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Tumor Necrosis Factor Gene Variation and the Risk of Mortality After Burn Injury: A Cohort Study

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Abstract

Infection risk and mortality after burn trauma are primarily determined by patient age, burn size and depth, and associated inhalation injury. Whether genetic differences contribute to otherwise unexpected variability in outcomes is unknown. We sought to determine whether there was an association between genetic variation in inflammation-related genes and outcomes after burn trauma. We evaluated patients with burns $\geq 15\%$ TBSA at a single regional burn center from October 2003 to December 2005. Blood was collected on admission and DNA genotyping was performed. We genotyped single nucleotide polymorphism (SNPs) in toll-like receptor 4 (TLR4) A896G, tumor necrosis factor alpha (TNF- α) G-308A, Interleukin-6 (IL-6) G-174C, interleukin-1 (IL-1) T-31C, and cluster of differentiation marker 14 (CD14) C-159T. We compared SNP genotypes between survivors and nonsurvivors by χ^2 analysis and logistic regression. Sixty-nine subjects with a median age of 38 years and mean TBSA of 34% were enrolled. The case fatality was 17%. Septic shock developed in 7 (10%) patients. After adjustment for age, percent full-thickness burns, and inhalation injury, carriage of the TNF- α -308 variant allele was associated with increased risk of mortality (OR 10.7, 95% CI = 1.2–95.5, $P = .034$). None of the other SNPs evaluated were associated with mortality. Mortality after burn trauma is primarily determined by clinical factors, but the TNF- α -308 A allele seems to contribute to an increased mortality risk.

Severe burns are often followed by sepsis, multiple organ dysfunction syndrome, and death.¹ Clinical risk factors for infection, organ failure, and death after burn trauma are well described.^{2–4} However, genetic differences contribute to variation in the inflammatory response and may influence outcomes after burn trauma. The most common genetic variation implicated in complex disease are single nucleotide polymorphisms (SNPs).^{5,6} A SNP is a single base pair substitution in genomic DNA for which sequence alternatives exist in normal individuals in some populations. SNPs do not cause disease per se but may affect the risk of developing a disease or the outcome of a disease. The majority of SNPs are thought to be nonfunctional, but some are believed to be functional leading to changes in protein amount or structure. The nonfunctional SNPs serve as useful markers where the presence of the SNP could potentially mark for other functional variants.⁷ We and others have previously reported on the association between SNPs of critical signaling genes in the innate immune response and risk of severe sepsis and mortality posttrauma. These SNPs are found in the genes central to endotoxin binding and downstream cytokine production. Specifically, the SNPs in toll-like receptor 4 (TLR4) A896G, tumor necrosis factor alpha

(TNF- α G-308A,⁸⁻¹⁰ interleukin-6 (IL-6) G-174C,^{11,12} interleukin-1 (IL-1) T-31C,¹³ and cluster of differentiation marker 14 (CD14) C-159T.¹⁴

In experimental murine models, genetic variations have been shown to influence variability in the immune-inflammatory response after major burn injury.¹⁵ Whether more subtle, naturally occurring variation seen in humans contributes to outcome is unknown. Few genetic association studies have been conducted in burn victims.^{14,16,17} In this study, we sought to determine whether an association exists between candidate SNPs and the risks of septic shock and mortality after severe burn injury. Furthermore, given that clinical factors have been utilized to predict mortality in patients with severe burns such as age, sex, inhalation injury, and percent TBSA,²⁻⁴ we sought to determine whether knowledge of these genetic differences might improve burn mortality prediction.

