Functional polymorphisms in the transcription factor *NRF2* in humans increase the risk of acute lung injury

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ABSTRACT **We recently used positional cloning to identify the transcription factor *Nrf2* (NF-E2 related factor 2) as a susceptibility gene in a murine model of oxidant-induced acute lung injury (ALI). NRF2 binds to antioxidant response elements (ARE) and up-regulates protective detoxifying enzymes in response to oxidative stress. This led us to investigate *NRF2* as a candidate susceptibility gene for risk of development of ALI in humans. We identified multiple single nucleotide polymorphisms (SNPs) by resequencing *NRF2* in ethnically diverse subjects, and one (−617 C/A) significantly ($P<0.001$) diminished luciferase activity of promoter constructs containing the SNP and significantly decreased the binding affinity ($P<0.001$) relative to the wild type at this locus (−617 CC). In a nested case-control study, patients with the −617 A SNP had a significantly higher risk for developing ALI after major trauma (OR 6.44; 95% CI 1.34, 30.8; $P=0.021$) relative to patients with the wild type (−617 CC). This translational investigation provides novel insight into the molecular mechanisms of susceptibility to ALI and may help to identify patients who are predisposed to develop ALI under at risk conditions, such as trauma and sepsis. Furthermore, these findings may have important implications in other oxidative stress related illnesses.—Marzec, J. M., Christie, J. D., Reddy, S. P., Jedlicka, A. E., Vuong, H., Lanken, P. N., Aplenc, R., Yamamoto, T., Yamamoto, M., Cho, H.-Y., Kleeberger, S. R. Functional polymorphisms in the transcription factor *NRF2* in humans increase the risk of acute lung injury. *FASEB J.* 21, 2237–2246 (2007)**

Key Words: antioxidant · reactive oxygen species · ALI · acute respiratory distress syndrome · trauma · oxidative stress · translational

Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), affect up to 150,000 patients annually in the United States alone (1). ALI is characterized by pulmonary edema and inflammation with resultant severe systemic hypoxemia. It is hypothesized that oxidant stress due to excessive reactive oxygen species (ROS) is a major contributor in the pathogenesis of ALI (2, 3). ROS can be generated by infiltrating inflammatory cells (4) or residential pulmonary cells, including endothelium (5), and can cause cellular damage by oxidizing nucleic acids, proteins, and membrane lipids (6). Cellular antioxidants (*e.g.*, glutathione, catalase, and superoxide dismutase) and antioxidant vitamins (*e.g.*, ascorbic acid and tocopherols) protect tissues against oxidative tissue damage. Imbalance between oxidant load and antioxidant defense enhances tissue susceptibility to oxidative stress.

The varied incidence of ALI in specific patient groups suggests that genetic factors may have a role in modulating development of this complex disorder (7–9). Our positional cloning studies in inbred mice (10, 11) identified *Nrf2* (NF-E2 related factor 2) as a candidate susceptibility gene for hyperoxia-induced lung inflammation and injury, a model of ALI. NRF2 is a "cap'n'collar" basic leucine zipper (CNC-bzip) transcription factor associated with Kelch-like ECH associating protein 1 (KEAP1) and is predominantly localized in the cytoplasm of unstressed cells (12). Oxidative stress disrupts sequestration of NRF2 by KEAP1 and permits NRF2 translocation to the nucleus (13). NRF2 then dimerizes with small Maf or the AP-1 family of proteins and binds to the consensus sequence 5′GTG-

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doi: 10.1096/fj.06-7759com

ACNNNGC-3′ [known as antioxidant response element (ARE)] to induce transcription of ARE-bearing detoxifying enzymes such as NAD(P)H:quinone oxidoreductase, certain glutathione-*S*-transferases, and γ-glutamate cysteine ligase regulatory subunit, as well as classical antioxidant enzymes (*e.g.*, catalase and superoxide dismutase), and heme oxygenase-1 (14, 15). Therefore, NRF2 appears to be an essential regulatory element in response to oxidant injury.

The primary objective of this study was to determine whether *NRF2* is an important determinant of susceptibility to ALI. To address this objective, we first identified novel single nucleotide polymorphisms (SNPs) in human *NRF2*; we then tested gene regulation and protein function of these novel SNPs; finally, we tested the association of functionally important SNPs with differential risk for ALI in patients after major trauma (see **Fig. 1**). This translational investigation is the first to test whether a functionally relevant candidate gene for susceptibility to ALI that was identified by positional cloning in mice is associated with susceptibility to ALI in human subjects.

MATERIALS AND METHODS

Polymorphism identification

NRF2 is located on chromosome 2 (2q31) and has five exons and four introns. We screened 16 mRNA samples (2 from tissue, 3 from human tracheobronchial epithelial cell lines, and 11 from blood donors) for SNPs in the coding region of *NRF2* without regard to lung injury status. We also screened commercially available DNA samples (n=40; Coriell Institute, Camden, NJ, USA) for SNPs in the promoter region of *NRF2*. Forty subjects were chosen to provide a detection rate of 98% for SNPs present in as little as 5% of the population (16). In addition, as these 40 samples included 10 subjects each from different ethnic backgrounds (10 each of European, African, Asian, and Native American descent), we had a detection rate of 90% for SNPs present in as little as 10% of each ethnic background (16). We designed primers (Sigma Genosys, The Woodlands, TX, USA) to span the entire coding region and the proximal 1 kilobases (kb) of the *NRF2* promoter. Nonsynonomous coding region polymorphisms alter amino acids in the translated protein and can have deleterious effects on function, conformation, and activity. Proximal promoter SNPs in regulatory regions and enhancer elements can affect transcription efficiency of the message, generating varying levels of mRNA and protein, and can impact critical signal transduction pathways within the cell or tissue. Sequence results were aligned and verified by overlapping reads using additional internal primers (Sigma Genosys).

