Extracellular Superoxide Dismutase Haplotypes Are Associated with Acute Lung Injury and Mortality

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Rationale: **Extracellular superoxide dismutase (EC-SOD) is a potent antioxidant that plays an important role in controlling oxidant-mediated stress and inflammation. High levels of EC-SOD are found in the lung. Acute lung injury (ALI) frequently occurs in patients with infection, and levels of EC-SOD have been shown to modulate severity of lung injury in transgenic animal models of endotoxemia-induced ALI. An R213G single nucleotide polymorphism (SNP) has been shown to alter levels of EC-SOD and patient outcomes in chronic obstructive pulmonary disease (COPD) and ischemic heart disease.**

Objectives: **To determine genetic variation in the promoter and EC-SOD gene and to examine whether EC-SOD haplotype blocks are associated with clinical outcomes.**

Methods: **We sequenced the EC-SOD promoter and gene to determine genetic variation and linkage disequilibrium (LD) patterns in a European American population. Two separate patient populations with infection-associated ALI were also evaluated to determine whether EC-SOD haplotypes were associated with clinical outcomes.**

Measurements and Main Results: **Sequencing resulted in the identification of 28 SNPs with relatively strong LD and 1 block consisting of 4691-5321-5360-5955-5982. This specific block was shown to be protective in two separate patient populations with infection associated ALI. In particular, patients with a GCCT haplotype had a reduced risk of time on the ventilator and mortality.**

Conclusions: **These results indicate that a GCCT haplotype may reduce inflammation in the lung, thereby decreasing the severity of lung injury and ultimately protecting patients from mortality associated with infection-induced ALI.**

Keywords: EC-SOD; haplotypes; acute lung injury; single nucleotide polymorphism

Acute lung injury (ALI) is a disease process that occurs frequently in patients with infection and is characterized clinically by an acute onset, Pa_{O_2}/FI_{O_2} ratio less than 250 mm Hg, and bilateral infiltrates on chest radiograph (1–3). In the setting of infection-induced ALI, activated neutrophils adhere to the pulmonary capillary endothelial surface and migrate into the interstitial and alveolar spaces where they secrete proinflammatory mediators, including cytokines such as tumor necrosis factor (TNF)-α and IL-1β, as well as reactive oxygen species (4–11). The release of these inflammatory mediators results in damage of the capillary endothelium and epithelium causing an increase in permeability and influx of protein-rich fluid (12).

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Extracellular superoxide dismutase (EC-SOD) is a potent antioxidant that plays an important role in protecting the lung against oxidant-mediated stress and inflammation. A R213G single nucleotide polymorphism in the EC-SOD gene has been shown to reduce susceptibility to chronic obstructive pulmonary disease.

What This Study Adds to the Field

These results indicate that a GCCT haplotype of EC-SOD may reduce inflammation in the lung, thereby decreasing the severity of lung injury and ultimately protecting patients from mortality associated with infection-induced ALI.

There are three human superoxide dismutases (SOD): a cytosolic form (CuZn SOD or SOD1), a mitochondrial form (Mn SOD or SOD2), and an extracellular form (EC-SOD or SOD3). EC-SOD is a potent extracellular antioxidant tetrameric enzyme that is expressed in the extracellular fluids of many tissues and plasma (13). EC-SOD is abundantly expressed in the lungs, where it plays a very important role in controlling oxidant stress and inflammation by regulating airway redox status through conversion of superoxide anion radicals to hydrogen peroxide and oxygen (14, 15). EC-SOD is post-translationally modified to form soluble EC-SOD (16–18). Binding EC-SOD has high affinity for the extracellular matrix, whereas soluble EC-SOD, lacking this binding region, is found in both plasma and lung airway surface lining fluids (17).

It has been shown that lung inflammation is increased with EC-SOD deficiency and is reduced with overexpression of EC-SOD (19–27). In particular, EC-SOD–deficient mice exposed to LPS had increased levels of neutrophils and proinflammatory cytokines in the lung (21). In contrast, mice that overexpressed EC-SOD had diminished levels of neutrophils and proinflammatory cytokines in the lung after exposure to LPS (21). The mechanism for EC-SOD's unique function in protecting against lung injury is hypothesized to be through scavenging reactive oxygen species at the airway lining surface, attenuating neutrophil accumulation in the lungs, and blunting oxidant-mediated release of proinflammatory cytokines by airway epithelial cells.

The EC-SOD gene is located in the 4p15 chromosomal region and consists of three exons. The coding region consisting of 720 base pairs is located in exon 3 of the gene. In humans, an R213G single nucleotide polymorphisms (SNP) in the EC-SOD gene has been shown to alter EC-SOD levels and function (28). This SNP occurs within the coding region and results in an arginine to glycine transition, which disrupts the binding properties of the protein to the extracellular matrix. As a result,

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a 10-fold increase in soluble EC-SOD occurs (28). Clinically, the R213G SNP was associated with a decreased susceptibility to chronic obstructive pulmonary disease (COPD) (29). Given the importance of EC-SOD's role in controlling oxidative stress and inflammation in the lung, we hypothesized that EC-SOD haplotypes might be associated with susceptibility to or outcome from infection-associated ALI.

