

# Genotypes and haplotypes of the VEGF gene are associated with higher mortality and lower VEGF plasma levels in patients with ARDS

R Zhai, M N Gong, W Zhou, T B Thompson, P Kraft, L Su, D C Christiani

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See end of article for authors' affiliations

Correspondence to:  
Dr David C Christiani,  
Harvard School of Public  
Health, 665 Huntington  
Avenue, Boston,  
Massachusetts 02115, USA;  
dchristi@hsph.harvard.edu

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**Background:** Endothelial injury is an important prognostic factor in acute respiratory distress syndrome (ARDS). Vascular endothelial growth factor (VEGF) plays a critical role in endothelial destruction and angiogenesis. Genetic variations of the VEGF gene have been associated with VEGF production. A study was undertaken to investigate the impact of VEGF gene polymorphisms on the clinical outcomes of ARDS.

**Methods:** Three VEGF polymorphisms ( $-460C/T$ ,  $+405C/G$  and  $+936C/T$ ) were determined in 1253 patients in an intensive care unit with risk factors for ARDS, 394 of whom developed ARDS. Patients were followed for assessment of 60 day survival. Plasma VEGF levels were measured in 71 patients with ARDS.

**Results:** The  $+936TT$  (OR 4.29, 95% CI 1.12 to 16.40,  $p=0.03$ ) and  $+936CT+TT$  (OR 1.98, 95% CI 1.14 to 3.42,  $p=0.01$ ) genotypes were significantly associated with increased mortality from ARDS. Plasma VEGF levels in patients with ARDS with the  $+936CT+TT$  genotype were significantly lower than in subjects with the  $+936CC$  genotype (median 49 (IQR 16–98) pg/ml vs 112 (IQR 47–162) pg/ml,  $p=0.02$ ). At the haplotype level, haplotype  $TCT$  ( $-460T+405C+936T$ ) was significantly associated with a higher rate of mortality (OR 2.89, 95% CI 1.30 to 6.43,  $p=0.009$ ) and haplotype  $CGT$  ( $-460C+405G+936T$ ) was associated less strongly with increased mortality (OR 1.90, 95% CI 0.94 to 3.83,  $p=0.07$ ) in patients with ARDS. Lower plasma VEGF levels were correlated with the probability of haplotype  $CGT$  (coefficient =  $-0.26$ ,  $p<0.05$ ), but the same trend of correlation was not significant to haplotype  $TCT$ .

**Conclusions:** VEGF polymorphisms may contribute to the prognosis and inter-individual variations in circulating VEGF levels in patients with ARDS.

Acute respiratory distress syndrome (ARDS) is lung injury characterised by alveolar injury and increased pulmonary vascular permeability.<sup>1,2</sup> The pathogenetic basis of ARDS is incompletely understood; however, emerging evidence has suggested that the severity and outcome of ARDS depend significantly on the balance between alveolar epithelial and/or vascular endothelial injuries and their repair mechanisms.<sup>2–6</sup>

Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen that promotes angiogenesis and mediates vascular permeability.<sup>6,7</sup> Overexpression of VEGF in the lungs induces increased pulmonary vascular permeability resulting in pulmonary oedema.<sup>8</sup> However, VEGF expression in human lung epithelial cells can also increase neovascularisation, thereby contributing to the repair of endothelial injuries.<sup>6,9</sup> In human lungs, low levels of VEGF were associated with the severity of ARDS while raised VEGF levels were associated with recovery from ARDS, indicating a role for VEGF in the repair process of lung injury.<sup>10–13</sup>

The VEGF gene is located on chromosome 6p31.3. Several functional single nucleotide polymorphisms (SNPs) in this gene have been described in the literature. The  $+936C/T$  polymorphism (rs3025039) has been related to lower levels of plasma VEGF,<sup>14,15</sup> whereas the  $-460C/T$  (rs833061) and  $+405C/G$  (rs2010963) polymorphisms have been shown to significantly increase VEGF production.<sup>16</sup> Recent studies have indicated that these three SNPs are implicated in the risk of several disorders in which vascular injury and acceleration of inflammation are critical in disease development.<sup>17,18</sup> However, the combined effect of these polymorphisms on the prognosis of ARDS has not been evaluated. Based on the biological function of these three VEGF polymorphisms on VEGF production and the pathological significance of VEGF in ARDS, we hypothesised