METHODS

Study Design and Data Collection

We enrolled patients admitted to the intensive care unit (ICU) at the University of Washington Regional Burn Center (Seattle, WA) with burns 15% TBSA or greater between October 2003 and December 2005. Patients were excluded if they were not resuscitated postinjury or if they had a major nonburn injury. The institutional review boards of the University of Washington approved this study and waiver of consent was obtained for use of discarded venous blood. Demographics and clinical data including admission data, percent TBSA, percent full-thickness TBSA, inhalation injury, hospital course, and complications were collected from the electronic medical record. The diagnosis of inhalation injury was made on clinical grounds, by the combination of history and physical findings on admission, as we do not use a specific management protocol based on the presence of inhalation injury. Data are reviewed both at admission and in subsequent patient charting, as there may be little information available upon admission, especially if the patient is on mechanical ventilation. This ensures data accuracy in the registry. For comorbid illnesses, a binary variable was created indicating whether or not subjects had none or one or more of these conditions: diabetes, cardiovascular disease, pulmonary disease, neurologic disease, obesity, and alcohol dependence.

Follow-up was until patients' hospital discharge or death. Primary outcome was mortality, and secondary outcomes were sepsis, septic shock, and ICU length of stay (LOS). The definitions for sepsis and septic shock established by the American Burn Association Conference were used thus did not assign a "severe" sepsis category.¹⁸

DNA Isolation and SNP Genotyping

Discarded venous blood samples collected in EDTA were obtained from each enrolled patient. DNA was isolated via the Qiagen QIAamp DNA Blood Midi Kit. DNA samples were then genotyped for the presence of mutations in TLR4 (A896G), TNF- α (G-308A), IL-6 (G-174C), IL-1 (T-31C), and CD14 (C-159T)—Table 1. All genotyping assays were designed by Applied Biosystems (Foster City, CA). The primer and probe sequences are provided in Table 2. Genotyping was done in a blinded fashion to the patients' clinical status. For each assay, the reaction utilizes two dual-labeled Taqman probes that specifically target the two alleles at the SNP site. The two probes are labeled with a fluorescent reporter dye (VIC or FAM) and a nonfluorescing quencher/minor groove binder. When a probe specifically binds to the SNP site, the 5' nuclease activity of Taq polymerase during PCR allows for the cleaving and subsequent fluorescence of the reporter dye. At the conclusion of PCR, samples are genotyped via analysis of the fluorescence of the two dyes. Each 5.0 μ l PCR contains the following: TaqMan®UniversalPCRMasterMix, NoAmpErase® UNG (2 $\times$);

Assays-on-Demand™(20×) or Assays-by-Design™ (40×) SNP Genotyping Assay Mix, and 1 ng of genomic DNA. Assays were conducted in 384-well format on the ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems). Reaction conditions were the following: initial de-naturation at 95°C for 10 minutes, followed by 40 cycles each of denaturation (95°C for 15 seconds), and annealing/extension (60°C for 60 seconds).

Data Analysis

Data were analyzed with SPSS 14.0 statistical software (SPSS, Chicago, IL). Continuous data are presented as medians and interquartile range (25th to 75th percentile) and were analyzed using Mann–Whitney *U* test. Categorical variables were compared using Pearson's χ^2 test. For each comparison, the exact *P* values are reported (considered significant if $P < .05$).

Multiple logistic regression analysis was used to evaluate independent risk factors for death postburn. The logistic regression model was constructed using variables with univariate values of $P \leq .2$, these included clinical factors that have been shown to be associated with death post severe burns: age, percent full-thickness TBSA, and inhalation injury.^{19,20} Variables were serially removed in a stepwise fashion and the quality of the model was assessed using the receiver operator characteristic curve and the Hosmer–Lemeshow goodness-of-fit test. Only variables that improved the quality of the model were retained.

RESULTS

Demographic and Clinical Outcomes

From August 2003 to December 2005, 81 subjects were enrolled. Nine subjects with severe associated traumatic injuries and three severely burned subjects who died within 24 hours were excluded. Demographic data and injury characteristics for the 69 patients included in analysis are summarized in Table 3. The most common cause of burns was flame injury occurring in 66 patients (85%). The most common comorbidity was cardiovascular disease in 22% of the patients, followed by pulmonary disease in 14% of the patients, and diabetes mellitus in 5% of the patients. Forty-four percent ($n = 30$) of patients did not have any comorbidity.

