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Genetic contribution of suppressor of cytokine signalling polymorphisms to the susceptibility to infection after traumatic injury

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Summary

Suppressor of cytokine signalling (SOCS) proteins are crucial negative regulators in many signalling pathways and are implicated in the pathogenesis of infectious diseases. The purpose of this study was to uncover possible associations of common polymorphisms within SOCS genes with infectious outcomes after traumatic injury. A total of 1087 trauma patients (Chongqing cohort 806 and Yunnan cohort 281) were recruited and followed-up for the development of infectious outcomes, such as sepsis and multiple organ dysfunction syndrome (MODS). Twelve selected single nucleotide polymorphisms (SNPs) were screened by pyrosequencing to determine their genotypes and associations with infectious complications. Among the 12 selected SNPs, only the cytokine-inducible Src homology (SH2) domain protein (CISH) promoter rs414171 polymorphism was found consistently to be associated statistically with the incidence of sepsis and MOD score in the two cohorts, despite analysing the SNPs independently or in combination. Further, patients with a T allele had significantly lower CISH expression and lower production of tumour necrosis factor (TNF)-α, but higher production of interleukin (IL)-10. Luciferase assay confirmed that the A→T variant in the rs414171 polymorphism inhibited the transcriptional activities of the CISH gene significantly. The CISH rs414171 polymorphism is associated significantly with susceptibility to sepsis and MODS in traumatic patients, which might prove to be a novel biomarker for indicating risk of infectious outcomes in critically injured patients.

Keywords: MODS, polymorphisms, sepsis, SOCS proteins, trauma

Introduction

Trauma is a leading factor threatening public health worldwide [1], and even with improved emergency care, patients can commonly survive an initial trauma but still have a high potential to progress to infectious complications, such as sepsis and multiple organ dysfunction syndrome (MODS), resulting eventually in morbidity and mortality [2]. Therefore, it is necessary to develop therapeutic strategies to increase patient survival successfully after trauma injury to control/treat sepsis, as well as MODS, and to establish diagnostic markers for such complications.

The suppressor of cytokine signalling (SOCS) family of proteins, including SOCS1–7 and cytokine-inducible Src homology (SH2) domain protein (CISH), were uncovered as suppressors of cytokine signalling, contributing significantly to the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway [3]. SOCS proteins play instrumental roles in many cellular processes, such as cell differentiation, maturation, proliferation, apoptosis and immune response. SOCS proteins have been demonstrated to limit the extent of Toll-like receptor (TLR) signalling indirectly by inhibiting autocrine cytokine responses in macrophages through IFN-γ and TNF-α [4] and interacting directly with TLR signalling cascades, consequently regulating TLR/nuclear factor kappa B (NF-κB) signalling via a mechanism distinct from an autocrine cytokine response [5]. CISH was the first uncovered SOCS protein and was found to be induced by interleukin (IL)-2, IL-3 and erythropoietin (EPO), indicating its critical role in T cell proliferation against microbial infection [6].

Hepatic messenger RNA expression of CISH, SOCS1 and SOCS3 have been shown to be increased transiently in Sprague–Dawley (SD) rats with sepsis and associated with the development of hepatic growth hormone (GH) resistance [7–9]. Serial, sequential investigations [10] in human neutrophil granulocytes of major trauma patients suggested that immune dysregulation was accompanied by a significant decrease in SOCS1 and SOCS3 mRNA expression 6–72 h after trauma, whereas IL-10 and STAT-3 levels increased. Accumulating evidence supports that SOCS proteins may be pivotal in regulating cytokine signalling in response to microbial challenges, and therefore may be considered as novel biomarkers for the early diagnosis of sepsis.

A growing body of evidence also suggests that genetic variations in the form of single nucleotide polymorphisms (SNPs) are associated with both inflammatory responses and clinical complications after trauma injury [11–13]. Variations within SOCS genes have been reported to associate strongly with susceptibility to autoimmunity, cancer and metabolic diseases, such as multiple sclerosis [14], asthma [15], type 2 diabetes [16], obesity [17], lipid metabolism and insulin resistance [18] and colorectal cancer [19]. In studies focusing on infectious diseases, worldwide screening has shown that variants within the CISH gene were correlated highly with susceptibility to diverse infectious diseases, such as malaria, tuberculosis, bacteraemia [20,21] and hepatitis B virus infection [22,23]. To date, however, there are few studies addressing the impacts of the genetic variants of SOCS genes on the susceptibility to post-traumatic infectious outcomes.

Therefore, given the pivotal role of SOCS in regulating inflammatory responses, we postulated that variations in SOCS genes might influence the susceptibility to sepsis or MODS after traumatic injury. To test this hypothesis, we examined a set of SNPs within the SOCS family (CISH, SOCS1–7) to evaluate their associations with sepsis and MODS in two trauma cohorts and their functional relevance.