Genotyping for –617 and –651 SNPs

We sequenced the –738 to –461 region of the *NRF2* promoter to detect the two polymorphisms we found to be functionally relevant using our reporter gene assays (see below). For adequate coverage of the polymorphic region, primers were designed at least 50 to 100 basepairs (bp) away from the SNP site. To obtain a GC clamp for robust sequencing, we amplified the region from −738 to −461. We amplified the site from 100 ng of genomic DNA using the Epicenter Failsafe system (Madison, WI, USA), purified products with the GenElute PCR cleanup kit (Sigma Chemical, St. Louis, MO, USA), and performed sequencing reactions with dGTP dye terminator kits (Applied Biosystems, Foster City, CA, USA). Purified products were analyzed on an ABI 377 Automated DNA Sequencer (Applied Biosystems). Primer sequences and PCR conditions are listed in **Table 1**.

Reporter gene assays

Reporter gene assays are used to determine effects of promoter mutations on transcriptional activity of the adjacent gene. The pGL3 basic reporter vector contains a genetically engineered firefly luciferase gene lacking a promoter. When promoter regions are cloned into the pGL3 vector upstream of the luciferase gene, there is strong transcriptional activation and expression of luciferase. Mutations (*e.g.*, insertions/deletions and SNPs) in the promoter of the target gene can influence transcriptional activity of the luciferase reporter gene that is detected fluorometrically *vs.* an internal control. We amplified the 5′-flanking region of genomic *NRF2* to generate two constructs [−727 to +131 bp, (727-Luc) and –538 to +131bp (538-Luc, lacking the SNPs)] and three polymorphic variants of the 727-Luc sequence and cloned products upstream of the luciferase reporter gene of pGL3 (Promega, Madison, WI, USA). To determine whether or not the SNPs influenced the message levels, the −727 to +131 region of *NRF2* containing the individual SNPs was cloned

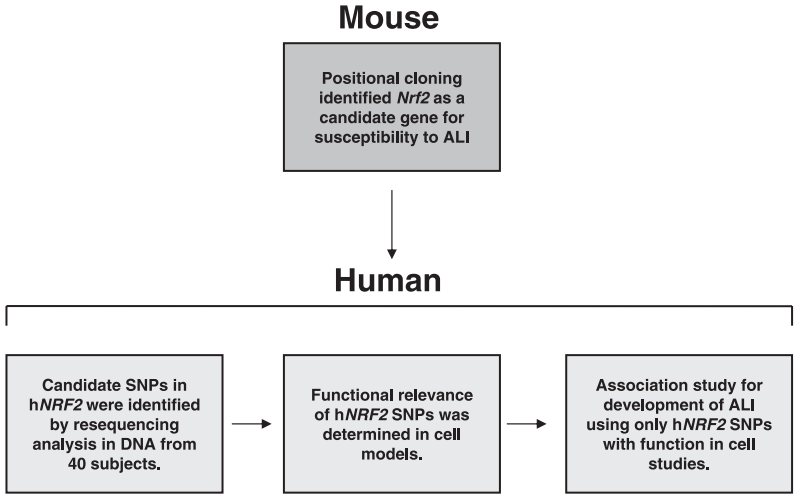

Figure 1. Translational study design. Schematic of translational study design that identified *NRF2* as a candidate susceptibility gene for ALI in mouse and human populations.

TABLE 1. *NRF2 primers for promoter and coding region polymorphisms, as well as EMSA primers*

	Primer	Amplified Size (bp)	Annealing Temp (°C)
Promoter Region			
−1058 to −464	5′-AGC ATA CTT GGA AGT AAC AAG GAG A-3′	593	57
	5′-CTT TTA TCT CAC TTT ACC GCC C-3′		
−741 to −318	5′-GAC CAC TCT CCG ACC TAA AGG-3′	423	59
	5′-CGA GAT AAA GAG TTG TTT GCG AA-3′		
−383 to +116	5′-CCA ACT GTT TAA ACT GTT TCA AAG C-3′	498	58
	5′-AGG CAG CTC CAA GTC CAT C-3′		
Coding Region			
16 to 1341	5′-CCG GGA CTC CCG TCC CAG CA-3′	1325	58
	5′-CAA GCG GCT TGA ATG TTT GT-3′		
367 (internal)	5′-ACA TTC CCG TTT GTA GAT GA-3′		
1268 to 2135	5′-CCA GAG AAA GAA TTG CCT GTA-3′	867	58
	5′-TTA GCC AGA TGT CAT ATC-3′		
1556 (internal)	5′-TAG TAG AAC TAG AGC AAG AT-3′		
ESMA Oligos			
−617F wt 26 bp	5′-CAC GAG CTG CCG GCG CTG TCC ACA TC-3′	26	95
−617R wt 26 bp	5′-GAT GTG GAC AGC GCC GGC AGC TCG TG -3′		
−617F var 26 bp	5′-CAC GAG CTG CCG G**A**G CTG TCC ACA TC-3′	26	95
−617R var 26 bp	5′-GAT GTG GAC AGC **T**CC GGC AGC TCG TG -3′		
−617F wt 62 bp	5′-TGG GAG TTC AGA GGA GGG CGT TCA GGG TGA CTG CGA ACA CGA GCT GCC GGC GCT GTC CAC AT-3′	62	95
−617R wt 62 bp	5′-ATG TGG ACA GCG CCG GCA GCT CGT GTT CGC AGT CAC CCT GAA CGC CCT CCT CTG AAC TCC CA -3′		
−617R var 62 bp	5′-ATG TGG ACA GC**T** CCG GCA GCT CGT GTT CGC AGT CAC CCT GAA CGC CCT CCT CTG AAC TCC CA -3′		

