Research Article

Association between heat shock protein 70 gene polymorphisms and clinical outcomes in intensive care unit patients with sepsis

Kartik Ramakrishna[1,2,3], Srinivasan Pugazhendhi[3], Jayakanthan Kabeerdoss[3], John Victor Peter[1]

Abstract

Objective: The objective of the following study is to evaluate the associations between single nucleotide polymorphisms (SNPs) in the Heat Shock Protein 70 (*HSP70*) gene, gene expression of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) and medical intensive care unit (MICU) stay and organ failure in sepsis. **Materials and Methods:** MICU patients with sepsis were genotyped for rs1061581, rs2227956, rs1008438 and rs1043618 polymorphisms in *HSP70* gene using polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis or allele-specific PCR. Messenger ribonucleic acid (mRNA) expression of IL-6 and TNF-α were quantitated in peripheral blood lymphocytes. Outcomes were recorded. **Results:** 108 patients (48 male) aged 40.7 ± 16.0 (mean ± standard deviation) years included H1N1 infection (36), scrub typhus (29) and urosepsis (12). Seventy-one (65.7%) had dysfunction of three or more organ systems, 66 patients (61.1%) were treated by mechanical ventilation, 21 (19.4%) needed dialysis. ICU stay was 9.3 ± 7.3 days. Mortality was 38.9%. One or more SNPs were noted in 101/108 (93.5%) and organ failure was noted in only 1/7 patients without a single SNP. The A allelotypes of rs1061581 and rs1008438 were associated with hematological dysfunction (P = 0.03 and 0.07) and longer ICU stay (P = 0.05 and 0.04), whereas IL-6 and TNF-α mRNA levels were associated with central nervous system dysfunction. **Conclusions:** *HSP70* genotypes may determine some adverse outcomes in patients with sepsis.

Keywords: Inflammatory response, innate immunity, interleukin-6, tumor necrosis factor-alpha

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Introduction

Sepsis, a syndrome characterized by a dysregulated systemic inflammatory response (SIRS) to an infection anywhere in the body, results in an initial outpouring of cytokines and chemokines leading to T cell exhaustion.[1] In SIRS, the ability of the host immune response to regulate itself is impaired leading to an imbalance between pro-inflammatory and anti-inflammatory cytokines. Individuals vary widely in their response to stimuli that elicit inflammation. Most such stimuli are microbial in nature and in recent years, there has been a focus on genetic factors that may determine this variability in response and therefore predispose to sepsis.[2]

Single nucleotide polymorphisms (SNPs) are single base mutations in deoxyribonucleic acid (DNA), which are present in more than 1% of the population. SNPs in genes involved in immune recognition or immune effector pathways may determine the nature of inflammatory and immune responses and can be associated with high cytokine output in response to noxious stimuli. Several genetic polymorphisms have been examined in patients with sepsis focusing largely on polymorphisms that influence innate immune responses.[3-8] Heat Shock Protein 70 (*HSP70*) genes are located in the HLA locus

From:
[1]Department of Intensive Care, [3]Department of Gastrointestinal Sciences, Wellcome Trust Research Laboratory, Christian Medical College, Vellore, Tamil Nadu, India, [2]Department of Internal Medicine, Unity Health System, Rochester, NY 14626, USA

Correspondence:
Dr. Kartik Ramakrishna, Department of Internal Medicine, Unity Health System, Long Pond Road, Rochester, NY 14626, USA.
E-mail: kartik_ramakrishna@hotmail.com

on the short arm of Chromosome 6. The *HSP70* protein is involved not only in chaperone function within cells, but is also involved in innate immune signaling. Polymorphisms in the *HSPA1B* and *HSPA1L* genes have been shown to be associated with altered cytokine output in response to inflammatory stimuli. In patients with severe multiple trauma, patients with the A allele for the *HSPA1B* 1538G > A polymorphism and the C allele for the *HSPA1L* 2437T > C polymorphism had higher blood levels of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) and the C allele was a significant risk factor for development of liver failure and higher organ failure scores.[9] Other mutations such as *HSP* 1267A > G, rs1008438 and rs1043618 have been shown to be associated with an increased susceptibility to high altitude pulmonary edema in Chinese railway construction workers.[10] The rs1008438 and rs1043618 SNPs of the *HSPA1A* gene have been shown to contribute toward the susceptibility to coronary heart disease.[11] Studies evaluating the role of *HSP70* mutations in sepsis have given conflicting results. In one study, two mutations in the *HSP70-hom* and *HSP70-2* regions were not associated with severe sepsis.[12] In a more recent study, in adult patients with community acquired pneumonia, the 1267A > G mutation in the *HSPA1B* gene was found to be associated with an increased risk of developing septic shock.[13,14] We hypothesized that, in a cohort of patients who develop sepsis, the presence of SNPs in the *HSP70* gene would be associated with severe sepsis and with longer hospital stays. This study was thus undertaken to evaluate the association between SNPs in the *HSP70* gene and organ dysfunction and duration of hospitalization in patients admitted with sepsis to the medical intensive care unit (MICU).