Sepsis developed in 56 patients (81%), of whom seven had septic shock. Median ICU LOS was 23 days (25th to 75th percentile was 10–42.5) and median hospital LOS was 40 days (21.5–61).

Clinical Risk Factors for Mortality

Ten (15%) study subjects died (Table 4). Age greater than or equal to 55 years, full-thickness burn size, and inhalation injury were associated with increased mortality risk ($P < .05$). Other clinical variables evaluated for risk of mortality and found not to be significant: female sex, coexistent trauma, and comorbid illness. The clinical factors associated with mortality risk are included in Table 5.

Association Between Innate Immune SNPs and Mortality After Burn Injury

Candidate SNP genotype frequencies among survivors and nonsurvivors are shown in Table 6. In this unadjusted analysis, none of the candidate SNPs were found to be significantly associated with risk of death.

Only after adjusting for age, percent full-thickness TBSA burn, and inhalation injury was carriage of the TNF- α 308 A allele associated with death (aOR 10.7, 95% CI 1.2–95.5, $P = .034$; Table 5). The 40% of the nonsurvivors and 22% of the survivors carried the TNF-

–308 A allele and no subjects were homozygous for the variant allele. Clinical risk factors did not differ significantly between alternate genotypes of TNF- –308 (Table 7) except for receiving a blood transfusion, which seems to be related to undergoing excision and grafting. Both occurred more frequently in the wild-type carriers subgroup compared to variant allele carriers. The clinical characteristics and TNF- –308 genotypes of each of the nonsurvivors are detailed in Table 8. None of the candidate SNPs were associated with sepsis or its most severe form, septic shock.

TNF- α Genotype Increases the Area Under the Receiver Operating Characteristic Curve Estimating Mortality

Using age, size of full-thickness burn, and inhalation injury, an area under the receiver operator characteristic curve of 0.875 ± 0.060 was obtained. Although this is a strong predictive model, its predictive ability was increased by the addition of patients' TNF- –308 promoter SNP genotype with an area under the receiver operator characteristic curve of 0.908 ± 0.041 (Figure 1).

DISCUSSION

Our aim was to examine the association between candidate SNPs involved in the innate immune response and septic shock and mortality in severely burned adults. We observed an association between the TNF- –308 A allele mortality after severe burn injury, but found no associations with other SNPs in genes important to innate immunity.¹⁴ We also observed that in the setting of well-described clinical risk factors,³ knowledge of genetic information provides little additional predictive value.

The TNF- –308 polymorphism is located in the promoter of the TNF- gene on chromosome 6 in a region containing the sequence for the activator protein-2 transcriptional factor.²¹ This is believed to influence TNF- transcriptional activity by modifying DNA confirmation and altering the binding of different nuclear proteins.²² In vitro studies have demonstrated that the TNF- –308 A allele increases transcriptional activation of TNF- leading to a “high producer” phenotype.²³ The latter phenotype is seen in healthy A-allele carriers' whole blood TNF- response to endotoxin^{24,25} and in patients after severe injury.¹⁰

This polymorphism is reported to be associated with autoimmune and inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, and acute pancreatitis.^{26–28} This association has also been reported—though not consistently—in patients with septic shock.^{8,10,29–32} In a previous study, we have demonstrated that carriage of the TNF- –308 A allele is associated with an increased risk for severe sepsis after traumatic injury but not death.⁹ Given the overwhelming burn-induced tissue damage, patients with large burns have an intense systemic inflammatory response and elevated circulating TNF- levels,³³ and it is plausible that they would demonstrate a clinically relevant effect of a gene polymorphism within the inflammatory response pathways even in the absence of an infection. The TNF- –308 gene polymorphism association with mortality after severe burns has biological plausibility in that nonsurvivors have higher circulating TNF- levels compared with survivors³⁴ and that death in burn patients occurs in the setting of multiple organ failure despite the clinical absence of uncontrolled infection.³⁵ Further supporting evidence is seen in the setting of acute pancreatitis where TNF- –308 gene polymorphism is associated with the risk of severe acute pancreatitis but not with the risk of septic shock.²⁸