F = 5′ forward primer; R = 3′ reverse primer; wt = wild type; var = variant; oligo = oligonucleotide; bp = base pair. Note: overlapping primer sets in the promoter, and internal primers in the coding region were used to verify initial sequencing results (see Materials and Methods for details).

upstream of a reporter gene, luciferase, and its expression was analyzed by transient transfection using reporter assays. The role of the −727 to –538 promoter region, which contains the two functionally relevant SNPs, was investigated by generating two constructs and comparing their promoter activity. Constructs were verified by sequencing and transiently transfected into A549 cells (American Type Culture Collection, Manassas, VA, USA) as described previously (17). Luciferase activity was assessed in three independent samples, and all experiments were repeated in triplicate. We tested for differences between groups by one-way ANOVA with Student-Newman-Keuls (SNK) *a posteriori* comparisons of means (Sigma Stat, SPSS Science, Inc., Chicago, IL, USA). A two-sided P value of <0.05 was considered statistically significant.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) allows visualization of transcription factor binding to promoter regulatory sequences and is used to assess effects of mutations at regulatory regions of genes. Radioactively labeled promoter DNA sequences are incubated with the nuclear proteins isolated from mammalian cells yielding DNA:protein complexes. Migration of the labeled probe on a polyacrylamide gel will be retarded or shifted due to the binding of a protein(s). Competition with the unlabeled probe or mutations or deletions in the DNA sequence of the probe will diminish or abolish the binding. The specificity of the shifted bands may be further confirmed using antibodies specific to the protein of interest. In the present study, an aliquot (5 μg) of human lung nuclear protein (Active Motif, Carlsbad, CA, USA) was incubated on ice with binding buffer for 15 min, followed by addition of 3×10^4 cpm [γ^{32}P]ATP (Amersham Biosciences, Piscataway, NJ, USA) end-labeled wild-type or variant probes (Table 1) and incubated for 30 min at room temperature. We used short (26 bp) and long (62 bp) fragment sequences for the EMSA experiments. The short fragment overlaps only the consensus sequence for NRF2, whereas the long fragment overlaps potential binding sites for AP-1, myeloid zinc finger-1 MZF1, and NRF2. The binding of transcription factors to their cognate sites is also influenced by the presence of flanking DNA sequences. Thus, to assess the influence of flanking MZF1 and AP-1 sites on the binding of NRF2 at the ARE-like element, we performed EMSA assays using a fragment containing all three sites. The protein binding of this fragment was compared with that of a shorter fragment, which contains only the ARE-like site. A higher degree of specificity is conferred with shorter oligonucleotide fragments, since there is a lower likelihood of nonspecific hybridization. To analyze specific binding of NRF2 at the –617 polymorphic site, nuclear proteins (5 μg) were preincubated with polyclonal rabbit anti-NRF2 antiserum (2 μl; ref 15) or control serum (2 μl) and processed for EMSA as described above. Samples were electrophoresed and autoradiographed with an intensifying screen at –70°C. We tested for statistically significant differences in DNA-NRF2-antibody complex formation between wild-type, heterozygous, and variant genotypes using one-way ANOVA with SNK *a posteriori* comparisons of means (Sigma Stat).

Association of SNPs with ALI in human subjects

Study participants

With the goal of testing the association of only the functional SNPs with development of ALI, we conducted a nested case