METHODS

EC-SOD Sequencing

Genomic DNA samples from 52 healthy European Americans were randomly selected from a pool of 100 healthy controls to determine EC-SOD gene variation. The study was approved by the Colorado Multiple Institutional Review Board, and each volunteer signed an informed consent document. Sequencing consisted of the 2-kb promoter and gene through the 3′ untranslated region of EC-SOD. Genomic DNA was amplified using specific forward and reverse primers for the 2-kb promoter and EC-SOD gene. The 50-μl PCR re1action contained approximately 50 ng of genomic DNA, 1× PCR Buffer, 100 pM of forward (5′-CCT CAG TAG TTT GGA CCA CAA GCA-3′) and reverse (5′-GGG TGA CTT AGT TAC CAG CAG GAG-3′) primers for the promoter or forward primer (5′-AAG GAC GGT AGG GTG GAA GG-3′) and reverse primer (5′-AAC CAA GAC CTC GAC GCA GT-3′) for the EC-SOD gene, 50 mM dNTPs, 100 pM $MgCl_2$ and 5 units of Hi-Fidelity Taq polymerase (Applied Biosystems, Foster City, CA). The polymerase chain reaction (PCR) cycling conditions to obtain the approximately 7 kb PCR product consisted of 96°C for 3 minutes and then 20 cycles of 30 seconds at 94°C, 1 minute at X° (where X decreases from 65 to 55°C by 0.5°C per cycle), followed by 15 minutes at 68°C extension and 15 cycles of 30 seconds at 94°C, 1 minute at 55°C, and 15 minutes at 68°C plus 20 seconds per cycle. After the PCR reaction, the PCR products were treated with 5 μl of ExoSap-IT (GE HealthCare, Uppsala, Sweden) to remove unused primers and nucleotides. The PCR product was separated by 0.75% agarose gel-electrophoresis (stained with 0.1% ethidium bromide) to confirm the size and quantity of the PCR product. After gel confirmation of the PCR product, sequencing was performed with 60 ng of PCR product and 5 pmol of forward and reverse primers specific for the EC-SOD promoter and gene (Table 1).

The sequencing reactions consisted of the following procedure: double-stranded DNA templates were sequenced by the University of Colorado Cancer Center DNA Sequencing and Analysis Core Facility using AB Prism kits from Applied Biosystems containing AmpliTaq DNA Polymerase FS in one of the following: BigDye Terminator Cycle Sequencing Ready Reaction kit (part number 4,336,776), or dGTP BigDye Terminator Cycle Sequencing Ready Reaction kit (part number 4,307,176). The standard cycle sequencing thermo-cycling parameters were: denaturation for 5 minutes at 94°C, followed by 30 cycles of denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds, and extension/termination at 60°C for 4 minutes, followed by incubation at 4°C until the samples were processed. The reaction products were analyzed on the ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). DNA Sequence Analyses of DNA sequences were done with the Sequencher program (Gene Codes Corp., Ann Arbor, MI).

Allelic Discrimination

Real-time PCR allelic discrimination assays were developed by the assay-by-design service offered by Applied Biosystems. Probe and primer combinations were designed for the following SNPs: rs2284659, rs13306703, rs8192287, rs699473, 1617, rs2695231, rs17881426, rs800444, rs1007991, rs8192291, rs1799895, rs2695232, and rs2855262, which capture all haplotypes with a frequency greater than 2% in the European American population. PCR reactions were performed in a final volume of 25 μl, which consisted of 1 to 25 ng of DNA diluted in dH_2O, 12.5 μl of 2× Taqman Universal PCR Master Mix and 1.25 μl of 20× TaqMan SNP genotyping Assay Mix. PCR was performed using an Applied Biosystems 7300 Real-time PCR system.

Control Selection

Healthy European American volunteers, 19 to 89 years of age, were recruited to determine EC-SOD polymorphisms. The sample included 74 males (mean age, 32.5 ± 9.9 yr; range, 19–70 yr) and 105 females (mean age, 38.4 ± 10.9 yr; range, 22–71 yr). The study was approved by the Colorado Multiple Institutional Review Board, and each volunteer signed an informed consent document.

TABLE 1. EXTRACELLULAR SUPEROXIDE DISMUTASE PROMOTER AND GENE SEQUENCING PRIMERS

	Sequencing Primers
Promoter Forward 1	5′- CCT CAG TAG TTT GGA CCA CAA GCA - 3′
Promoter Reverse 1	5′- GGG TGA CTT AGT TAC CAG CAG GAG - 3′
Promoter Forward 2	5′- ATT TAC AAC TGG CAT TCC TG - 3′
Promoter Reverse 2	5′- AGC TAC TAG GAA GGC TAA GG - 3′
Forward 1	5′- TCC CTC TTA TCT CGC AGA ATG CCT G-3′
Reverse 1	5′-TTT GCT GCA TGG AAA TGG GCA C-3′
Forward 2	5′-TTG TAC ACC TCT CCA AAC AGG CGA-3′
Reverse 2	5′-TCT GCT TTC CTA AGG AGG TGG CTT-3′
Forward 3	5′-AGG AGA TAA AGG CTT GGT GCC TCT-3′
Reverse 3	5′-CTC TGC TGC TTA GAA AGT TCT CCC-3′
Forward 4	5′-AGG AGG AAG GTG TCT TTG ATG ATG GG-3′
Reverse 4	5′-TGA ATC TAA AGC CTG ACT CTG CCA C-3′
Forward 5	5′-TCT TTG GAG GCA GCA TCA ATC CCT-3′
Reverse 5	5′-ACC CAA GCA GGA AAT GAA GGC T-3′
Forward 6	5′-AAA CTC CTA CCC ATC TTG TGG AAC CC-3′
Reverse 6	5′-TGC TGT GGC TGT AAA GAT GGG TGA-3′
Forward 7	5′-ACG TGA CTA AGC CTC ACT CTG CC-3′
Reverse 7	5′-CGT TCC CGT TCT CCA CGC T-3′
Forward 8	5′-ACT CAG AGC GCA AGA AGC GG-3′
Reverse 8	5′- AAC CAA GAC CTC GAC GCA GT-3′

