LETTER

An Association Study of CASQ1 Gene Polymorphisms and Heat Stroke

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Received 19 March 2014; accepted 31 March 2014
Available online 2 June 2014
Handled by Jun Yu

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
immunocompromised individuals, such as the very young and
the elderly [2]. The inability to properly predict, diagnose
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 proteo-
mic 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 pro-
duces 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 dis-
ease 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 ex-
perienced heat stroke [13,14]. In this study, we aim to discover
sequence variations in CASQ1 gene and carry out an associa-
tion 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 amplifica-
tion 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 indi-
cated 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
<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 pos-
bility, 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 electrophore-
sis (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 contain-
ing 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 leuko-
cyte 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 signifi-
cance 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 sug-
gest 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

| Nucleotide variation | Reference sequence | Variant sequence | Location | dbSNP ID | Chr position | Amino acid variation |
|---------------------|-------------------|------------------|----------|----------|-------------|---------------------|
| g. 175A > G         | 5’...TACAAAGATGT...3’ | 5’...TACAAAGATGT...3’ | Exon 1   | –        | 160190926    | N59D               |
| g. 2968C > T        | 5’...ACTACCCCCACCC...3’ | 5’...ACTACCTCACCC...3’ | Intron 2 | rs3747623 | 160193719     | –                   |
| g. 3015C > T        | 5’...GACAGATGTAT...3’ | 5’...GACAGATGTAT...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.
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
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 

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

Table 2 Blood physiological indices of heat stroke patients

|                      | Genotype NN (n = 35) | Genotype ND (n = 72) | Genotype DD (n = 43) | P value |
|----------------------|----------------------|----------------------|----------------------|---------|
| Leukocyte (count/µL) | 10.6 × 10^3 ± 0.10 × 10^3 | 11.1 × 10^3 ± 0.25 × 10^3 | 13.3 × 10^3 ± 0.22 × 10^3 | -0.05   |
| Platelet (count/µL)  | 9.6 × 10^3 ± 0.67 × 10^3 | 9.3 × 10^3 ± 0.50 × 10^3 | 7.7 × 10^3 ± 0.61 × 10^3 | *0.016  |
| Creatinine (mM)      | 39.12 ± 3.20         | 37.17 ± 9.20         | 57.79 ± 5.60          | #0.016  |
| Ca^{2+} (mM)         | 6.11 ± 1.06          | 7.52 ± 0.98          | 8.04 ± 0.88           | #0.016  |
| Urea nitrogen (mM)   | 0.80 ± 0.31          | 0.82 ± 0.21          | 0.89 ± 0.30           | #0.016  |

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 | 0.016* |
| 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).
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 indispensable 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-K2) anticoagulant tubes, stored 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.

Acknowledgements

We are grateful for the support from the Department of Emergency of Beijing Electric Power Hospital and the Department of Molecular Biology of Chinese PLA Medical School. Special thanks also goes to Dr Haihan Zhang for critical reading.

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.

References

[1] Kosgallana AD, Mallik S, Patel V, Beran RG. Heat stroke induced cerebellar dysfunction: a “forgotten syndrome”. World J Clin Cases 2013;1:260–1.
[2] Knollmann BC. Casq2 deletion causes sarcoplasmic reticulum volume increase, premature Ca2+ release, and catecholaminergic polymorphic ventricular tachycardia. J Clin Invest 2006;116:2510–20.
Priori SG, Napolitano C, Tiso N, Memmi M, Vignati G, Bloise R, Ming Q, Wang A, Cheng C, Tomasi M, Canato M, Paolini C, Dainese M, Reggiani C, Volpe O'Malley PG. The environment of health care: primum nonnocere. Protasi F, Paolini C, Dainese M. Calsequestrin-1: a new candidate protein in acute-care settings. Robinson R, Carpenter D, Shaw MA, Halsall J, Hopkins P, Zhao JJ, Zhou JJ, Hu J, Zhou FH, Kang HJ, Liu H, et al. Clinical effects of intravenous calcium release mediator 1 (CASQ1) rescue. Wedel DJ, Quinlan JG, Iaizzo PA. Heat stroke in intensive care and anesthesia. Br J Anaesth 2002;88:700.

Kourtis N, Nikoletopoulou V, Tavernarakis N. Small heat-shock proteins protect from heat-stroke-associated neurodegeneration. Nature 2013;490:213–8.

Tobin JR, Jason DR, Challa VR, Nelson TE, Sambuughin N. Malignant hyperthermia and apparent heat stroke. JAMA 2001;286:168–9.

Wedel DJ, Quinlan JG, Iaizzo PA. Clinical effects of intravenously administered dantrolene. Mayo Clin Proc 1995;70:241-6.

Tomas M, Canato M, Paolini C, Dainese M, Reggiani C, Volpe P, et al. Calsequestrin (CASQ1) rescues function and structure of calcium release units in skeletal muscle of CASQ1-null mice. Am J Physiol Cell Physiol 2012;302:C575–86.

Daw SK, Chu W, Zhang Z, Hasstedt SJ, Elbein SC. Calsequestrin 1 (CASQ1) gene polymorphisms under chromosome 1q21 linkage peak are associated with type 2 diabetes in Northern European Caucasians. Diabetes 2005;53:3300–6.

Thomas J, Crowhurst T. Exertional heat stroke, rhabdomyolysis and susceptibility to malignant hyperthermia. Intern Med J 2013;43:1035–8.

Patel DR, Gyamfi R, Torres A. Exertional rhabdomyolysis and acute kidney injury. Phys Sportsmed 2009;37:71-9.

Sanchez EJ, Lewis KR, Danna BR, Kang C. High-capacity Ca^{2+} binding of human skeletal calsequestrin. J Biol Chem 2012;287:11592–601.

Zhao X, Min CK, Ko JK, Parness J, Kim do H, Weisleder N, et al. Increased store-operated Ca^{2+} entry in skeletal muscle with reduced calsequestrin-1 expression. Biophys J 2010;99:1556–64.

Charles BA, Shriner D, Rotimi CN. Accounting for linkage disequilibrium in association analysis of diverse populations. Genet Epidemiol 2014;38:265–73.

Rosich Del Cacho M, Pareja Grande J, Martinez Jimenez MD, Latorre Latorre JF, Bejarano Ramirez N, et al. Heat stroke related to the use of topiramate. The importance of prevention. An Pediatr 2013;88:424–30.

Leon LR. Heat stroke and cytokines. Prog Brain Res 2007;162:481–524.

Grogan H, Hopkins PM. Heat stroke: implications for critical care and anesthesia. Br J Anaesth 2002;88:700.

Leon LR, Dineen S, Blaha MD, Rodriguez-Fernandez M, Clarke DC. Attenuated thermoregulatory, metabolic, and liver acute phase protein response to heat stroke in TNF receptor knockout mice. Am J Physiol Regul Integr Comp Physiol 2013;305:1421–32.