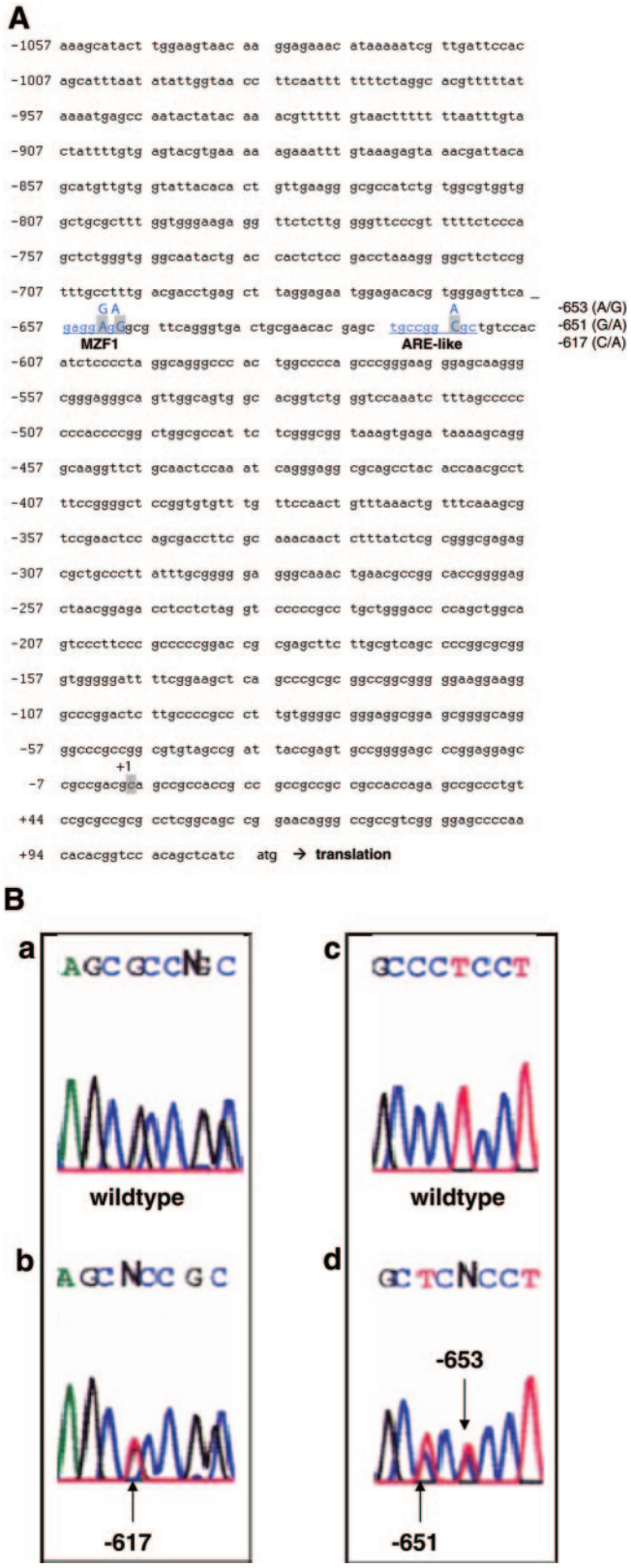

Figure 2. Proximal 1 kilobases (kb) of the *NRF2* promoter and identification of −617, −651, and −653 polymorphisms. *A)* Proximal 1 kb of the human NRF2 promoter sequence (wild-type sequence shown). Three novel polymorphisms at positions −653(A/G), −651(G/A), and −617(C/A) are indicated in bold. Putative binding sites for MZF1 (AGAGGAGG) and NRF2 (TGCCGGCGC) are underlined, transcriptional start site is highlighted, and ATG start codon is in bold. *B)* Representative electropherograms of *NRF2* promoter region between −656 to −614 (reverse strand shown). *a)* −617 GG; *b)* −617 GT; *c)* −651 CC and −653 TT; *d)* −651 CT and −653 TC.

control study of subjects with major trauma. These human data are part of a prospective cohort study to determine the biological risk factors and biomarkers for development of ALI after major trauma. Subjects in this trauma cohort study met the following inclusion criteria: *1)* they were admitted to a surgical intensive care unit (SICU) as a result of acute trauma directly from the hospital's emergency department; and *2)* they had an injury severity score (ISS) ≥ 16 as calculated on the basis of information available during their first 24 h of hospitalization (18). Exclusion criteria were death or discharge from the SICU in the first 24 h, age <14 yr, current or prior congestive heart failure or recent myocardial infarction, severe chronic respiratory disease, morbid obesity, burns over body surface area of 30% or more, lung transplantation, and bone marrow transplantation. The subjects for this study were recruited, and data were collected between July 1, 1999 and July 1, 2001. During this time period, there were 711 subjects screened, of which 164 were eligible for the study. The vast majority of exclusions (>98%) were due to subjects not having an ISS of >16. Patients were followed for 28 days.

To qualify as an ALI case, subjects had to meet all ALI criteria within the first 5 days after major trauma. We used the standard criteria of the American European Consensus Conference (AECC) definition while tracheally intubated and receiving assisted ventilation: acute onset; bilateral pulmonary infiltrates on chest radiograph consistent with pulmonary edema; no evidence of left atrial hypertension; and a ratio of arterial oxygen tension to fraction of inspired oxygen (PaO_2/FiO_2) ≤300 (19). Study subjects were screened daily from the time of admission, and physician investigators reviewed the arterial blood gases and chest radiographs for classification of ALI. Although this cohort study was designed with ALI as the outcome variable, 86% of subjects with ALI also met AECC criteria for ARDS (PaO_2/FiO_2≤200) (19) at the time of identification, and >97% of ALI subjects met this criterion for ARDS during the first 5 days after a traumatic event. Thus, in this study population, the term "ALI" also describes subjects with ARDS.

Trained study coordinators prospectively collected all clinical data using standardized research case report forms designed for the trauma cohort study without knowledge of genotype. We collected blood samples from residual EDTA blood samples otherwise drawn for clinical purposes at the time of admission to the emergency department. Samples were centrifuged, and we extracted genomic DNA from thawed buffy coat aliquots using the Qiagen Qiamp 96 DNA blood kit (Qiagen, Valencia, CA, USA). Negative controls were included in all DNA extraction runs. The institutional review board of the University of Pennsylvania approved the study protocol. A waiver of consent was granted for the following reasons: *1)* the study would be impossible to do with consent; *2)* the use of otherwise discarded materials constitutes a minimal risk study; *3)* the only potential risk to participants was potential loss of confidentiality, and this was protected by encryption and removing all possible patient identifiers from the sample; *4)* at the time of publication there were no clearly established disease associations with our candidate gene; and *5)* we had neither the ability nor the intent to contact participants in the event that *NRF2* (or any other gene) became clearly associated with human disease in the future.

We selected 30 subjects with ALI as cases (the first 15 sequentially accrued subjects within each of 2 ancestry strata: African descent and European descent). Two controls (*i.e.*,

subjects who did not develop ALI but met the same inclusion and exclusion criteria for enrollment in the trauma cohort) for each case were matched on ancestry and ISS stratum (16–19, 20–29, 30–39, 40 and above). Thus half of the matched sets in the study were African descent and half were European descent (each with 15 cases and their 30 matched controls), as ascertained and classified by study coordinators.