Materials and Methods

Study population and definitions

Consecutive patients admitted to the MICU of a tertiary care university hospital between August 2009 and February 2010 with a diagnosis of sepsis or severe sepsis were considered for inclusion. Exclusion criteria were hematological malignancy, febrile neutropenia, human immunodeficiency virus infection, connective tissue disorders and failure to get consent. Sepsis was defined as the presence of a confirmed or strongly suspected infection and the presence of at least two SIRS criteria during the first 24-h of MICU admission:[15] (a) Core temperature >38.3°C or <36°C, (b) heart rate >90 beats/min, (c) respiratory rate >20 breaths/min, $PaCO_2$ <32 mmHg or need for mechanical ventilation, (d) white blood cells count >12,000/mm^3 or <4000/mm^3 or >10% immature (band) forms. Severe sepsis was defined as sepsis associated with dysfunction of organs distant from the site of infection. Organ dysfunction was characterized as the cardiovascular system dysfunction (systolic blood pressure ≤90 mmHg or mean arterial pressure ≤70 mmHg for 1 h, despite adequate fluid resuscitation, or the need to administer vasopressors in order to maintain systolic blood pressure ≥90 mmHg or mean arterial pressure ≥70 mmHg); central nervous system (CNS) dysfunction (acute deterioration of neurological condition not attributable to start of sedation or CNS disease in the 24 h before admission or during the period of observation); acute renal dysfunction (urine output <0.5 ml/kg/h for at least 6 h, despite adequate fluid resuscitation, or serum creatinine ≥177 μmol/l not attributable to chronic kidney failure); respiratory system dysfunction (a ratio of PaO_2 to FiO_2 <200 if the source of infection was not pulmonary and <250 if infection source was pulmonary, or if invasive or non-invasive ventilation was indicated because of clinical respiratory insufficiency within the first 24 h of admission); hematological dysfunction (platelet count <80,000/mm^3, or a 50% decrease in the 2 days preceding admission; international normalized ratio [INR] >1.5 or activated partial thromboplastin time >60s); metabolic dysfunction (metabolic acidosis with pH ≤7.30 or base deficit ≥5.0 mmol/l in association with a plasma lactate level >3.0 mmol/l); liver dysfunction (bilirubin >43 μmol/l, or alanine aminotransferase >50 U, or partial thromboplastin time more than 1.5 times normal or INR >1.5 in the absence of systemic anticoagulant agents).[15]

Outcomes

The primary outcome of interest was organ dysfunction (as defined above). Secondary outcomes included mortality and duration of MICU and hospital stay. Relevant clinical and laboratory data were recorded in the case report forms. All patients were followed up until discharge from MICU or until death. A volume of 15-ml of venous blood was collected within 24-h of MICU admission and used for genetic studies and gene expression studies after obtaining written consent from the next of kin. The study was approved by the Institutional Review Board and the Institutional Ethics Committee.

Polymorphic analysis

The four SNPs studied were *HSPA1B* 1267A > G (rs1061581), *HSPA1L* 2437C > T (rs2227956) and *HSPA1A* (rs1008438 and rs1043618). DNA was isolated from venous blood using phenol: Chloroform extraction. The primers used, amplicon sizes, the restriction enzymes used and the restriction digest

product sizes in relation to genotype are shown in Table 1.[16,17] The polymerase chain reaction (PCR) consisted of 2-ml of ×10 PCR buffer, 1-ml Jumpstart Taq DNA polymerase (Sigma, USA), 250-nm primers and 200-mm deoxynucleotide triphosphates (dNTPs). For the rs1061581 polymorphism, initial denaturation was at 95°C for 1-min, followed by 39 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 1-min and final extension at 72°C for 1-min and cooling at 15°C for 10-min. For the rs2227956, the conditions were identical except for annealing temperature of 57°C. For the other two polymorphisms, cycle denaturation was at 95°C, annealing was 63°C, final extension was for 8-min and cooling was at 10°C for 10-min. Restriction digestion was done using the same protocols described earlier.