Population I: ARDS Network Patient Population

All patients included in this study had been enrolled in NIH ARDS Network studies and had received low tidal volume ventilation using previously published protocols (30). All patients were European American with infection-associated ALI, characterized by either systemic or local-

TABLE 2. SINGLE NUCLEOTIDE POLYMORPHISMS IDENTIFIED BY SEQUENCING THE EXTRACELLULAR SUPEROXIDE DISMUTASE PROMOTER AND GENE

Position*	Location	RS#	Base Change	MAF
−1735	Promoter	rs2239585	G → A	0.019
−1294	Promoter	new	A → G	0.010
−1257	Promoter	rs2284659	G → T	0.394
−541	Promoter	rs13306703	C → T	0.125
−86	Promoter	new	G → A	0.010
574	Exon 1	rs8192287	G → T	0.058
598	Exon 1	new	T → C	0.010
684	Intron 1	rs8192288	G → T	0.058
809	Intron 1	rs699473	T → C	0.385
1385	Intron 2	new	T → C	0.010
1617	Intron 2	new	A → C	0.058
1824	Intron 2	rs17878863	A → G	0.096
2054	Intron 2	new	C → T	0.010
2187	Intron 2	rs2695231	T → C	0.356
2203	Intron 2	rs2536511	G → A	0.356
2606	Intron 2	new	C → T	0.010
3203	Intron 2	new	G → A	0.010
3506	Intron 2	rs17881426	T → A	0.106
3576	Intron 2	new	T → C	0.010
3707	Intron 2	rs17880362	C → T	0.019
3869	Intron 2	rs800444	A → G	0.038
4691	Intron 2	rs1007991	G → C	0.317
4717	Intron 2	new	C → T	0.010
5321	Exon 3	rs2536512	A → G	0.356
5360	Exon 3	rs8192291	C → T	0.183
5955	Exon 3	rs2695232	T → C	0.327
5982	Exon 3	rs2855262	C → T	0.327
6418	Exon 3	rs8192290	T → C	0.038

Definition of abbreviations: RS = reference SNP; MAF = minor allele frequency.

* The promoter position includes base pairs upstream of exon 1 and the gene position includes base pairs starting at exon 1.

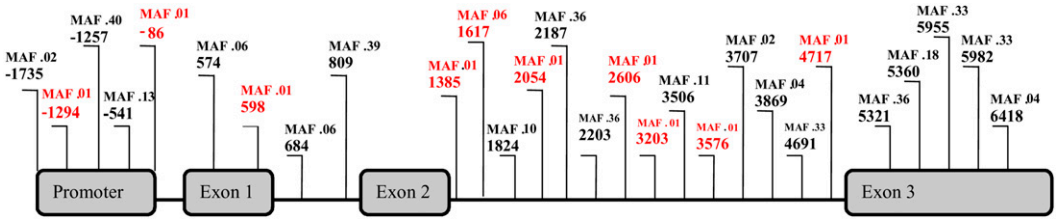

Figure 1. Location of single nucleotide polymorphisms (SNPs) in the extracellular superoxide dismutase promoter and gene identified by sequencing. *Red*: new SNPs not previously described. MAF = minor allele frequency.

ized pulmonary infection as the investigator-identified primary etiology for ALI. ALI was defined by standard criteria. Septic shock was defined as a systolic blood pressure of less than 90 mm Hg for at least 30 minutes despite fluid replacement or the use of inotropic support to maintain blood pressure. Exclusion criteria included the following: below 18 years of age, a neurologic condition that could impair weaning from mechanical ventilation, severe chronic respiratory disease, severe chronic liver disease (defined as a Child-Pugh score of >10), burns over more than 30% of total body surface area, malignancy or other irreversible condition for which the mortality at 6 months was estimated to be greater than 50%, use of high-dose immunosuppressive therapy, or a history of lung or bone marrow transplantation.

This genetic analysis study was approved by the University of Colorado Health Sciences Center Institutional Review Boards. Consent was obtained from all patients or their surrogates before enrollment as part of their acceptance into the National Institutes of Health protocols.

Population II: WaTTCH Patient Population

All patients with sepsis were obtained from the Canadian Waveform Abnormalities of activated partial thromboplastin time (aPTT) in Critically Ill Hospitalized Patients (WaTTCH) trial. This study was designed to evaluate the association between waveform abnormalities in aPTT and the subsequent development of sepsis, mortality, and duration of intensive care unit stay. A total of 597 patients were enrolled, of which 33% developed sepsis with acute organ failure, as defined above and in previous trials of novel interventions for sepsis. All patients were European Americans. Consent was obtained from all patients or their surrogates before enrollment as part of their enrollment into the WaTTCH study.