Statistical analysis of the human study

We tested allelic distributions at each locus for Hardy-Weinberg equilibrium via the STATA 8.0 "genhw" command (STATA Data Corp, College Station, TX, USA). Potential haplotype effects were assessed by testing nonrandom associations using multilocus tests to measure total pairwise disequilibrium between any two loci, using the "hapipf" command in STATA 8.0 (20, 21). The association between individual genotype and ALI was conditioned on matched pair grouping using conditional logistic regression methods, which extends McNemar's test to multiple controls. To assess the potential confounding effects of clinical variables in the relationship of *NRF2* genotype with ALI risk, we used multivariable conditional logistic regression models. Because the number of cases was 30 we added potential confounders to the base model (containing genotype and ALI case designation) one at a time. Adjusted odds ratios were reported for the following potential confounder variables: age, gender, mechanism of trauma (categorized as blunt or penetrating), and APACHE II score (22). For the purposes of this analysis, the APACHE II score was calculated without the PaO_2 and FiO_2 variables. In addition, we evaluated the potential confounding effects of fluid and blood resuscitation by including the following variables as potential confounders: volume of packed red blood cells, crystalloid fluids, and fresh frozen plasma administered within the first 24 h of trauma. A two-sided *P* value of 0.05 was considered significant in all analyses. To fulfill asymptotic assumptions in the regression models, LogXact v2.1 (Cytel Software, Cambridge MA, USA) was used to generate exact *P* values from the conditional logistic regression models. As all clinical and genotyping data were complete, no imputation strategies were employed.

RESULTS

SNP discovery

We identified three SNPs in the coding region of *NRF2*: Thr72Ala (A/G), Thr92Ile (C/T), and Leu177Val (T/G). Thr72Ala was previously published (rs1135118) with two additional coding SNPs, Ser-99Pro (T/C) (rs5031039) and Arg517Lys (G/A) (rs1057044) that were homogeneous in the screened populations. The Leu177Val (T/G) variation was present (14%) in the screen but homogeneous among ALI study subjects. We observed no variation between ethnic groups for the Thr72Ala, Thr92Ile, Ser99Pro, or Arg517Lys polymorphisms. We identified three new *NRF2* promoter polymorphisms at positions −617 (C/A), −651 (G/A), and −653 (A/G) (**Fig. 2*A***). Polymorphism allelic frequencies for −617 and −653 were >10% (2 mutant alleles/20 screened) in the four ethnic groups (**Table 2**).

We used transcription factor motif analysis (TRANSFAC; ref23) to assess transcription factor binding potential in the promoter regions that contain the polymorphisms and whether the substitutions may disrupt transcription factor binding. These analyses revealed that variations at −653/-651 and −617 may potentially alter the consensus recognition sequences for MZF1 and NRF2, respectively (Fig. 2*B*), suggesting that these polymorphisms may affect *NRF2* transcription. To examine this concept, we transiently transfected the 727-Luc (or −617C allele) and 538-Luc (which lack the polymorphisms) into A549 cells and basal level activity of the *NRF2* promoter with or without polymorphisms was determined. Compared to the basic pGL3-vector, the 538-Luc displayed a significantly higher level of activity ($P<0.001$; **Fig. 3**). The −538 to +131 bp region of *NRF2* (538-Luc) contains regulatory elements that drive the promoter activity above the levels seen with the promoter-less basic vector. However, the inclusion of −538 to −727 region to the 538-Luc construct further enhanced the reporter activity. These results indicate that the −538 to +131 bp region acts as a basal promoter, while the −538 to −727 region acts as an enhancer. Relative to 538-Luc, the 727-Luc reporter had 4-fold higher luciferase activity ($P<0.001$; Fig. 3), indicating that the −538 to −727 region most likely contains DNA sequences required for high level *NRF2* promoter activity.

SNP function

We cloned the 727 bp *NRF2* promoter bearing the indicated polymorphisms upstream of the Luc gene to determine functional significance of *NRF2* promoter polymorphisms. Variants and putative cis-elements, such as AP-1-like and ARE-like sites, encompassed the SNP region (−659 to −608; **Fig. 4**). The MZF1, AP1-like, and ARE-like sites mediate transcriptional responses and have been shown to play key regulatory roles in various cellular responses after toxic and oxidant insults (13, 24, 25). Given the significance of these sites in the regulation of gene expression, we examined

TABLE 2. *Allelic frequencies of NRF2 promoter polymorphisms in four ethnic groups*

Allele	European Descent, %*	African Descent, %	Native American, %	Asian , %
-617A	20.0 (4/20)	10.0 (2/20)	55.0 (11/20)	40.0 (8/20)
-651A	10.0 (2/20)	5.0 (1/20)	ND	ND
-653G	25.0 (5/20)	10.0 (2/20)	40.0 (8/20)	60.0 (12/20)

ND = not detected. *Numbers of mutant alleles per 20 alleles screened are in parentheses. DNA samples (Coriell Institute) were initially screened to identify *NRF2* polymorphisms and are included for comparison [n = 40 (10/group)].

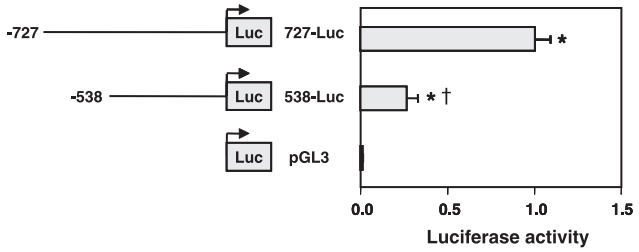

Figure 3. Effect of the −727 to −538 *NRF2* region on *NRF2* promoter activity. Luciferase activity of 2 *NRF2* promoter constructs: 727-Luc containing SNP region and 538-Luc without SNP region, compared to pGL3 vehicle control. Intact promoter with polymorphic region exhibited 4-fold higher luciferase activity compared to 538-Luc reporter. $^{*}P < 0.001$ compared with pGL3; $^{\dagger}P < 0.001$ compared with 727-Luc. *P* values were calculated from a Student-Newman-Keuls *a posteriori* comparisons of means test after one-way analysis of variance; group sizes = 4–7.

whether they regulate *NRF2* gene expression. In transient transfection assays, luciferase activity was significantly ($P<0.001$) higher in –617 C wild-type compared to promoter constructs bearing –617 A and –651 A variants (Fig. 4). Basal activity of cells transfected with a promoter construct bearing –651 A and –653 G polymorphisms differed significantly ($P=0.014$) from that of cells transfected with –651 A alone. Thus, it is likely that promoter polymorphisms at positions −617 (C to A) or −651 (G to A) affect basal level expression of NRF2, thereby resulting in attenuation of ARE-mediated gene transcription.