Materials and methods

Study population

A total of 1156 Chinese adults who were of Han ethnicity from different families without blood relationships, including 1087 blunt trauma patients and 69 healthy voluntary blood donors without any recent records of acute illness, chronic illness or sepsis, were enrolled prospectively into our study. The criteria used for trauma patient enrolment has been described previously [13]. Among them, 806 patients from the Chongqing district during the period of January 2005 to July 2013 (Chongqing Emergency Medical Center and the department of Trauma Surgery of Daping Hospital, Chongqing, China) were used for SNP screening to reveal clinical relevance, and 281 patients from the Yunnan district between January 2007 and April 2012 (the Department of Trauma and Emergency in Kunming General Hospital, Yunnan, China) constituted the validation cohort. Healthy donors were used to analyse CISH mRNA expression. Informed consent signed by participants and ethical approval from their next of kin were obtained from all subjects. All experiments were in agreement with the Helsinki declaration and approved by the Ethical and Protocol Review Committee of Third Military Medical University. Subject confidentiality was preserved according to guidelines for studies of human subjects.

Clinical evaluation

Demographic and clinical data were collected from electronic medical records, as described previously [13]. Our primary outcome measure was the occurrence of sepsis and organ dysfunction during hospitalization. Sepsis was identified as infection-induced systemic inflammatory response syndrome (SIRS), according to the guidelines from the American College of Chest Physicians/Society of Critical Care Medicine Consensus Committee [24]. The clinical diagnosis of infection was confirmed by positive cultures from biological samples or clinical features. The diagnosis of SIRS is appropriate where at least two of the following signs are present: temperature < 36°C or > 38°C; respiratory rate faster than 20 breaths per min or $PaCO_2$ less than 32 mmHg or requiring mechanical ventilation; heart rate faster than 90 beats per min; and leucocyte count > 12 000/ml or < 4000/ml or > 10% band forms. Organ dysfunction was assessed using multiple organ dysfunction (MOD) scores, which were measured by the well-validated Marshall scoring system [25]. The score was calculated excluding the neurological component (as measured by the Glasgow Coma Scale score). A Marshall score of > 4 over 48 consecutive hours was classified as MODS based on the studies reported by Sauaia [26]. Clinical evaluation was carried out by trained specialists who were blinded to the genotype data.

SNP selection

Full sequences of the human SOCS genes, including 3 kb upstream/downstream from the transcription start site (TSS)/stop codon, were retrieved from GenBank (www.ncbi.nlm.nih.gov/genebank, Accession number: GRCh37.p13). Genetic variation data of Chinese Han in Beijing (CHB) was obtained from HapMap project phase III (https://hapmap.ncbi.nlm.nih.gov, release 28). Haplotype

blocks and tag SNPs were constructed according to our previous report [11]. Due to the different chromosome loci of SOCS genes, tagSNPs for each of the SOCS genes were selected separately.

To explore the functions of SNPs within the 5′-flanking region of SOCS genes, the TFSEARCH algorithm (www.cbrc.jp/research/db/TFSEARCH.html) was used to evaluate their potential effects on transcription factor-binding sites.

Genotyping

Whole blood was collected during enrolment, and genomic DNA was obtained as per the manufacturer's protocol in the Wizard genomic DNA purification kit (Promega, Madison, WI, USA). DNA concentration and quality were determined by ultraviolet (UV) spectrophotometry. DNA was diluted to 30 ng/μl using sterile distilled water and kept at −80°C. Genotyping was performed by blinded specialists without knowledge of clinical features using the pyrosequencing method as described in a previous report [13], and 10% of the samples were sequenced in duplicate to validate genotyping quality.

Transcription activity

The transcriptional effect of rs414171 (−237A/T) resided in 5′-flanking of the CISH gene on promoters, as revealed by a reporter gene assay system [12]. As reported previously [13], a 1078 base pairs (bp) sequence (position −923/+155) of the CISH gene with an A or a T allele at position −237 were cloned into a pGL3-Basic plasmid (Promega) that contained firefly luciferase. Human embryonic kidney cells (HEK)293T cells were maintained according to our previous report [13]. A total of 1.5×10^5 cells per well were seeded into 24-well plates (BD Biosciences, Bedford, MA, USA). After culture for 24 h, cells were co-transfected with 0·8 μg of constructed plasmids or pGL3-Basic vector and 20 ng of Renilla luciferase reporter plasmid pRL-cytomegalovirus (CMV) using Fugene HD transfection reagent (Promega). At 48 h post-transfection, luciferase activity was determined by a luciferase assay system (Promega), according to the manufacturer's instructions, using a Luminoskan Ascent luminometer (Thermo Labsystems, Helsinki, Finland). The results are described as relative fold changes in the constructed vector compared with the pGL3-Basic vector. Each condition was performed in triplicate.

Measurement of cytokine levels

Cytokine assays were conducted according to our previous report [27]. Briefly, after incubating an aliquot of whole blood with 100 ng/ml lipopolysaccharide (LPS) (*Escherichia coli* O55:B5; Difco Laboratories, Detroit, MI, USA) at 37°C for 4 h, plasma was centrifuged carefully and kept at −80°C. Concentrations of TNF-α and IL-10 in plasma from trauma patients were measured via a human enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA), as per the manufacturer's protocol. Duplicate performance was conducted to minimize bias.