Statistical Analysis

Analysis for the haplotypes in EC-SOD cases and controls involved the use of SAS version 9.1 (SAS Institute Inc., Cary, NC) and Haploview version 4.0 (http://www.broad.mit.edu/node/443) (31) statistical packages. Haplotype frequencies were estimated using the expectation-maximization algorithm assuming Hardy-Weinberg equilibrium (HWE) (32–34). These estimates were used to assign probabilities to each individual for having a particular haplotype pair. Deviation from HWE was tested by χ^2 (35). Linkage disequilibrium (LD) assessed by D′ was used to determine the degree of allelic association between SNPs (31). Haplotype blocks were defined by the confidence interval method (36).

To evaluate association between dichotomous traits (case-control status, mortality [yes/no] and ventilator-free days [VFD] ⩽14 or >14) haplotype based hypothesis testing was conducted. An omnibus test was performed over all haplotypes using a likelihood ratio test (37). Haplotype frequencies were compared between groups by 2 × 2 contingency tables testing the frequency of each particular haplotype versus all other haplotypes using χ^2 statistics as well as calculating exact *P* values through permutation (37). In addition, haplotype trend and logistic regression models in which weights or posterior probabilities are assigned to haplotypes were used to test haplotypes as independent variables for the ARDSNet patient population (38). The logistic model also incorporated the covariates age, sex, hospital, pneumonia, and comorbidities (diabetes, cancer, immunosuppression, and AIDS). Exact

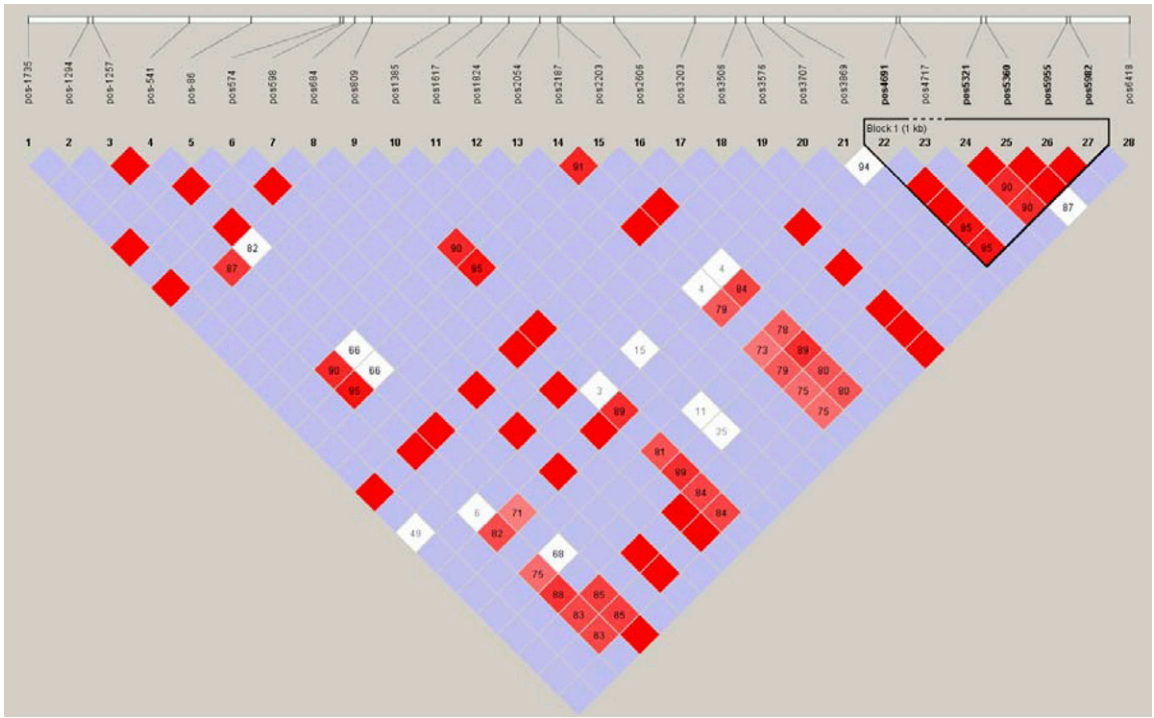

Figure 2. D′ pairwise linkage disequilibrium (LD) plot of 52 healthy European Americans. *Red*: D′ = 1 (LOD ⩾ 2.0); *blue*: D′ = 1 (LOD < 2.0); *pink*: D′ < 1 (LOD ⩾ 2.0); *white*: D′ < 1 (LOD < 2.0). LOD = logarithm of odds.

TABLE 3. CHARACTERISTICS OF ALI IN THE ARDS NETWORK PATIENT POPULATION

Characteristics	ARDSNet Patients (*n* = 251)
Age, yr	51.3 ± 15.6, (17–90)
Male, %	50.0
APACHE III, n	96.2 ± 29.7
Mechanical ventilation, %	100
Mortality, %	25.0
Male	26.0
Female	24.0
Source of infection, %	
Lung	55.0
Peritoneum	4.0
Vascular line	4.0
Skin	2.0
GI/biliary tract	2.0
Other	33.0

Definition of abbreviations: ALI = acute lung injury; APACHE III = acute physiology, and chronic health evaluation III.

Values are means ± SD, (range), or %.

logistic regression was used for the WaTTCH patient population. Survival curves were computed in cases to determine time-to-death by using the Kaplan-Meier and Cox's regression models. Odds ratio (OR) and 95% confidence intervals (CI) were used to determine the magnitude of significant associations ($P \leq 0.05$).