Five hour treatment of cells with oxidative-stress agents hydrogen peroxide (H_20_2, 200 μM), cadmium chloride ($CdCl_2$, 30 μM), and butylated hydroxytoluene (BHT, 100 μM) did not significantly affect luciferase expression driven by wild-type and polymorphic constructs in A549 cells (Kleeberger *et al.*, unpublished data). *NRF2* mRNA and protein levels are highly abundant in various tissues/cell types including airway epithelial cells, alveolar type II cells, and macrophages (15). Because these stress inducing agents that activate antioxidant enzyme expression failed to further enhance *NRF2* mRNA levels above the basal level, our data are consistent with the contention that the −617 and −651 SNPs affect the basal *NRF2* message levels.

Importantly, formation of protein-DNA complex was significantly diminished in heterozygotes ($P<0.001$) and variants ($P<0.001$) for the −617 polymorphism in the ARE-like sequence (**Fig. 5**). The significant reduction of protein-DNA complex indicates that the −617 SNP affects efficient binding of proteins, such as NRF2, to the ARE-like site. In other words, NRF2 binds less efficiently to ARE-like sequences that contain the −617 polymorphism, as compared with the wild-type allele. Supershift assays with anti-NRF2 antiserum revealed binding of NRF2 to the wild-type ARE-like sequence, suggesting that NRF2 autoregulates transcription through this promoter region. In support of this notion, Kwak *et al.* (26) showed that NRF2 binds to the ARE-like element of the mouse *Nrf2* promoter (−754) and up-regulates its transcription.

Association of functional SNPs with risk of ALI

Based on these functional data, we next asked whether the –617 and –651 polymorphisms increased the risk for ALI in our sample of 90 patients with major trauma. The −653 polymorphism was not tested because it had relatively little functional importance compared to the other SNPs (see Fig. 4). Combined allelic frequencies in subjects of African and European descent were within the range established by the initial screen: −617A, 11.9% (21/180); −651A, 5.1% (9/180). No significant differences in allelic frequency at the two loci were found between the European and African descent subjects. Both SNPs satisfied Hardy-Weinberg proportions in both the African-American and European populations ($P>0.05$). The –617 polymorphism was associated with increased risk of ALI (**Table 3**). Relative to wild type (−617 C), subjects with at least one A allele at position −617 were at significantly greater risk of ALI (OR 6.44; 95% CI 1.34, 30.8; $P=0.021$). No significant confounding of this association was found with adjustment for clinical variables, including age, gender, mechanism of trauma, APACHE II score, and volume of fluids and blood products administered (**Table 4**). Analyses of potential haplotype effects among the two promoter SNPs did not add any explanatory information to the individual SNP association, as no subjects had variants at both the –617 and –651 loci.

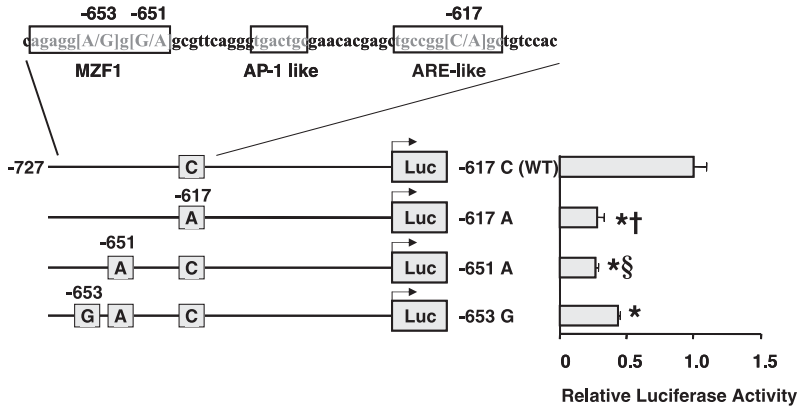

Figure 4. Effects of *NRF2* polymorphisms on *NRF2* promoter activity. Luciferase activity of four *NRF2* promoter constructs: −617C (wild-type sequence, 727-Luc), −617 A, −651 A, and −653 G. Remaining SNP sites are indicated when double variants are used. Wild-type 727-Luc reporter exhibited >50% higher luciferase activity compared to polymorphic variants. $^{*}P < 0.001$ compared with –617 C (WT); $^{\dagger}P = 0.014$ compared to −651 A; $^{\S}P = 0.019$ compared to −653 G. *P* values were calculated from a Student-Newman-Keuls *a posteriori* comparisons of means test after one-way analysis of variance; group sizes = 4.