Barber et al^{14,17} evaluated the association between candidate SNPs and severe sepsis and mortality in 233 burn victims with 15% TBSA burns. The TNF- –308 variant A allele was associated with risk for severe sepsis but not mortality, whereas CD14 –159 C allele was associated with increased risk for mortality. The differences in our findings may be due

to our population demographics being predominantly Caucasian (81%) compared with 56% Caucasian patients in the cohort studied by Barber et al. Furthermore, we enrolled fewer subjects who could account for different observations, an unfortunately common feature of many genetic association studies.³⁶ The small sample size explains our wide confidence intervals and possibly the lack of association between other reported clinical risk factors and outcomes after severe burns such female sex and comorbid illness.^{2–4} Such limitation in burn studies could be mitigated through large collaborative multicenter efforts.^{37–39} Despite our study limitation, the association of TNF- α -308 A allele with mortality provides supporting evidence that this allele has strong relevance in vivo as a marker for mortality risk in this setting.^{8,28,40}

We have confirmed that clinical risk factors capture much of the variability in risk for death after burn injury. Improvement in this predictive model by adding the TNF- α -308 genotype was small. Nevertheless, this is a single variant, and perhaps with a more widespread genotyping approach (including genome-wide methods), and additional important SNPs are found, their cumulative effect will be relevant to risk stratification and important to patient care.

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Figure 1.

Comparative receiver operating characteristic (ROC) curves of two models used to predict the mortality among burned patients. The clinical risk factors model included age ≥ 55 years old, size of full-thickness burn, and inhalation injury. The addition of TNF- α genotype to the clinical risk factor model modestly improves the area under the curve TNF- α , tumor necrosis factor alpha.

Table 1

Single nucleotide polymorphisms evaluated and their reported clinical associations

SNP	Reference SNP ID#	Major Allele	Minor Allele	Chromosome	Gene Location	Reported SNP Associations
TLR4 (A896G)	4986790	A	G	9	Exon 3	Risk of complicated sepsis in trauma & burn patients ^{17,41}
TNF- (G-308A)	1800629	G	A	6	Promoter	Risk of complicated sepsis in trauma & burn patients ^{9,10,17}
IL-6 (G-174C)	1800795	G	C	7	Promoter	Risk of severe sepsis ¹¹ and risk severity of appendicitis ⁴²
IL-1 (T-31C)	1143627	T	C	2	Promoter	Risk of death after severe sepsis ¹³
CD14 (C-159T)	2569190	C	T	5	Promoter	Risk of death after burns ¹⁴

TLR4, toll-like receptor 4; *TNF*^ε, tumor necrosis factor alpha; *IL*₆, interleukin; *CD14*, cluster of differentiation marker 14.

Table 2

Primer and probe sequences used in SNP detection

SNP	Sequence * (5' to 3')
	Forward primer
	Reverse primer
	Probe (wild type)
	Probe (variant)
TLR4 (A896G)	TGACCATTGAAGAATTCCGATTAGCA ACACTCACCAGGGAAAATGAAGAA FAM-TACCTCGATGATATTATT-MGB VIC-CCTCGATGATATTATT-MGB
TNF- (G-308A)	CCAAAAGAAATGGAGGCAATAGGTT GGACCCTGGAGGCTGAAC FAM-CCCGTCCCATGCC-MGB VIC-CCCGTCCCATGCC-MGB
IL-6 (G-174C)	GACGACCTAAGCTGCACTTTTC GGGCTGATTGGAAACCTTATTAAGATTG FAM-CTTTAGCATCGCAAGAC-MGB VIC-CCTTTAGCATCGCAAGAC-MGB
IL-1 (T-31C)	CAGCCTCCTACTTCTGCTTTTGA AGGTTTGGTATCTGCCAGTTTCTC VIC-TCGCTGTTTTATAGCTT-MGB FAM-CGCTGTTTTATGGCTT-MGB
CD14 (C-159T)	CTAGATGCCCTGCAGAATCCTT CCCTTCCTTTCCTGGAAATATTGCA VIC-CTGTTACGGCCCCCT-MGB FAM-CTGTTACGGCCCCCT-MGB