RESULTS

EC-SOD Sequencing

We identified a total of 28 SNPs within the EC-SOD gene and 2 kb promoter by sequencing, of which 10 were not previously described (Table 2). The allele frequency of all new SNPs identified were approximately 1% in the population with the exception of SNP 1617 at 6%. Figure 1 shows the position the SNP occurs within the promoter and gene.

Pairwise LD across the EC-SOD gene was relatively strong in the European American population (Figure 2). One haplotype block was defined in the 3′ end of the EC-SOD gene. This block consisted of 5 SNPs: 4691-5321-5360-5955-5982. Sequencing the EC-SOD promoter and gene gave us information regarding haplotype structure and tagging SNPs. Thus, to capture greater than 2% haplotypes in the control and patient populations, 13 SNPs were genotyped to assess whether a particular haplotype was associated with case-control or outcome in patients with sepsis. These included: −1257, −541, 574, 809, 1617, 2187, 3506, 3869, 4691, 5360, 5840, 5955, and 5982.

ARDS Network Patient Population

Characteristics of the patient population. A total of 252 European American patients with infection-associated ALI were available for analysis (Table 3). The primary source of infection was the lungs, involving 55% of these patients. Men and women were equally represented in the patients with ALI. An additional 179 healthy volunteers were recruited for comparison. Table 4 shows the comparisons between the GCCT haplotype and all other haplotypes.

TABLE 4. COMPARISONS BETWEEN THE GCCT HAPLOTYPE AND ALL OTHER HAPLOTYPES IN THE ARDS NETWORK PATIENT POPULATION

EC-SOD Haplotype	All Other Haplotypes	G-C-C-T
N	232 (92)	20 (8)
Age, yr	51.7 ± 15.3	45.8 ± 17.8
APACHE III	96.1 ± 29.6	97.2 ± 25.8
60-D mortality	63 (27)	1 (5)
VFD	17 (0–28)	22 (4–26)
Shock	114 (49%)	9 (45%)

Definition of abbreviations: APACHE III = acute physiology, and chronic health evaluation III; EC-SOD = extracellular superoxide dismutase; VFD = ventilator-free days.

Values are n (%), mean ± SD, or median (range).

Comparison of EC-SOD haplotypes in healthy control and ALI populations and outcomes in patients. Blocks were identified by the confidence interval method proposed by Gaberial and colleagues (36). Two blocks were defined and consisted of block 1: −1257, −541, 574, 809, 2187, and 3506 and block 2: 4691, 5360, 5955, and 5982. LD plot of block 2 in the ARDS network patient population is shown in Figure 3. These two blocks were analyzed for haplotype association between cases and controls and outcomes in cases. HWE was tested using the approaches described by Wigginton and colleagues (35) as implemented in Haploview. When tested on cases and controls, all SNPs in blocks 1 and 2 were in HWE.

We first examined the relationship between haplotype frequencies in block 1 and block 2 with either being a case or control. There was a significant association between cases and controls in block 2 and haplotype frequency (Table 5). In particular, CTCT haplotype frequency was shown to be significantly increased (OR, 2.04; 95% CI, 1.12–3.71) in cases, after controlling for age and sex. There were no significant associations in block 1.

VFD is the measure of the time that a patient requires mechanical ventilation, with fewer VFD indicating more time on the ventilator. Patients with the GCCT haplotype had a reduced risk (OR, 0.25; 95% CI, 0.05–0.77) of being on the ventilator when compared with all other haplotypes after adjusting for age and diabetes (Table 5). No significant differences were found in the logistic model when the covariates of sex, site of infection, pneumonia, hospital, cancer, immunosuppressives, and AIDS were evaluated. Additionally, there was no significant difference between haplotypes in block 1 and time on the ventilator.

We next examined the relationship between haplotypes and 60-day mortality. There was a significant association between the GCCT haplotype in block 2 and mortality after adjusting for age and immunosuppression (Table 5). In particular, patients with this haplotype had a reduced risk (OR, 0.153; 95% CI, 0.008–0.773) of dying from infection-induced ALI. Kaplan-Meier survival analysis supported this finding that patients with the GCCT haplotype had a reduced risk of mortality when compared with all other haplotypes. After adjusting for age, using a Cox regression analysis, patients with a GCCT haplotype had a significant ($P = 0.03$) reduction in risk of death when compared with all other haplotypes. Additionally, there was no significant difference between haplotypes in block 1 and mortality.

WaTTCH Patient Population

Characteristics of the patient population. A total of 157 European American patients with infection-associated ALI were available for analysis (Table 6). This patient population was evaluated to validate the findings found in the ARDS network patient population for block 2. The LD plot of block 2 in the WaTTCH network patient population is shown in Figure 4. The primary source of infection was the lungs, involving 31% of these patients. There were more men (58%) in this patient population. The 179 healthy volunteer subjects, as described above, were also used to compare haplotype frequencies between cases and control subjects. Table 7 shows the comparisons between the GCCT haplotype and all other haplotypes.