Quantitative real time–polymerase chain reaction (RT–qPCR)

CISH mRNA expression in peripheral blood leucocytes from healthy blood donors after LPS stimulation was quantified by RT–qPCR with a SYBR Green assay kit (Toyobo, Osaka, Japan), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primers used for CISH were forward 5′-CCTTCGGGAATCTGGCTGGTA-3′ and reverse 5′-CGGAAGCTGGAGTCGGCATAC-3′ and for GAPDH were forward 5′-CCAAAAGGGTCATCATCTCTG-3′ and reverse 5′-CATGAGTCCTTCCACGATACC-3′, respectively. Relative expression levels were calculated using the $2^{-\Delta CT}$ method. All experiments were performed in triplicate.

Statistical analysis

Differences between observed genotype frequencies with expected frequencies were calculated to determine deviations from Hardy–Weinberg equilibrium (HWE) using χ^2 analyses. Three different genetic models, including dominant, recessive and allele-dose models, were used for analysing SNP relevance with clinical outcomes. The relationship between genotypes and sepsis incidence rate was also determined by χ^2 analysis. Multivariable logistic regression models were used to calculate odds ratios (ORs) with 95% confidence intervals (CIs) to access sepsis risk, and the models were adjusted for age, sex and injury severity for confounding effects. The association of polymorphisms with MOD scores was determined by linear regression analysis, adjusting by age, sex and injury severity for confounding effects. For each comparison, the exact *P*-values are reported and considered significant if $P < 0{\cdot}05$. Data were analysed using SPSS version 11.5 statistical software (SPSS Inc., Chicago, IL, USA).

Results

SNP selection

In total, 145 SNPs with minor allele frequencies (MAFs) 5% within the SOCS family were obtained from the HapMap database, except for the SOCS1 gene, in which no SNP has been identified. Based on the analysis of SNP haplotypes in each block and tagging threshold of r^2, a total of nine

tagSNPs were selected as candidates, including CISH (rs2239751T/G), SOCS2 (rs10777530C/T), SOCS4 (rs1209087T/C), SOCS5 (rs3829835C/T, rs17771255G/A, rs3768720T/G), SOCS6 (rs9646604A/G, rs3809954G/A) and SOCS7 (rs3748726T/C). In addition, another three SNPs, rs414171A/T, rs8064821C/A and rs1351887T/A, located in the 5′flanking regions of CISH, SOCS3 and SOCS6, respectively, were also selected based on their potential effects on the corresponding promoter (Supporting information, Table S1).

Clinical features of trauma patients

The trauma patients from two districts were mainly young (mean age = 41·1 ± 13·3 and 39·9 ± 12·4) and suffered from severe injuries [Mean Injury Severity Score (ISS) = 22·2 ± 9·3 and 21·3 ± 8·5], while 41·9 and 35·2% of patients progressed to sepsis, respectively. The median time point for sepsis occurrence was 6 days (interquartile range = 5·0–8·5 days) and 5 days (interquartile range = 4·0–9·5 days). Respiratory tract infection accounted in approximately 43·6 and 44·0% of documented infections, respectively. Pathogen testing in blood cultures revealed that Gram-negative infection occurred in 18·0 and 16·7% of patients, Gram-positive infectious in 6·7 and 8·7% and mixed-Gram infection in 29·0 and 26·9%, respectively. Organ dysfunction occurred in 50·7 and 45·7% of trauma patients, of which 16·3% (131 patients) and 15·5% (44 patients) exhibited dysfunction in ≥2 organs (Table 1). The median time point for MODS occurrence was 7 days (interquartile range = 5·5–11·0 days) and 5·5 days (interquartile range = 3·5–8·0 days).

Clinical relevance of SNPs with infectious complications in patients from the Chongqing district

We first investigated the clinical relevance of the 12 SNPs selected from SOCS genes in 806 trauma patients from the Chongqing district. Genotyping success rates of patients ranged from 99·8 to 100%, and genotyping results in 10% of the randomly selected samples were 100% in the duplicates. All the genotype distributions were consistent with HWE ($P > 0·05$). There were no differences in comparing overall MAF in trauma patients with that in 139 healthy unrelated Chinese Han Beijing (CHBs) from the HapMap database (Supporting information, Table S2).

By comparing clinical features among different genotype patients of each SNP, we did not find significant changes in age, sex or ISS (Table 2). Overall, the strongest significant association was found in the CISH promoter polymorphism rs414171A/T. Patients carrying the variant T allele (TT+AT) revealed a significant association with decreased sepsis incidence and MOD scores using dominant model analysis compared with wild-type homozygotes (AA) ($P = 0·044$ and $P = 0·029$, respectively). There was also a significant difference in the sepsis incidence rate using recessive model analysis ($P = 0·023$). After adjustments in age, sex and ISS, the associations of rs414171A/T in the allele-dose model analysis with sepsis and MOD scores remained significant (OR = 0·73, 95% CI = 0·58–0·91, $P = 0·005$ and $P = 0·026$, respectively). Another polymorphism, rs3748726T/C, located in the 3′UTR region of the SOCS7 gene, also showed a significant association with susceptibility to sepsis. Patients with the variant C allele had a significantly lower sepsis incidence rate ($P = 0·017$ in the dominant model) compared with the wild-type model. There were no statistical associations observed with the other 10SNPs.