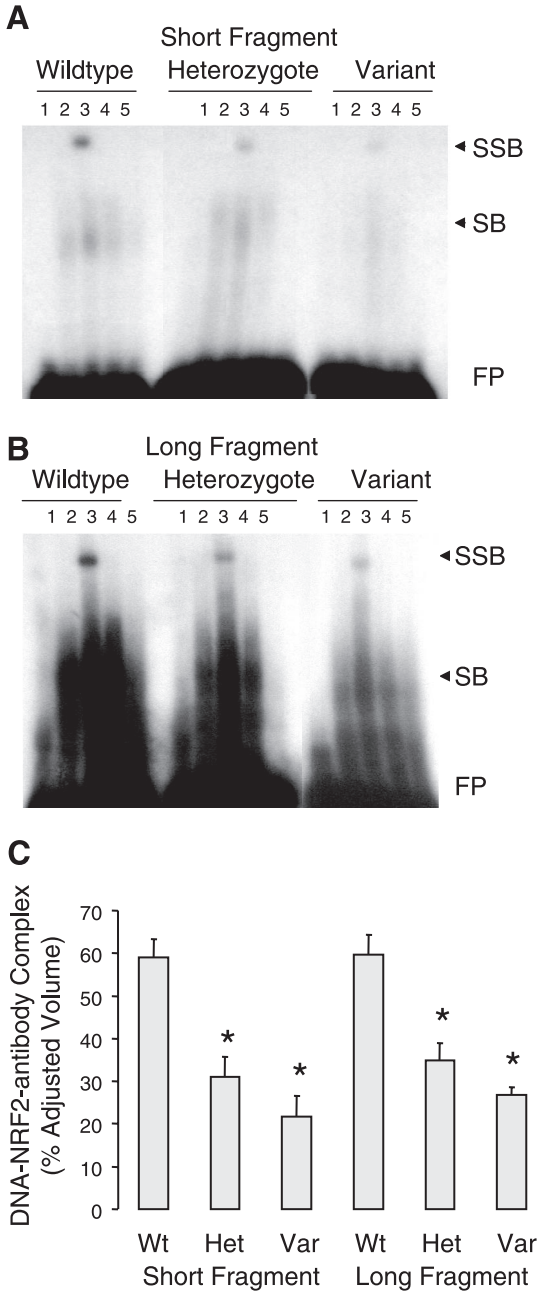

Figure 5. Effects of the −617 polymorphism in *NRF2* on NRF2-DNA complex formation. EMSA was used to evaluate effects of the −617 polymorphism on protein-DNA complex formation. *A)* 26 basepair wild-type, heterozygote and homozygous variant oligonucleotides (short fragment) spanning the −617 polymorphic region. *B)* 62 basepair wild-type and heterozygote oligonucleotides (long fragment) spanning the −617 polymorphic region. Lane 1: free probe; lane 2: reaction without antibody; lane 3: reaction with anti-NRF2 antiserum (2 μl); lane 4: control serum (2 μl); and lane 5: competition without antibody (40×). *C)* Densitometry results for supershifted bands (DNA-NRF2-antibody complex) are represented as percent adjusted volume for short and long fragments. Means (±SE) are presented; group sizes = 3–9. *$P < 0.001$ compared with Wt. *P* values were calculated from a Student-Newman-Keuls *a posteriori* comparisons of means test after one-way analysis of variance.

DISCUSSION

Understanding the genetic basis of complex diseases is a challenge because of multiple etiologies and genetic heterogeneity of the populations under study. ALI does not lend itself to traditional family-based studies to identify susceptibility genes because individuals must have an extreme physiological insult to be at risk. Therefore, investigations of genetic predisposition to ALI have focused on traditional epidemiological studies in at risk populations (27, 28). However, candidate gene studies can be plagued by limits of theoretical assumptions of SNP function and potential multiple comparisons. We specifically designed our study to address these concerns by first using positional cloning in mice to identify a candidate susceptibility gene. We then resequenced individuals of diverse ethnic backgrounds, tested SNPs for functional relevance, and tested only those SNPs with potential function for association with ALI in a separate human population. This careful, hypothesis-driven approach limits the likelihood that our association of candidate SNPs with ALI is due to random chance alone or is a spurious type I error.

We identified *Nrf2* as a susceptibility gene by using a positional cloning approach in inbred mice to identify genes that account for a significant portion of the genetic variance in susceptibility to hyperoxic lung injury and that may be tested in ALI patients (10). Positional cloning in rodent models has proved useful to identify disease-causing genes, including those for arthritis severity (29), polycystic kidney disease (30), and bacterial infections (31, 32). Because the candidate susceptibility gene *Nrf2* is essential to protection against oxidative pulmonary injury in mice (11, 33–35), we hypothesized that polymorphisms in *NRF2* resulting in decreased function similarly predispose humans to ALI.

To address this hypothesis, we initially resequenced *NRF2* in four ethnically diverse populations. The genomic sequence of human *NRF2* is not well characterized, and only a few SNPs have been localized to coding regions within the gene. We identified six novel SNPs in *NRF2*. Three promoter polymorphisms were predicted to have functional significance, and one [−617 (C/A)] significantly affects basal *NRF2* expression and function. These polymorphisms were also found in a Japanese population by Yamamoto *et al.*

TABLE 3. *Association of ALI risk in relation to NRF2 −617 and −651 polymorphisms in a population of patients with major trauma*

Polymorphism	Genotype	Odds Ratio*	Confidence Interval	*P* Value
−617	CC	referent	–	–
	CA	6.44	1.34, 30.8	0.021
−651	GG	referent	–	–
	GA	0.40	0.04, 3.42	0.182

*Odds ratio for matched pairs derived from conditional logistic regression analysis, conditioned on racial ancestry and ISS.

TABLE 4. *Multivariable analysis of the association of NRF2 −617 genotype and ALI risk*

Clinical Variable	Adjusted Odds Ratio for NRF2 -617 CA (95% CI)	*P* Value
Unadjusted (base model)	6.44 (1.34, 30.8)	0.021
Adjusted for:		
Age	6.37 (1.26, 32.1)	0.025
Gender	6.51 (1.31, 32.2)	0.020
APACHE II score	5.37 (1.03, 28.1)	0.046
Mechanism of trauma	5.26 (1.08, 25.7)	0.040
Total crystalloid fluid, ml	9.16 (1.04, 80.8)	0.046
Packed red blood cells, units	6.46 (1.25, 33.3)	0.026
Fresh frozen plasma, ml	5.70 (1.16, 27.9)	0.032

CI, confidence interval; APACHE II, Acute Physiology and Chronic Health Evaluation II (22). The reported odds ratio is for the association of *NRF2* -617 CA genotype and ALI, adjusted for each clinical variable, matched on race and ISS strata using conditional logistic regression methods. Clinical variables are baseline variables collected during the first 24 h after trauma.