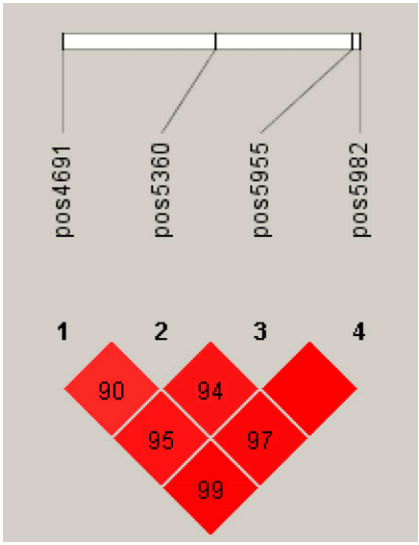

Figure 3. Linkage disequilibrium plot (block 2) ARDS net patient population.

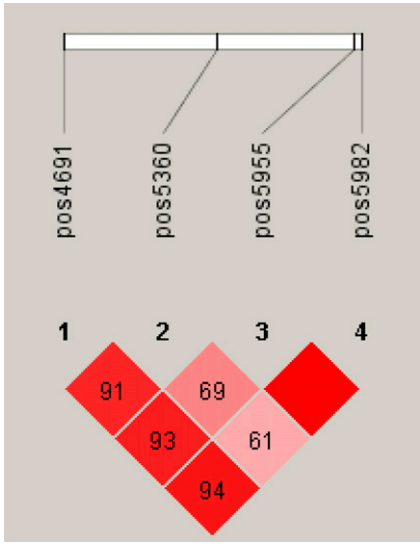

Figure 4. Linkage disequilibrium plot (block 2) WaTTCH patient population.

Comparison of EC-SOD haplotypes in healthy control and ALI populations and outcomes in patients. We first determined the relationship between haplotype frequencies in block 2 between cases and controls. There was a significant association (P = 0.05) with the GCCT haplotype between cases and controls. However, when adjusting for sex, the haplotype association was no longer significant.

VFD ($\leq$14 or $>$14) was next examined in the WaTTCH patient population by the methods proposed by Zhao and colleagues (37) and exact logistic regression. There was a reduced risk of time on the ventilator with the GCCT haplotype. However, use of exact logistic regression showed a trend with the GCCT haplotype and reduced risk of time on the ventilator. Multivariate logistic regression analysis was not performed because of the low frequency of the haplotype in this patient population.

Finally, we assessed haplotypes in block 2 with mortality. There was a significant relationship between the GCCT haplotype with 28-day mortality (Table 8). Patients with the GCCT haplotype were shown to have a reduced risk of 28-day mortality. Use of exact logistic regression showed a trend with the GCCT haplotype and a reduced risk of 28-day mortality. When time to death was evaluated by Kaplan-Meier survival, a trend was found with respect to the GCCT haplotype and survival (Figure 5B). Cox regression analysis showed a trend in a reduced risk of mortality (P = 0.10) in patients with a GCCT haplotype when compared with all other haplotypes. Again, multivariate logistic regression analysis was not performed because of the low frequency of the haplotype in this patient population.

TABLE 5. HAPLOTYPE ALLELE FREQUENCIES IN CONTROL SUBJECTS AND PATIENTS WITH INFECTION-INDUCED ALI

	ARDS Network Population								
Block 2	Case Control			Ventilator-free Days			28-Day Mortality		
Haplotype*	Case	Control	*P* Value	⩽14	>14	*P* Value	Dead	Alive	*P* Value
G-C-T-C	0.625	0.616	NS	0.619	0.629	NS	0.627	0.623	NS
C-C-C-T	0.175	0.210	NS	0.195	0.159	NS	0.167	0.177	NS
C-T-C-T	0.142	0.066	0.02	0.167	0.125	NS	0.190	0.126	0.09
G-C-C-T	0.034	0.037	NS	0.009	0.061	0.01	0.008	0.050	0.02

Definition of abbreviations: ARDS = acute respiratory distress syndrome; ALI = acute lung injury.

* Extracellular superoxide dismutase block 2 haplotype consists of 4691-5360-5955-5982. Logistic regression models, in which weights or posterior probabilities are assigned to haplotypes, were used to test haplotypes as independent variables (38).

DISCUSSION

We sequenced the EC-SOD gene to characterize genetic variation within the promoter and gene as well as to assess LD patterns across the genes in the European American population. To our knowledge, the determination of genetic variation involving the entire 2-kb promoter and gene has not been previously investigated. Sequencing of the promoter and gene resulted in the identification of a total of 28 SNPs, of which 10 have not been previously described. There were a total of 16 (57%) intron spanning SNPs, 7 (25%) exon SNPs, and 5 (18%) promoter SNPs.

Previously, the EC-SOD gene was isolated and characterized, which led to the identification of many unique transcriptional regulatory elements (39). Many of these transcriptional regulatory elements are situated within the promoter region of the EC-SOD gene and include the metal regulatory element, the cAMP-responsive element, the AP-1 binding site, and a glucocorticoid response element (39). The promoter SNPs identified in this study are located upstream of these regulatory elements. Of note, SNP 574 (rs8192287) and SNP 598 (new), both 5′ UTR SNPs, are located 45 bp and 19 bp, respectively, upstream of the EC-SOD transcription start site. Additionally, a CAAT box element is located antisense at position 598 where a T→C transition was identified in 1% of the population. A total of five SNPs were identified in exon 3 where the entire 720-bp coding region is located. The 5321 (rs2536512) SNP is a nonsynonymous SNP and results in an amino acid change Thr→Ala at amino acid position 58. This SNP has a potential of altering protein function. Another SNP (synonymous SNP) in the coding region was identified at position 5360 (rs8192291) resulting in a C→T. The remaining three SNPs in exon 3 were located in the 3′ UTR. Whether genetic variation in the promoter, 5′ UTR, or 3′ UTR influences levels of EC-SOD gene expression, remains to be determined.