Gene expression studies

Peripheral blood mononuclear cells were isolated by layering blood on a Ficoll-Hypaque gradient and centrifugation. Ribonucleic acid (RNA) was isolated from the peripheral blood mononuclear cells using Tri reagent (T9424, Sigma, USA) and converted into complementary deoxyribonucleic acid (cDNA) using reverse transcriptase core kit (RT-RTCK-05, Eurogentec, Belgium). The reverse transcriptase mix contained final concentration of ×1 buffer, 5 mM $MgCl_2$, 500 µM of each dNTPs, 2.5 µM random nonamer, 0.4 U/µl of RNase inhibitor, 1.25 U/µl of EuroScript RT, 200 ng of total RNA and RNase free water to adjust the final volume to 10 µl. The cDNA conversion was carried out in an Eppendorf Mastercycler gradient (Eppendorf, Germany) with an initial step for 10-min at 25°C, followed by reverse transcription for 30 min at 48°C and final inactivation for 5-min at 95°C. Real time PCR was carried out in a Chromosome 4 cycler (Bio-Rad, USA) using MESA GREEN quantitative PCR MasterMix Plus kit (Eurogentec, Belgium). The reaction volume of each assay was 20 µl, containing final concentration of ×1 master mix and 200 nm of primers. Table 1b lists the primers used for amplifying the target messenger RNA. We have evaluated several genes as housekeeping genes for studies of gene expression in peripheral blood mononuclear cells and chosen human acidic ribosomal protein on the basis of its performance.

Table 1 A: Primer composition and product sizes for the HSP genotyping

SNP	Forward primer	Reverse primer	Product size	Restriction enzyme	Restriction fragments
HSP A1267G	CAT CGA CTT CTA CAC GTC CA	CAA AGT CCT TGA GTC CCA AC	1117	Pst1	AA -1117 GG - 936, 181
HSPA1L C2437T	GAT CCA GGT GTA TGA GGG	GTA ACT TAG ATT CAG GTC TGG	705	Nco1	CC - 705 TT - 550, 155
rs1008438	CAG GAC GGG AGG CGA AAC (FC) CAG GAC GGG AGG CGA AAA (FA)	CAC AGG TTC GCT CTG GGA A	219		
rs1043618	GCT CGG TGA TTG GCT CAG AA	CTG CTC TCT GTC GGC TCG CTG CTC TCT GTC GGC TCC	282		

SNP: Single nucleotide polymorphisms, HSP: Heat shock protein

Sample size calculations

The previous studies showed that the inflammatory *HSPA1B* 1538G > A genotype was present in 55% of the population.[18] Since we were looking only for a "modifier" effect of the mutation on the course of sepsis, it was assumed that 55% of patients with sepsis would also have the inflammatory genotype. Failure of more than three organs (associated with a worse prognosis) occurs in 64% of patients with sepsis in MICU.[15] We assumed a real odds ratio of 4 for the mutation being associated with more than three organ failure and calculated a sample size of 108 subjects with 80% power of 80% and 5% type I error.

Statistics

Categorical data were compared using the Pearson Chi-square test, while continuous data were compared using the two-tailed independent *t*-test or the Mann-Whitney test as appropriate. SPSS version 15 was used for analysis.

Results

A total of 108 patients of which 48 were male and 60 were female with aged range 40.7 ± 16.0 (mean ± standard deviation) years formed the study cohort. Specific causes

Table 1 B: Primer composition and product sizes for the cytokine gene expression

Gene	Forward	Reverse	Amplicon size (bp)
HuPO	5'-GCTTCCTGGAGGGTGTCC-3'	5'-GGACTCGTTTGTACCCGTTG-3'	105
IL-6	5'- CATTTGTGGTTGGGTCAGG-3'	5'- AGTGAGGAACAAGCCAGAGC-3'	112
TNF-α	5'-CCTGCCCCAATCCCTTTATT-3'	5'-CCCTAAGCCCCCAATTCTCT-3'	81