TLR4, toll-like receptor 4; *TNF*, tumor necrosis factor alpha; *IL*, inter-, leukin; *CD14*, cluster of differentiation marker 14; *MGB*, minor groove, binder.

* Polymorphic nucleotide is underlined.

Table 3

Demographic data of burn patients (N = 69)

Variable	Median/Count
Age (yr)	38 (24–50)
Male	54 (78)
Ethnicity	
Caucasian	56 (81)
Hispanic	5 (7)
African American	3 (4)
Other	5 (7)
Comorbidities	
Cardiovascular disease	17 (22)
Asthma, COPD, or bronchitis	11 (14)
Diabetes mellitus	4 (5)
Mechanism of burn	
Flame	66 (85)
Flash	7 (9)
Scald	4 (5)
Electrical	1 (1)
Location of burns	
Head and/or face	51 (65)
Chest and/or abdomen	53 (68)
Back and/or buttocks	43 (55)
Upper extremities	70 (90)
Lower extremities	54 (69)
Perineum	6 (8)
% TBSA	34 (24–44)
% TBSA full-thickness burn	25 (10–40)
Inhalation Injury	20 (29)
Died	10 (15)
Sepsis	56 (81)
Septic shock	7 (10)
Underwent excision and grafting	48 (72)
Number of operations	2 (0–4)
Area of excision and grafting (cm ²)	2190 (2190–5282)
Blood transfusion during hospitalization	44 (64)
ICU length of stay (d)	23 (10–43)
Hospital length of stay (d)	40 (22–61)

ISS, Injury Severity Score; *COPD*, chronic obstructive pulmonary disease; *TBSA*, total body surface area.

Continuous variables are presented as median (25th–75th percentile). Discrete variables are presented as no. (%).

Table 4

Demographics and characteristics according to survival status

	Survivors (n = 59)	Nonsurvivors (n = 10)	P
Age	33 (21–47)	52 (41–77)	.002
Sex (male)	46 (78)	8 (80)	.885
Ethnicity			
Caucasian	48 (81)	8 (80)	.082
Non-Caucasian	11 (19)	2 (20)	
Comorbid illness	32 (54)	7 (70)	.352
% TBSA	33 (24–40)	40.5 (25–58)	.154
% TBSA full-thickness burn	22 (3–38)	40 (24–61)	.020
Inhalation injury	14 (24)	6 (60)	.019
Sepsis	50 (88)	6 (60)	.064
Septic shock	3 (5)	4 (40)	.001
Underwent excision and grafting	46 (78)	5 (50)	.063
Area of excision and grafting (cm ²)	2870 (300–5200)	400 (0–6375)	.631
Blood transfusion during hospitalization	37 (66)	5 (50)	.327
ICU length of stay (d)	27 (11–43)	8 (5–23)	.006
Hospital length of stay (d)	46 (27–61)	9 (5–35)	.003

TBSA, total body surface area.

Continuous variables are presented as Median (25th–75th percentile). Discrete variables are presented as no. (%).