(36). Interestingly, these mutations in *NRF2* are predicted to have different effects than the *Nrf2* T-336C SNP in the mouse that we found to correlate with susceptibility to hyperoxic lung injury (10). The human −617 and −651/−653-SNPs are predicted to affect *NRF2* ARE-like and MZF1 promoter binding sites (see Results), respectively, while the mouse −336 SNP is predicted to add an SP-1 binding site to the *Nrf2* promoter (10).

Importantly, we found that the −617 SNP was associated with an increased risk of developing ALI in a nested case-control study of at-risk patients with major trauma. This suggested a role for *NRF2* in development of the syndrome. The mechanism through which *NRF2* confers protection against oxidative stress likely relates to the ability of this transcription factor to regulate antioxidant and phase II enzyme genes that bear promoter AREs in their regulatory (promoter and/or enhancer) regions (37–39). It may be postulated that individuals with functional polymorphisms in NRF2 that alter basal expression of NFR2, or the ability of *NRF2* to translocate from the cytoplasm to nuclear binding sites, are at enhanced risk of oxidative stress and ALI. Consistent with this hypothesis, targeted disruption of *Nrf2* significantly decreased antioxidant capacity in mice, and thus enhanced susceptibility to prooxidant, -fibrotic, and -carcinogenic agents (35, 40, 41). Investigations are ongoing to determine whether NRF2 transcript levels (or activity) from lavaged cells or biopsy cells from ALI patients is different from those who do not develop ALI or ARDS.

Oxidative stress has been implicated in multiple biological processes and diseases including carcinogenesis, aging, atherosclerosis, and inflammatory disorders such as asthma and chronic obstructive pulmonary disease (42). Because NRF2 is critical to regulation of antioxidant defense, polymorphisms that affect NRF2 activity may have fundamental importance to these other diseases. An important implication of our findings is the potential use of the functional *NRF2* SNPs as identifiers of increased risk to oxidative lung injury and thus identify susceptible individuals. Specific to the trauma population, if our findings are confirmed in other trauma populations, *NRF2* genetic variation could potentially be used to identify at risk individuals for pharmacogenomic prevention trials with antioxidant strategies. Our study represents the first step toward this goal (43). In addition, a more complete understanding of the mechanisms through which NRF2 confers protection against oxidant injury may also lead to alternative intervention strategies. Further studies are therefore needed to examine *NRF2* transcripts in other susceptible populations, as well as putative downstream target genes involved in cellular ROS detoxification.

Potential limitations of the clinical portion of our study include small sample size, uncontrolled confounding, and potential bias introduced by selection of controls. Effects of small sample size may be manifest in an error due to multiple comparisons leading to a spurious association (type I error). However, we specifically designed our study to minimize this effect by limiting our study to only the two polymorphisms that had evidence of decreased function in our *in vitro* studies. Small sample size may also affect the generalizability of our clinical associations to other populations, and our results need to be confirmed in other at-risk populations. Although small sample size would not be expected to produce a false positive (spurious) association, there is a reasonable chance any negative associations may be due to relatively low power (*i.e.*, a type II error) therefore, the lack of association of the −651 SNP in the human study should be interpreted with caution.

Our results were robust when adjusted for the potential confounding effects of important baseline clinical variables; however, the odds ratios had wide confidence intervals and thus the point estimates should be interpreted with caution. Uncontrolled confounding due to population admixture is a possibility in our population; however, prior publications in our source population indicate that this effect appears to be minimal with matching on ancestry (44–46). Although selection bias may be an important factor in many case control studies, we minimized selection bias by performing a nested case control study drawing our entire study

population from an existing cohort study. In a complex disorder such as ALI, a SNP association could be due to the effects of other polymorphisms, both within NRF2 and at loci nearby. Although this may be true in our study, given the clear functional evidence for the −617 variant, we do not believe undetermined haplotype effects will diminish the importance of our findings.

In conclusion, we identified functional polymorphisms in the promoter of *NRF2* that are found in relatively high frequency among multiple ethnic populations. We also present epidemiological evidence of an association of an *NRF2* polymorphism with ALI risk in a well-characterized clinical at-risk group. This study demonstrates the utility of inbred mouse models for identification of candidate genes in human disease processes when traditional family-based approaches are not feasible and illustrates a candidate gene association approach based on solid laboratory evidence of altered gene function. Future investigations will be instrumental in confirming our observations in additional populations at risk for ALI, as well as investigating the role of *NRF2* in other oxidant-related disease processes such as carcinogenesis, aging, atherosclerosis, and chronic inflammatory disorders. FJ

The authors acknowledge the contributions of B. Finkel for clinical project management and data collection, C. Gaughan and A. R. Localio for assistance with data management and analysis, B. Fuchs for participation in ALI phenotyping and advice on study interpretation, T. Rebbeck for guidance on genetic epidemiology study design and interpretation, and A. Fisher for guidance on oxidant stress physiology and grant administration. This study was supported by National Institutes of Health grants R01 HL-66109 (H. Vuong, S. Reddy), P50 HL-60290–01 (J. Christie and P. Lanken), K23 HL-04243 (J. Christie), the Doris Duke Charitable Foundation (R. Aplenc), and the Intramural Program at the National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services (J. Marzec, H. Cho, and S. Kleeberger). The funding agencies had no role in the design and conduct of the study, the collection, analysis, and interpretation of the data, or the preparation, review, or approval of the manuscript.

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Received for publication November 28, 2006.
Accepted for publication February 8, 2007.