for sepsis were identified in the majority of patients. H1N1 infection (n = 36) was the major single category of patients admitted with sepsis during this period. A total of 29 patients were diagnosed with scrub typhus infection, 12 with urosepsis, 4 with community acquired pneumonia and 3 with dengue. Eleven patients had an acute febrile illness for which the etiology could not be identified, whereas 13 patients were diagnosed with other infections that included leptospirosis, tropical pyomyositis, melioidosis, malaria, liver abscess, puerperal sepsis and hollow viscus perforation. The pattern and distribution of organ dysfunction are shown in Figure 1. Of note, 71 patients (65.7%) had dysfunction of 3 or more organ systems. Respiratory system dysfunction (84.3%) was the most frequent organ dysfunction. More than half the patients manifested renal, cardiovascular, hepatic and hematologic dysfunction [Figure 1]. Sixty-six (61.1%) patients required invasive mechanical ventilation, 25 (23.1%) were managed solely on non-invasive ventilation, whilst 17 (15.7%) patients did not require ventilatory support. Twenty one (19.4%) patients required dialysis. The duration of MICU and hospital stay were 9.3 ± 7.3 days and 14.4 ± 10.1 days respectively. Hospital mortality was 38.9% (n = 42).

Table 2 lists the frequencies of the various polymorphisms, showing a range of frequency of genotype from 0% to 80.5%. The heterozygous genotypes were widely prevalent, whereas the homozygous genotypes of *HSPA1L* (CC genotype) and rs1008438 (AA genotype) were virtually absent. Linkage patterns between the four polymorphisms were studied in the entire cohort of patients and are shown in Figure 2. In the entire group as well as in the subgroup with H1N1 infection, there was strong linkage disequilibrium between rs1008438, rs1043618 and rs1061581 while there was little or no linkage between rs2227956 (*HSPA1L*) and the other 3 SNPs.

Associations between the *HSP* gene polymorphisms and the various clinical outcomes are also shown in Table 2. Hematological dysfunction was significantly associated (P = 0.03) with the rs1061582 polymorphism, while there was a trend (P = 0.07) toward an association with the rs1008438 polymorphism. The duration of MICU stay was significantly associated with the rs1008438 polymorphism (P = 0.04) as well as with the A allelotype of the rs1061582 (P = 0.05). There was no association of mortality or multiorgan failure with individual polymorphisms. The presence of one or more polymorphisms was positively associated with the development of hematologic dysfunction (P = 0.02) as well as the need for invasive mechanical ventilation (P = 0.04).

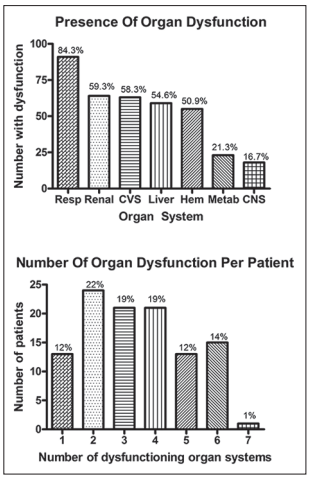

Figure 1: Frequency of organ dysfunction in 108 patients with sepsis. Panel A depicts the frequency of dysfunction of individual organ systems, while panel B depicts the distribution of the numbers of organs with dysfunction in these patients

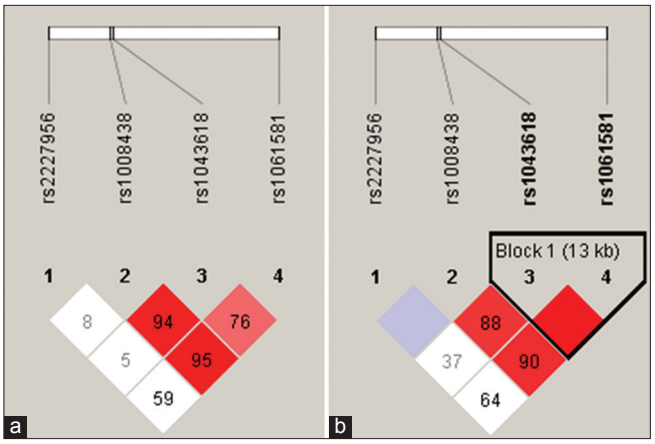

Figure 2: Linkage patterns between the four studied polymorphisms in the entire cohort of patients (a) and in the sub-group of patients with H1N1 infection. (b) In both groups of patients, there is strong linkage disequilibrium between rs1008438, rs 1043618 and rs1061581 (A1267G) while there is little or no linkage between rs2227956 (HSPA1L) and the other three single nucleotide polymorphisms