Whether the observation that CISH rs414171 and SOCS7 rs3748726 genotypes altered the susceptibility to sepsis is specific to certain bacteria is unknown. Therefore, we next tested for genetic association of altered susceptibility to sepsis by five pathogen categories, including Gram-positive bacteria (GPB), Gram-negative bacteria (GNB), fungus, mixed GPB and GNB, and negative cultures in the Chongqing derivation cohort. For rs414171A/T, the minor T allele was associated more significantly with decreased susceptibility to sepsis by fungi ($P = 0.013$ in the dominant model, $P = 0.021$ in the recessive model and OR = 0·31, 95% CI = 0·14–0·68, $P = 0·004$ in the allele-dose model) and sepsis by negative culture ($P = 0·017$ in the recessive model, and OR = 0·66, 95% CI = 0·48–0·91, $P = 0·011$ in the allele-dose model). Another polymorphism, SOCS7 rs3748726T/C, also showed significant association with susceptibility to negative culture sepsis ($P = 0·032$ in the dominant model and OR = 0·57, 95% CI = 0·38–0·85, $P = 0·006$ in the allele-dose model) (Supporting information, Table S3).

Validation of the rs414171 polymorphism in trauma patients from the Yunnan district

To validate our results, another cohort from the Yunnan district ($n = 281$) was processed in the same way. Due to the locations of the two cohorts (both were in the western region of China), SNP distributions and clinical features of trauma patients were similar (Table 1). As expected, we found similar SNP associations with clinical outcomes in the Yunnan cohort. The sepsis incidence rate in patients with the variant T allele was significantly lower than that in the wild-type ($P = 0·01$ in the dominant model). Multiple logistic regression analysis indicated that the association was significantly allele-dose dependent (OR = 0·64, 95% CI = 0·44–0·93, $P = 0·019$). However, although no significant difference was found among subjects with different genotypes, there was a decreasing trend for MOD scores in association with T allele copy number.

Table 1. Overall clinical characteristics of patients with major trauma.

Variables	Screening cohort Chongqing (***n*** = 806)	Validation cohort Yunnan (***n*** = 281)	*P*-value
Age (years)	41·1±13·3 (18-65)	39·9±12·4 (18-65)	0·257
Male/female, *n*	650/156	231/50	0·565
Injured body regions, *n* (%)			
Head	424 (52·6)	157 (55·5)	0·345
Thorax	513 (63·6)	152 (53·7)	0·005
Abdomen	313 (38·8)	96 (33·9)	0·164
Extremities	469 (58·2)	165 (58·3)	0·877
Number of regions injured, n (%)			
Two	298 (37·0)	124 (43·8)	0·034
Three	205 (25·4)	62 (21·9)	0·258
All four	69 (8·6)	13 (4·6)	0·032
ISS	22·2±9·3	21·3±8·5	0·546
16, < 25, n (%)	479 (59·4)	178 (63·3)	
25, n (%)	327 (40·6)	103 (36·7)	0·248
Organ dysfunction, n (%)	408 (50·7)	129 (45·7)	0·174
One	277 (34·4)	85 (30·2)	0·207
Two	108 (13·4)	32 (11·3)	0·386
Three or above	23 (2·9)	12 (4·2)	0·247
Sepsis, n (%)	338 (41·9)	99 (35·2)	0·050
Source of infection, %			0·906
Respiratory tract infection	43·6	44·0	
Primary bloodstream infection	19·7	19·1	
Urinary tract infection	17·5	16·8	
Catheter associated infection	9·7	10·5	
Wound infection	7·6	8·2	
Others*	1·9	1·4	
Pathogens, % (positive blood cultures)			0·233
Gram-negative	18·0	16·7	
Gram-positive	6·7	8·7	
Fungi	7·0	5·9	
Mixed Gram-negative and Gram-positive	29·0	26·9	
Negative blood cultures	39·3	41·8	

ISS = Injury Severity Score. *Other sites of infection included soft tissue infection, bone and ear infection. Continuous data are reported as the mean ± standard deviation. Categorical data are reported as percentage frequencies. Statistical significance was determined using Student's *t*-test for continuous variables and χ^2 test for categorical data. Exact *P*-values are reported and considered significant if $P < 0{\cdot}05$.

By pooling the two cohorts, we observed an even stronger relevance of rs414171 polymorphism with sepsis incidence (OR = 0·71, 95% CI = 0·58–0·85, $P = 0{\cdot}0003$ in the allele-dose model) and MOD scores ($P = 0{\cdot}003$ in the allele-dose model) (Table 3). Due to the small sample size of the Yunnan sepsis patients who had positive culture (Gram-positive $n = 9$, Gram-negative $n = 16$, fungus $n = 6$, mixed $n = 27$, total $n = 58$), we did not analyse the association with different pathogen sepsis in the Yunnan cohort.