Table 5

Adjusted odds ratios for known and suspected risk factors for mortality postburn injury as determined by stepwise logistic regression (N = 69)

Factor	OR (95% CI)	P
Age ≥ 55 yr	58 (3.4–985.8)	.005
% Full-thickness TBSA	1.06 (1.01–1.11)	.019
Inhalation injury	13.1 (1.08–159.1)	.044
TNF- α -308 variant (A allele)	10.7 (1.2–95.5)	.034

TBSA, total body surface area.

Full-thickness TBSA was evaluated as a continuous variable with odds ratio for each additional one percent increase in full-thickness burn size.

Table 6

Comparison of genotype frequencies of candidate innate immune SNPs according to survival status

Genotypes	Survivors (n = 59)	Nonsurvivors (n = 10)	P
TNF- γ -308 G A			.223
Homozygous G (n = 52)	46 (78)	6 (60)	
A-allele carriers (n = 17)	13 (22)	4 (40)	
TLR4 +896 variant A G			.874
Homozygous A (n = 63)	54 (91.5)	9 (90)	
G-allele carriers (n = 6)	5 (8.5)	1 (10)	
IL-6 -174 variant G C			.732
Homozygous G (n = 31)	27 (45.8)	4 (40)	
C-allele carriers (n = 38)	32 (54.2)	6 (60)	
IL-1 β -31 variant T C			.210
Homozygous T (n = 18)	17 (28.8)	1 (10)	
C-allele carriers (n = 51)	42 (71.2)	9 (90)	
CD14 -159 C T			.560
Homozygous C (n = 19)	17 (28.2)	2 (20)	
Heterozygotes (n = 33)	28 (47.5)	5 (50)	
Homozygous T (n = 17)	14 (23.7)	3 (30)	

Values are expressed as no. (%). Exact *P* values are presented for unadjusted analysis of genotype frequencies between the two groups by χ^2 analysis.

Table 7

Demographics and injury characteristics by TNF- genotype

	Homozygote G (n = 52)	A-Allele Carrier (n = 17)	P
Age	38 (24–50)	33 (16–52)	.775
Sex (male)	39 (75)	15 (88)	.251
% TBSA	34.5 (25–44)	27 (22–44)	.233
% TBSA full-thickness burn	25 (14–45)	19 (0–31)	.093
Inhalation injury	16 (31)	4 (24)	.568
Blood transfusion	37 (71)	7 (41)	.026
Underwent excision and grafting	48 (83)	10 (50)	.004
ICU length of stay (d)	26.5 (10–43)	15 (7–41)	.329
Hospital length of stay (d)	45 (26–62)	30 (15–61)	.215

TBSA, total body surface area; *TNF- α* , tumor necrosis factor alpha. Continuous variables are presented as median (25th–75th percentile). Discrete variables are presented as no. (%).

Table 8

Characteristics and TNF- genotype for nonsurvivor patients (n = 10)

Age	Sex	%TBSA	Inhalation Injury	TNF- Genotype	MODS	Length of Stay (d)	Cause of Death
83	Female	15	No	GG	Yes	113	Septic vs cardiogenic shock, unknown source of sepsis
52	Male	25	No	GA	Yes	10	Sudden cardiopulmonary arrest
76	Male	25	No	GG	Yes	7	Worsening respiratory failure
38	Male	41	Yes	GG	Yes	8	<i>Streptococcus pneumoniae</i> sepsis, stroke
23	Male	55	Yes	GG	Yes	3	Sudden asystolic arrest
43	Male	65	Yes	GA	Yes	5	Sudden cardiorespiratory arrest
68	Female	44	Yes	GG	Yes	23	Enterobacter septic shock
52	Male	35	Yes	GA	Yes	5	<i>S. pneumoniae</i> septic shock
51	Male	85	Yes	GG	Yes	13	Enterococcus septic shock
80	Male	40	No	GA	No	55	<i>Pseudomonas Aeruginosa</i> sepsis

SIRS, systemic inflammatory response syndrome; *MRSA*, methicillin-resistant *Staphylococcus aureus*; *MODS*, multiple organ dysfunction syndrome.