Assessing patterns of LD is important in mapping genes that may have a causative role in many diseases. In this study, we showed that pairwise LD across the EC-SOD gene in the European American population was relatively strong, indicating

TABLE 6. CHARACTERISTICS OF ALI IN THE WaTTCH PATIENT POPULATION

Characteristics	WaTTCH Patients (*n = 157*)
Age, yr	64.5 ± 13.5, (19–89)
Male	58.0
APACHE II	19.7 ± 7.5
Mechanical ventilation	94
Mortality	25.0
Male	25.0
Female	25.0
Source of infection	
Lung	31.0
Peritoneum	4.0
Vascular line	1.0
Skin	3.0
GI/biliary tract	0.0
Other	57.0

Definition of abbreviations: ALI = acute lung injury; APACHE III = acute physiology, and chronic health evaluation III; WaTTCH = waveform abnormalities of aPTT in critically ill hospitalized patients.

Values are mean ± SD, (range), or %.

that this gene is somewhat conserved in this population. However, although pairwise D′ is near 1 for many of the alleles, the CI for these estimates are large, making it difficult to assess the amount of historical recombination within this gene in this population. There was one clear block (pairwise $D' > 0.90$) identified by the CI method proposed by Gabriel and colleagues (36) and consisted of 4691-5321-5360-5955-5982.

In this study, we used a block approach to determine associations between cases and controls and outcomes in cases. A significant relationship was demonstrated between the GCCT haplotype in block 2 and time on the ventilator and mortality in two separate patient populations with infection-associated ALI. These findings indicate that the EC-SOD haplotype GCCT may have a protective effect in reducing time on the ventilator and mortality in the setting of infection induced ALI. Kaplan-Meier survival curves supported the findings that this haplotype is protective against mortality in both the ARDSNet (n = 20 [5% mortality]) and WaTTCH (n = 4 [0% mortality]) patient populations. There were no significant associations with outcome in patients with infection-induced ALI or case-control status in block 1.

High levels of EC-SOD are produced in the lung where it is postulated to play a very important role in controlling inflammation through scavenging superoxide free radicals (14, 15). Studies using animal models have demonstrated that levels of EC-SOD influence the degree of inflammatory cells in the lung and the production of many proinflammatory mediators. In particular, mice lacking EC-SOD have a significant increase in lung inflammation in models of hemorrhagic shock (19, 20) and endotoxemia (21), whereas overexpression of lung EC-SOD attenuates lung inflammation in these models. These studies show that lung EC-SOD levels are of critical importance in controlling inflammation in acute mouse models of lung injury. The result that the GCCT haplotype may be protective against ALI and mortality, suggests that patients with this particular haplotype may have higher levels of EC-SOD in the lung, resulting in a decrease in lung inflammation.

TABLE 7. COMPARISONS BETWEEN THE GCCT HAPLOTYPE AND ALL OTHER HAPLOTYPES IN THE WaTTCH PATIENT POPULATION

EC-SOD Haplotype	All Other Haplotypes	G-C-C-T
N	154 (97)	4 (3)
Age, yr	64.6 ± 13.6	65 ± 19.5
APACHE II	19.8 ± 7.5	16.3 ± 10.2
60-d mortality	39 (25)	0 (0)
VFD	23 (0–28)	20 (14–23)
Shock	80 (52)	4 (75)

Definition of abbreviations: APACHE III = acute physiology, and chronic health evaluation III; EC-SOD = extracellular superoxide dismutase; VFD = ventilator-free days; WaTTCH = waveform abnormalities of aPTT in critically ill hospitalized patients.

Values are n (%), mean ± SD, or median (range).

TABLE 8. HAPLOTYPE ALLELE FREQUENCIES IN CONTROL SUBJECTS AND PATIENTS WITH INFECTION-INDUCED ALI

	WaTTCH Patient Population								
	Case Control			Ventilator-free Days			28-Day Mortality		
Block 2 Haplotype*	Case	Control	*P* Value	⩽14	>14	*P* Value	Dead	Alive	*P* Value
G-C-T-C	0.670	0.616	NS	0.685	0.634	NS	0.696	0.587	0.08
C-C-C-T	0.184	0.210	NS	0.181	0.193	NS	0.172	0.225	NS
C-T-C-T	0.083	0.066	NS	0.082	0.081	NS	0.079	0.088	NS
G-C-C-T	0.010	0.037	NS	0.005	0.024	0.02	0.004	0.029	0.01

Definition of abbreviations: ALI = acute lung injury; WaTTCH = waveform abnormalities of aPTT in critically ill hospitalized patients.

* Extracellular superoxide dismutase block 2 haplotype consists of 4691-5360-5955-5982. Haplotype frequencies were compared between groups by 2 × 2 contingency tables testing the frequency of each particular haplotype versus all other haplotypes using χ^2 statistics as well as calculating exact *P* values through permutation (37).