Effect of the rs414171 polymorphism on CISH expression

To evaluate the effect of the rs414171 (–237A/T) polymorphism on the promoter activity and mRNA expression of the CISH gene, we co-transfected HEK293T cells with constructed plasmids/control vector with a luciferase reporter plasmid and found that homozygous T at position –237 attenuated promoter activity compared significantly with homozygous A ($P = 0{\cdot}001$), indicating that the T variation could affect promoter activity, consequently changing mRNA expression of CISH (Fig. 1).

To assess further the association of the rs414171 polymorphism with CISH mRNA expression, 69 healthy subjects were selected to determine the expression of CISH mRNA in peripheral blood after LPS stimulation *in vitro*. RT–qPCR (Fig. 2) revealed that LPS-induced CISH mRNA expression in peripheral blood was associated significantly with rs414171 genotypes. mRNA expression in the subjects with the T allele was decreased significantly compared

Table 2. Association of the SOCS SNPs among trauma patients in the Chongqing district

SNP	Genotype	*n*	Age (years)	Sex M/F	ISS	Sepsis (%)	MOD score
CISH	AA	278	41·6 ± 13·2	224/54	22·1 ± 9·3	130 (46·8)	4·6 ± 0·2
rs414171A/T	AT	408	40·9 ± 13·5	331/77	22·5 ± 9·5	169 (41·4)	4·1 ± 0·1
	TT	120	40·6 ± 12·8	97/23	22·0 ± 8·9	39 (32·5)	4·0 ± 0·2
						a1, b1, c1	a2, c2
CISH	TT	301	41·4 ± 13·1	246/55	22·5 ± 9·4	139 (46·2)	4·4 ± 0·2
rs2239751T/G	TG	387	41·6 ± 13·6	304/83	22·3 ± 9·4	153 (39·5)	4·2 ± 0·2
	GG	118	38·8 ± 12·9	101/17	21·5 ± 8·8	46 (39·0)	4·2 ± 0·2
SOCS2	GG	281	41·5 ± 13·3	225/56	22·0 ± 8·9	111 (39·5)	4·2 ± 0·2
rs10777530G/A	GA	388	40·9 ± 13·2	311/77	22·8 ± 9·8	170 (43·8)	4·3 ± 0·2
	AA	137	40·9 ± 13·9	115/22	21·4 ± 8·9	57 (41·6)	4·4 ± 0·2
SOCS3	CC	461	40·7 ± 13·1	378/83	22·4 ± 9·4	185 (40·1)	4·3 ± 0·1
rs8064821C/A	CA	301	41·9 ± 13·5	239/62	22·3 ± 9·5	135 (44·9)	4·2 ± 0·1
	AA	44	40·0 ± 14·1	34/10	20·3 ± 7·2	18 (40·9)	4·0 ± 0·4
SOCS4	TT	489	40·7 ± 13·3	399/90	22·4 ± 9·4	204 (41·7)	4·3 ± 0·1
rs1209087T/C	TC	274	41·8 ± 13·7	218/56	22·1 ± 9·4	114 (41·6)	4·3 ± 0·2
	CC	43	40·9 ± 11·6	34/9	21·2 ± 8·1	20 (46·5)	4·3 ± 0·5
SOCS5	CC	641	41·4 ± 13·4	520/121	22·4 ± 9·2	269 (42·0)	4·2 ± 0·1
rs3829835C/T	CT	158	39·9 ± 13·0	125/33	21·8 ± 9·7	65 (41·1)	4·4 ± 0·3
	TT	5	41·2 ± 13·3	3/2	18·0 ± 9·2	3 (60·0)	4·3 ± 1·2
SOCS5	CC	770	40·9 ± 13·3	619/151	22·3 ± 9·4	319 (41·4)	4·3 ± 0·1
rs17771255C/T	CT	36	44·1 ± 14·7	32/4	21·0 ± 7·3	19 (52·8)	4·3 ± 0·3
	TT	0	–	–	–	–	–
SOCS5	CC	230	40·1 ± 13·1	187/43	22·5 ± 8·8	96 (41·7)	4·2 ± 0·2
rs3768720C/A	CA	413	41·3 ± 13·1	336/77	22·1 ± 9·6	173 (41·9)	4·4 ± 0·1
	AA	163	41·9 ± 14·3	128/35	22·3 ± 9·4	69 (42·3)	4·1 ± 0·2
SOCS6	AA	661	41·2 ± 13·4	534/127	22·1 ± 9·3	285 (43·1)	4·3 ± 0·1
rs1351887A/T	AT	134	40·6 ± 13·0	108/26	22·6 ± 9·3	47 (35·1)	4·2 ± 0·2
	TT	11	39·0 ± 11·4	8/3	25·8 ± 9·2	6 (54·5)	5·1 ± 0·8
SOCS6	AA	443	40·5 ± 13·4	354/89	22·5 ± 9·2	187 (42·2)	4·2 ± 0·1
rs9646604A/G	AG	304	41·6 ± 13·0	249/55	22·0 ± 9·2	124 (40·8)	4·3 ± 0·2
	GG	59	42·8 ± 14·5	48/11	21·7 ± 10·9	27 (45·8)	4·6 ± 0·4
SOCS6	GG	705	40·8 ± 13·2	566/139	22·3 ± 9·5	296 (42·0)	4·2 ± 0·1
rs3809954G/A	GA	98	43·7 ± 13·8	82/16	21·9 ± 7·7	42 (42·9)	4·8 ± 0·4
	AA	3	27·3 ± 8·5	3/0	13·7 ± 4·0	0	2·0 ± 0·0
SOCS7	TT	483	41·4 ± 13·6	388/95	21·8 ± 9·2	219 (45·3)	4·4 ± 0·1
rs3748726T/C	TC	290	40·7 ± 12·9	235/55	22·9 ± 9·4	109 (37·6)	4·2 ± 0·2
	CC	33	44·4 ± 13·6	28/5	22·2 ± 11·0	10 (30·3)	3·6 ± 0·4
						a3	