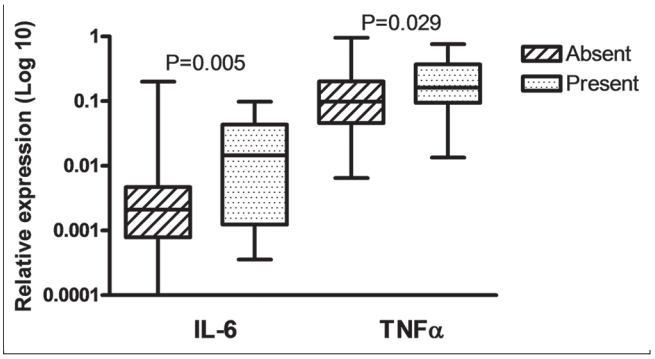

Figure 3: Messenger ribonucleic acid expression of interleukin-6 and tumor necrosis factor-alpha in patients with and without central nervous system dysfunction. Expression of mRNA for these genes was normalized to expression of mRNA for the housekeeping gene human acidic ribosomal protein. The box plots show the median, interquartile range (box) and total range (whiskers)

In the subgroup of patients with H1N1, liver dysfunction was significantly associated with the rs1043618 polymorphism (P = 0.03) and showed a trend to the association with rs2227956 polymorphism (P = 0.06). Two SNPs, rs1061582 (P = 0.007) and rs1008438 (P = 0.005), were strongly associated with duration of MICU stay while rs1043618 (P = 0.04) was also significantly associated with duration of MICU stay.

Gene expression studies for IL-6 and TNF-α showed that the two were highly correlated (r = 0.637, P = 0.000), which was expected since both are Th1 cytokines. Higher cytokine gene expression was significantly associated with CNS dysfunction [Figure 3] but not with other parameters including the *HSP* genotype, dysfunction of organs other than the CNS, length of MICU stay or mortality.

Discussion

Polymorphisms in the *HSP70* gene occur commonly in many populations[16-18] and their presence may influence the type and quantity of cytokines that are released into circulation in response to inflammatory stimuli. The present study examined these polymorphisms in a cohort of patients who had already developed sepsis and supports our hypothesis that these polymorphisms predispose to prolonged MICU stays in hospitalized patients with sepsis. Our study explored polymorphisms of the *HSP70* gene in a population of patients with a wide range of infectious diseases common to the developing world.

The frequency of occurrence of these polymorphisms was different in our study compared to populations from other countries. Comparison of the allelic frequency in our cohort with other populations shows a distinct population variation in alleles. For instance, rs1008438 A allele frequency was 40% of our cohort compared to 64% in Chinese, 8% in sub-Saharan Africans, 47% in Hispanics and 88% in Europeans.[11] The rs1043618 C allele frequency was 57% in our cohort compared with 24% in Chinese, 19% in Japanese, 34% in Caucasians and 9% in Africans.[11] However, our data is from hospitalized patients and may not truly reflect the prevalence in the community.

In another study from the same center in India that examined *HSPA1B* and *HSPA1L* genotypes among patients from the same population admitted for care of diabetic foot ulcers, the pro-inflammatory *HSPA1B* A allele (rs1061581) was noted in 70 of 100 (70%) patients,[18] similar to the 86 of 108 (80%) patients in our cohort. In the same study, the pro-inflammatory *HSPA1L* C allele (rs2227956) accounted for 21% of their cohort, again similar to the present cohort where it was 31%. This would suggest that patients with an inflammatory process requiring hospitalization in our population

Table 2. Distribution of HSP70 genotypes and their associations with outcomes in the patients with sepsis

Outcome	rs1061581				rs2227956			
	AA (n=29)	AG (n=57)	GG (n=22)	P value	CC, (n=2)	CT (n=32)	TT (n=74)	P value
Cardiovascular	15	35	13	0.69	1	21	14	0.60
Central nervous	5	11	2	0.55	1	7	10	0.25
Renal	14	36	14	0.37	1	23	40	0.22
Respiratory	23	49	19	0.69	1	26	64	0.32
Hematological	11	36	8	0.03*	1	16	38	0.99
Metabolic	4	15	4	0.36	1	8	14	0.47
Liver	17	30	12	0.87	1	14	44	0.32
No. with >3 organ dysfunction	10	31	9	0.18	1	18	31	0.39
Mortality	10	22	10	0.73	1	11	30	0.79
MICU stay, mean (SD) days	10 (7.9)	9.7 (8.0)	7.4 (4.1)	0.38	5 (1.4)	9.6 (7.9)	9.2 (7.1)	0.42
Hospital stay, mean (SD) days	14.1 (8.7)	14.9 (10.5)	13.5 (10.9)	0.84	7.5 (2.1)	15.8 (10.6)	13.9 (9.9)	0.28
Outcome	**rs1008438**				**rs1043618**			
	AA (n=0)	AC (n=87)	CC (n=21)	P value	CC (n=31)	CG, (n=62)	GG (n=15)	P value
Cardiovascular	0	49	13	0.38	16	39	8	0.53
Central nervous	0	16	2	0.32	4	12	2	0.68
Renal	0	50	14	0.44	18	36	10	0.82
Respiratory	0	72	19	0.38	25	52	14	0.53
Hematological	0	48	8	0.07*	13	36	6	0.22
Metabolic	0	19	4	0.77	6	14	3	0.93
Liver	0	47	12	0.79	20	30	9	0.30
No. with >3 organ dysfunction	0	41	9	0.72	13	29	8	0.76
Mortality	0	33	10	0.67	12	22	8	0.44
MICU stay, mean (SD) days	0	9.7 (7.8)	7.3 (4.0)	0.04*	10.3 (7.8)	9.2 (7.6)	7.4 (4.4)	0.84
Hospital stay, mean (SD) days	0	14.5 (9.9)	13.8 (11.0)	0.77	14.3 (8.5)	14.5 (10.7)	13.8 (10.6)	0.16