There are several possibilities for the biological effect in which the GCCT haplotype may be protective in patients with infection-induced ALI. First, this haplotype block is in very strong LD with several promoter SNPs; therefore, it is plausible that a protective allele in the promoter may lead to increased production of EC-SOD. In particular, this block is in strong LD with the two promoter SNPs −1257 and −541. In addition, block 2 is in strong LD with the 5′ UTR SNP at position 574 in exon 1, which is located 45 bp upstream of the transcription start site. Second, individuals with this haplotype may have an increase in mRNA stability leading to more EC-SOD being translated to protein. Two of the SNPs, 5955 and 5982, are within the 3′ UTR, and the combination of these two alleles may provide greater stability to the mRNA. Finally, the nonsynonymous SNP at position 5321, which results in a Thr→Ala at amino acid position 58, is a haplotype tag SNP with 4691, 5955, and 5982. Because this SNP is within this block and in strong LD, the amino acid change may alter EC-SOD function. However, with these results, it is somewhat difficult to determine the possible mechanism in which this haplotype may be protective. More functional studies are warranted to determine the biological effect of this haplotype and to further identify the causative allele(s) that led to the positive association in the two patient populations.

Individuals with a R213G SNP in exon 3 have been shown to have 8 to 10 times greater EC-SOD plasma levels than individuals lacking this genetic mutation (28). The amino acid change from arginine to glycine in the extracellular matrix-binding region alters protein function by disrupting the binding of the protein to extracellular matrix, resulting in higher plasma levels (28). Clinically, it has been shown that individuals with the R213G SNP had a reduced risk of developing COPD (29). Given the clinical importance of this SNP in lung disease and the association with high plasma levels of EC-SOD, we evaluated this SNP in the ARDS network population and did not see an association with time on the ventilator or mortality (data not shown). In addition, pairwise LD with block 2 showed a lack of

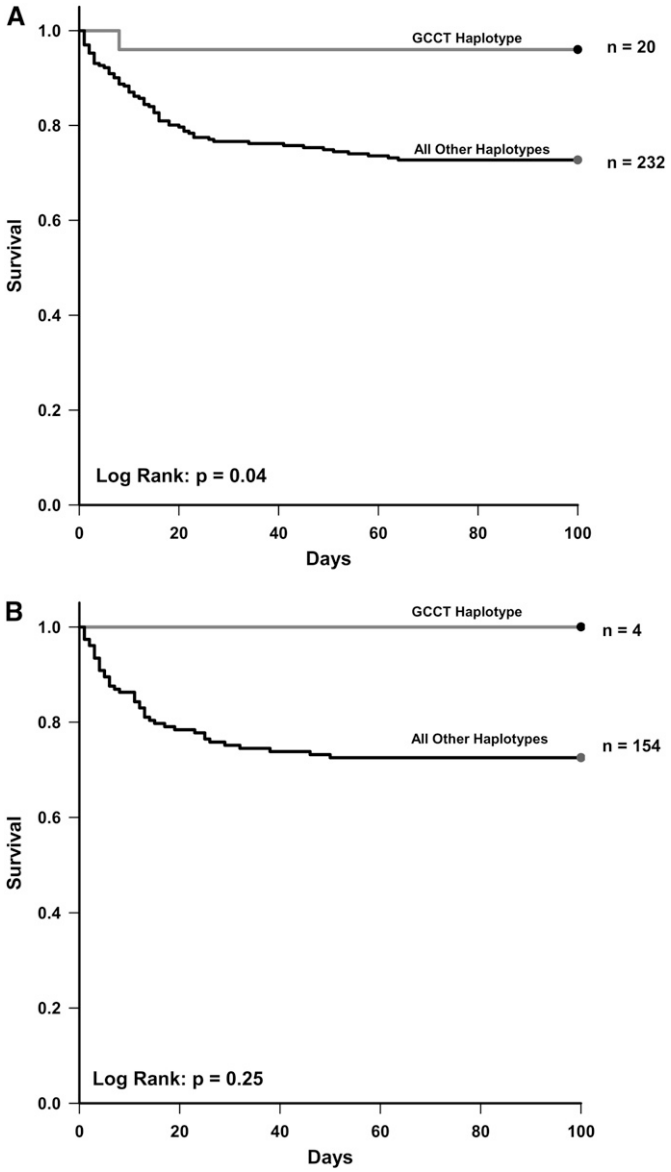

Figure 5. Kaplan-Meier survival analysis by extracellular superoxide dismutase promoter haplotype. Patients with the GCCT haplotype had a reduced risk of mortality in (*A*) the ARDS network patient population and (*B*) WaTTCH patient populations when compared with all other haplotypes.

association with the R213G and 4691-5360-5955-5982 with an average D' of 12. This suggests that the association found with the GCCT haplotype in ARDS and WaTTCH patient populations with time on the ventilator and mortality are not the result of the R213G SNP.

In this study, we sequenced the EC-SOD promoter and gene in a European American population to determine genetic variation and to assess LD patterns. Sequencing resulted in the identification of 28 SNPs with relatively strong LD and 1 block consisting of 4691-5321-5360-5955-5982. This specific block was shown to influence outcomes in two separate patient populations with infection-associated ALI. In particular, the GCCT (4691-5360-5955-5982) haplotype was shown to reduce the risk of time on the ventilator and mortality. Because EC-SOD plays a critical role in modulating the inflammatory response in the lung, these results suggest that patients with this specific haplotype may have reduced inflammation in the lung, which ultimately protects them from infection-induced ALI–associated time on the ventilator and death. However, it is important that additional functional studies, as well as larger association studies, be completed to validate these findings.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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