CISH = cytokine-inducible Src homology 2 (SH2) domain protein; ISS = Injury Severity Score; M/F = male/female; MOD = multiple organ dysfunction; SNP = single nucleotide polymorphism; SOCS = suppressor of cytokine signalling.

[a1]P = 0·044, [a2]P = 0·029, [a3]P = 0·017 for dominant association (variant homozygotes + heterozygotes *versus* wild-type homozygotes).

[b1]P = 0·023 for recessive association (variant homozygotes *versus* heterozygotes + wild-type homozygotes).

[c1]P = 0·005 odds ratio (OR) = 0·73, 95% confidence interval (CI) = 0·58–0·91), [c2]P = 0·026 for allele–dose association.

with subjects carrying the wild-type A allele (P = 0·033 in the dominant model; P = 0·013 in the allele-dose model).

Association between the rs414171 polymorphism and cytokine levels induced by LPS in trauma patients

Given the important role of CISH in cytokine processing and inflammatory responses, we therefore assessed how the rs414171 variant influenced inflammatory cytokine levels, such as IL-10 and TNF-α (Fig. 3). Using ELISA, we found that IL-10 levels were increased significantly in patients with the T allele (TT+AT) in rs414171 compared with rs414171AA patients (P = 0·031 in the dominant model), whereas TNF-α levels were decreased significantly (P = 0·013 in the recessive model) after LPS stimulation. Using linear regression analysis in the allele-dose model, a significant association of rs414171 polymorphism with IL-10 was also revealed in the same manner in different genotypes of trauma patients (P = 0·035). However, without LPS stimulation no significant changes were observed in TNF-α or IL-10 levels between rs414171 genotypes (P > 0·05, data not shown).

Table 3. Clinical relevance of the rs414171A/T polymorphism among trauma patients in the two cohorts

	Chongqing (*n* = 806)			Yunnan (*n* = 281)			Merged (*n* = 1087)		
Genotype	*n*	Sepsis n (%)	MOD score	*n*	Sepsis *n* (%)	MOD score	*n*	Sepsis *n* (%)	MOD score
AA	278	130 (46·8)	4·6 ± 0·2	103	46 (44·7)	4·9 ± 0·3	381	176 (46·2)	4·7 ± 0·2
AT	408	169 (41·4)	4·1 ± 0·1	128	38 (29·7)	4·6 ± 0·3	536	207 (38·6)	4·3 ± 0·1
TT	120	39 (32·5)	4·0 ± 0·2	49	14 (28·6)	3·9 ± 0·5	169	53 (31·4)	4·0 ± 0·2
		a1, b1, c1	a2, c2		a3, c3			a4, b2, c4	a5, c5

Merged = two cohorts combined from the Chongqing and Yunnan, MOD = multiple organ dysfunction.

^{a1}P = 0·044, ^{a2}P = 0·029, ^{a3}P = 0·01, ^{a4}P = 0·003, ^{a5}P = 0·01 for dominant association (variant homozygotes + heterozygotes versus wild-type homozygotes).

^{b1}P = 0·023, ^{b2}P = 0·011 for recessive association (variant homozygotes versus heterozygotes + wild-type homozygotes).

^{c1}P = 0·005 [odds ratio (OR) = 0·73, 95% confidence interval (CI) = 0·58–0·91], ^{c2}P = 0·026, ^{c3}P = 0·019 (OR = 0·64, 95% CI = 0·44-0·93); ^{c4}P = 0·0003 (OR = 0·71, 95% CI = 0·58–0·85); ^{c5}P = 0·003 for allele-dose association.

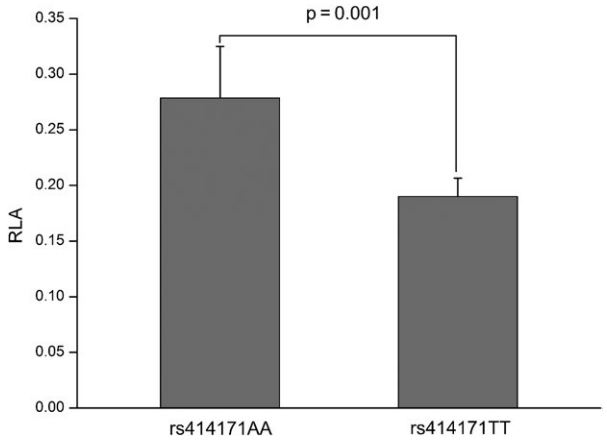

Fig. 1. Effect of the rs414171 polymorphism on cytokine-inducible Src homology (SH2) domain protein (CISH) promoter activity. Relative luciferase activity (RLA) was measured in human embryonic kidney cells (HEK)293T cells transfected with rs414171-237AA or TT constructed plasmid. Luciferase activity was normalized for transfection efficiency by using a control plasmid, cytomegalovirus (CMV) immediate early enhancer/promoter region (pRL-CMV)]. The A→T variation significantly attenuated promoter activities (TT *versus* AA: P = 0·001, Student's *t*-test).