*P<0.05 was considered statistically significant, SD: Standard deviataion, MICU: Medical intensive care unit, HSP: Heat shock protein

have a similar frequency of the polymorphism. A study on polymorphisms of innate immune response genes done in Utah found the frequency of the A allele of the *HSP70* 1267A > G polymorphism to be significantly higher in those with puerperal sepsis than matched controls.[19] This may suggest a higher frequency of polymorphisms in those with dysregulated inflammation as in sepsis.

The study group included patients with a wide mix of infectious diseases-scrub typhus, H1N1, malaria, leptospirosis, meliodosis and dengue. The most common causes were H1N1 infection and scrub typhus. Mortality in H1N1 infection was 58%, while mortality in scrub typhus was 10%. Thus, there was heterogeneity between groups with respect to MICU course. MICU stay was longer for H1N1 patients than for patients in other groups.

Organ failure was frequent in our cohort of patients. Consistent with a recent series of patients with sepsis from several MICUs in the Netherlands,[15] respiratory dysfunction was dominant. The profile of other organ dysfunctions was also similar. MICU stay was 9.3 ± 7.3 days in the present series of patients compared to 13.3 ± 12.7 days in the Netherlands series.

We found that there were several associations that suggest a modifier effect of *HSP70* gene polymorphisms on the MICU course of patients with sepsis. The A allelotypes of rs1061581 and rs1008438 were associated with a higher prevalence of hematological dysfunction. These allelotypes were also associated with longer MICU stay in the entire group and this association was more pronounced in H1N1 infection. In H1N1 infection, the C allelotypes of *HSPA1L* and rs1043618 were associated with liver dysfunction. The C allelotype of rs1043618 was also associated with longer MICU stay in patients with H1N1 infection. The former is similar to the association found in a study on patients with severe trauma where the C allele was found to be a risk factor for a higher incidence of liver failure and higher organ failure score.[9] In another study, the *HSPA1B* AA (rs1061581) genotype was the strongest predictor of septic shock in patients with community acquired pneumonia.[13] That the study did not examine patients with other causes of sepsis nor did they look at other polymorphisms. Taken in conjunction with their findings, it appears that the A allele is a predictor of more severe illness in patients with sepsis admitted to MICU. The rs1061581 polymorphism has been noted to be in strong linkage disequilibrium with a *HSPA1A* and *HSPA1B* promoter region polymorphism that influences *HSPA1A* and *HSPA1B* protein production.[14] This, as well as the known association of these SNPs with increased levels of IL-6 and TNF-α, suggests that there could be multiple mechanisms to explain an effect of these polymorphisms on sepsis outcomes.[12] IL-6 expression, in our study, was associated with CNS dysfunction in patients with sepsis. Elevated IL-6 at discharge has been shown to predict all-cause mortality in patients with sepsis.[20] While we did not find an association with mortality, this study was not powered to detect such an association. The heterogeneity of the cohort was a limitation of the study, although we were looking for much broader responses which were not specific to particular infectious agents.

Conclusion

SNPs of the *HSP70* gene were frequently found among patients with sepsis. Within this cohort of patients, the presence of these polymorphisms was associated with hematological dysfunction as well as longer MICU stay particularly in patients with H1N1 infection. Replication of these findings in other populations and settings may allow management strategies to be tailored based on detection of these polymorphisms.

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