investigation of the molecular detail of the CASQ1 N59D variation is of importance to elucidate the roles of CASQ1 in heat stroke.

Materials and methods

Patients

A retrospective study was performed for patients who were diagnosed with heat stroke and admitted to the PLA hospital or Beijing Electric Power Hospital between 2007 and 2010. Patients with other diseases, such as cancer, liver and renal inflammation, or central nervous system disorders, that may complicate the data interpretation were excluded. After exclusion, in total 150 patients who suffered or died from heat stroke were recruited in this study, including 83 males and 67 females aged 18–41 years old. All these patients had no kinship or genetic relationship with each other. Heat stroke was diagnosed by the body temperature above 40 °C and apsychia after strenuous exercise. The Hospital Ethics Committee approved this study and written informed consents were acquired from all the patients.

Sample collection and DNA extraction

Blood samples were collected from 150 heat stroke patients. The procedure was approved by the institutional internal review board and informed consents were signed before sample collection. Blood samples were collected into ethylene diamine tetraacetic acid dipotassium salt (EDTA-K_2) anticoagulant tubes, stored at −20 °C and used within a week. DNA was extracted using the Tiangen DNA extraction kit (Beijing Tiangen Biotechnology, Beijing, China) as instructed by the manufacturer and stored frozen at −20 °C (final concentrations of 60–90 ng/μl).

SNP discovery

We designed 6 pairs of PCR primers using Primer 5.0 to amplify all 11 exons of *CASQ1* (Table S1). Equal amount of PCR products from all patients using the same primers were pooled together before sequencing. Sequencing was performed by Beijing Sunbiotech to detect potential SNPs.

We employed the PCR-restriction fragment length polymorphism (RFLP) assay for genotyping [38]. Briefly, *CASQ1* Exon 1 was amplified using primers designed for exon1 (Table S1). The resulting PCR products were separated on agarose gel and purified using UltraClean® Tissue DNA Isolation Kit. Purified DNA was then digested with BstU I (New England Biolabs, Ipswich, MA, US) and the resulting bands were resolved on 1% agarose gel.

Statistical method

Chi-square test was performed to evaluate the relationship between the blood physiological index of the heat stroke patients and their genotypes. Especially, the recurrence of heat stroke among patients was investigated based on a linear model. All statistical procedures were calculated by using SAS 9.0. The significance threshold is set to a P value of 0.05.

Bioinformatics analysis

BioMart (http://www.biomart.org/) and DAVID 6.7 were used to detect potential genes located 0.5 Mb upstream and downstream of the SNP position or the locus (the total region is 1 Mb). All genes in the defined region were annotated based on information from the NCBI database. We selected the mouse as the homologous species to humans due to the wider coverage of the annotated genes.

Authors' contributions

LM design the study; YL and YW performed the experiments and analyzed the data; YL and LM wrote the manuscript. All the authors read and approved the final manuscript.

Competing interests

The authors declared that there is no competing interests.

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Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.gpb.2014.03.004.

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