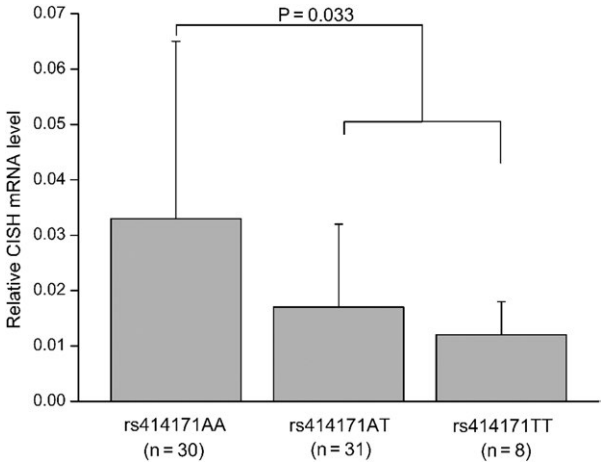

Fig. 2. Association of the rs414171 polymorphism with lipopolysaccharide (LPS)-induced expression of cytokine-inducible Src homology (SH2) domain protein (CISH) in the healthy cohort. Expression levels of CISH mRNA in human peripheral blood leucocytes with different genotypes in the rs414171 polymorphism were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression, shown as the mean and standard deviation. Significant differences were observed among AA, AT and TT genotypes under LPS stimulation (TT+AT *versus* AA: P = 0·033, Student's *t*-test).

Discussion

Our study revealed a significant association of rs414171, a polymorphism located in the CISH promoter region, with the susceptibility to sepsis and MODS in trauma patients from two independent cohorts, analysed individually and in combination. Moreover, the rs414171 polymorphism showed significant effects on CISH expression and LPS-induced activation of peripheral blood leucocytes. Compared with other studies, our study was distinctive. First, we targeted a trauma population to avoid potential bias in population stratification when comparing with inappropriate controls, such as healthy blood donors, helping to maximize the likelihood of finding a meaningful genetic association. Secondly, instead of screening SNPs spanning the whole genome, we evaluated only tagging and potential functional SNPs from the whole SOCS family genes, and for the first time found one significantly associated polymorphism with post-traumatic sepsis susceptibility. Thirdly, we revealed the potential mechanism underlying the association between the rs414171 polymorphism and the incidence rate of post-traumatic sepsis.

CISH is a suppressor in cytokine signalling, which inhibits interactions of cytokine receptors with signalling mediators, thus affecting signal mediator function [3]. Up-regulated CISH strongly promotes cytokine production

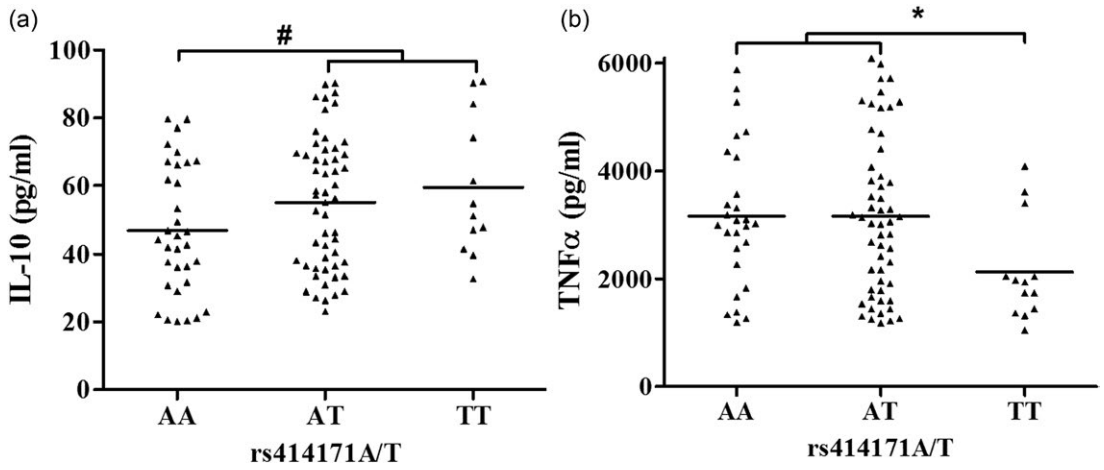

Fig. 3. Association of the rs414171 polymorphism on lipopolysaccharide (LPS)-induced cytokine production by peripheral blood leucocytes. The whole-blood samples collected immediately from trauma patients were incubated with 100 ng/ml LPS (*Escherichia coli* O55:B5) at 37°C for 4 h. The levels of interleukin (IL)-10 (a) and tumour necrosis factor (TNF)-α (b) in the plasma were assayed by a sandwich enzyme-linked immunosorbent assay. Student's *t*-test was used to assess statistical significance. #Dominant effect (TT + AT *versus* AA), $^{\#}P = 0{\cdot}031$, *recessive effect (TT *versus* AT + AA), $^{*}P = 0{\cdot}013$.

and T cell proliferation and prolongs the activated T cell life span in humans. The bi-directional functions of CISH in cytokine and T cell responses are important mechanisms for controlling homeostasis and regulating immune responses [28]. Several prior studies found the rs414171SNP of CISH to be associated with multiple infectious diseases, including hepatitis B virus infection [22,23,29], bacteria [20], tuberculosis [20,21,30,31] and malaria [20] among multiple populations, highlighting the importance of this genetic variant in infectious diseases. However, our results were inconsistent with another study [20] in which the rs414171 T allele in CISH accounted for the major responsibility for increased susceptibility to bacteraemia. One possibility was that susceptibility to infection was caused by the combined effects of several SNPs. However, in this study, we selected only 12 SNPs and focused on rs414171 among multiple CISH polymorphisms, which was one of the major differences between the two studies. The second possibility might be that CISH SNPs could tag informative variants in different structures of the population.

Using bioinformatics analysis, the –237 A allele in rs414171 was shown to have transcription factor AML-1a binding sites. Luciferase analysis indicated that the transcriptional activity of the T allele in rs414171 was decreased significantly compared with that of the A allele. These findings were consistent with Hu's study [23], which suggested that the variant allele at rs414171 may decrease the activity of the CISH promoter. In addition, Khor *et al*. [20] demonstrated that IL-2 stimulation decreased rs414171 T allele transcriptional activity. These findings confirmed our results, indicating that rs414171 is a functional SNP, and the substitution of T for A at position –273 could reduce CISH promoter transcriptional activity and mRNA expression. In addition, by evaluating the variant effects in inflammatory responses, we found that subjects who carried the variant T allele had lower levels of the proinflammatory cytokine TNF-α and higher levels of the anti-inflammatory cytokine IL-10. All these findings suggest that the presence of the T allele in rs414171 can significantly decrease CISH transcriptional activity and gene expression by altering the dynamic balance in pro-inflammatory responses to an anti-inflammatory state after LPS exposure, thus ultimately inhibiting the development of sepsis. Therefore, the rs414171 polymorphism might serve as a potential biomarker for indicating post-traumatic complications upon clinical examination.

However, this study had several potential limitations. First, we recruited only trauma patients in the Chinese Han population, which is different from other ethnic populations in some respects, and further studies in other populations should be included to explore rs414171 polymorphism function fully. Secondly, the sample size, although being adequate for the Chongqing cohort, was not large enough in the Yunnan cohort. The values of power for CISH rs414171 are 89 and 67%, respectively, in the Chongqing and Yunnan cohorts at a significance of 0·05. Additional large-scale studies are needed for the validation of clinical relevance of the rs414171 polymorphism. Thirdly, this study had territorial limits; as both the Chongqing and Yunnan districts are located in the southwest of China they have similarly genetic characteristics, so further studies should include populations from other parts of China to minimize bias caused by regional differences. Fourthly, we

did not obtain a sufficient amount of blood samples to assess the polymorphism effects and CISH protein levels at other time-points; therefore, the functions of the rs414171 polymorphism need to be confirmed further. Finally, the exact mechanisms of SOCS in the pathogenesis of post-traumatic complications remain incompletely understood; therefore, further investigation of these findings, both *in vivo* and *in vitro*, are merited.

Conclusion

In our study, genetic and functional evidence indicated that the rs414171 polymorphism, located in the promoter region of the CISH gene, correlated significantly with the incidence of post-traumatic sepsis and MODS in the Chinese Han population. Our findings supported the concept that SOCS variants play important roles in trauma progress, and these variants can add significantly predictive value to infectious development. Larger-scale samples from more diverse populations are warranted to obtain a compelling conclusion.

Ethics approval and consent to participate

The study protocol was approved by the Ethical and Protocol Review Committee of the Third Military Medical University (no. TMMU2012009). Informed consent was obtained from the patients or their next-of-kin.

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Disclosure

All authors declare that they have no competing interests.

Author contributions

A.-Q. Z. was the main researcher for this study and contributed to writing this paper. W. G., H.-X. L., L.-Y. Z., D.-Y. D. and J. H. were involved in the collection of blood samples and clinical data. L. Z., D.-L. W. and X. W. performed the technical work. J. -X. J. planned the study, wrote the protocol and was involved in the genetic and clinical aspects of data analyses and revised the paper.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Table S1. Characteristics of the selected single nucleotide polymorphisms (SNPs) from suppressor of cytokine signalling (SOCS) genes.

Table S2. Distribution of the selected 12 SNPs among trauma patients in the Chongqing district.

Table S3. Association of cytokine-inducible Src homology (SH2) domain protein (CISH) rs414171 and suppressor of cytokine signalling (SOCS7) rs3748726 polymorphisms with sepsis of certain bacteria in Chongqing trauma patients.