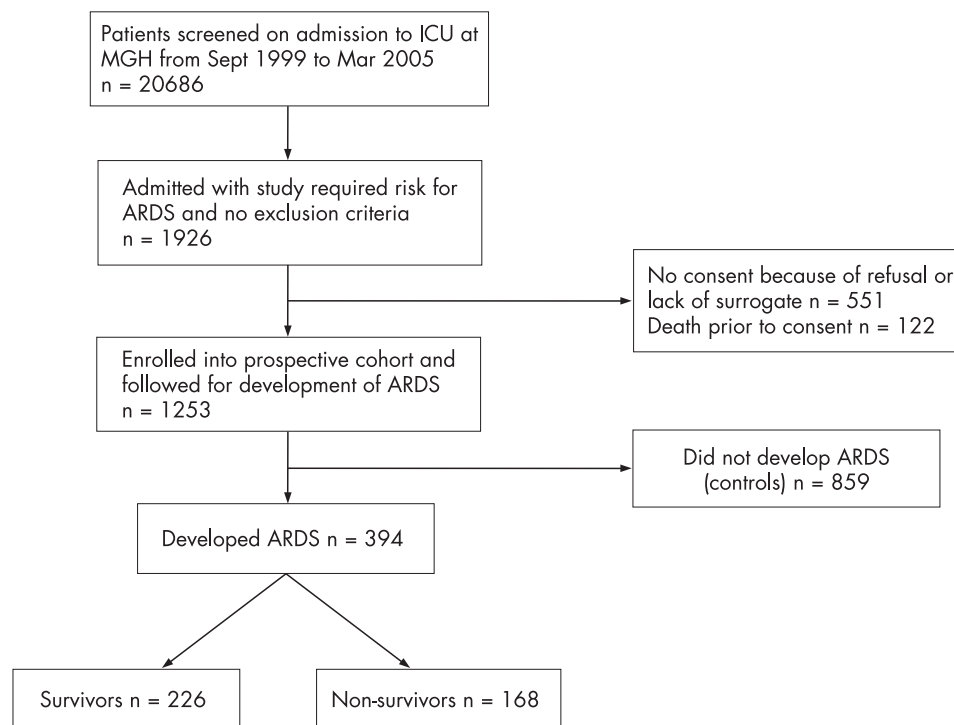
that VEGF polymorphisms may contribute to the poor outcomes in ARDS, and correlate with circulating VEGF levels in patients with ARDS.

## METHODS

### Study subjects

The present study was part of an ongoing research investigating the effects of multiple genetic variants on the risks or prognosis of ARDS. Recruitment of subjects was started in September 1999 and will continue until 2010. Details of the study have been described previously.<sup>19,20</sup> Briefly, all admissions to the intensive care unit (ICU) at Massachusetts General Hospital were screened daily for study-defined clinical risk factors for ARDS such as sepsis, trauma, aspiration or massive transfusions and were followed prospectively during their stay in the ICU for the development of ARDS (fig 1). Subjects were classified as having ARDS if they were intubated on positive ventilation and met the American European Consensus Committee (AECC) criteria for ARDS: (1) arterial oxygen tension/fraction of inspired oxygen ratio of  $\leq 200$  mm Hg; (2) bilateral infiltrates on chest radiographs; and (3) pulmonary arterial occlusion pressure  $\leq 18$  mm Hg or no clinical evidence of left atrial hypertension. All cases of ARDS were followed for all-cause 60 day mortality. The study was approved by the Human Subjects Committees of Massachusetts General Hospital and the Harvard School of Public Health, Boston, and informed written consent was obtained from all subjects or their appropriate surrogates.

**Abbreviations:** ARDS, acute respiratory distress syndrome; SNP, single nucleotide polymorphism; VEGF, vascular endothelial growth factor



**Figure 1** Flow diagram of study design and patient selection for the case-control study. ICU, intensive care unit; MGH, Massachusetts General Hospital, Boston, Massachusetts, USA. Patients who had a previous history of acute respiratory distress syndrome (ARDS) and those who had previously been admitted to the ICU were excluded from being controls. The subjects were all Caucasians. Non-Caucasians were also enrolled but they were excluded from the analysis because they accounted for only 4% of all enrolment.

### DNA isolation and genotyping assays

Genome DNA was extracted from peripheral blood samples using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, Minnesota, USA) following the manufacturer's protocol. The allelic discrimination of the *VEGF* gene was assessed with the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, California, USA) using the fluorogenic 5' nuclease assay with Taqman Minor Groove Binder (MGB) probes. The wide-type Taqman MGB probes were FAM-labelled and the mutant probes were VIC-labelled. The primers and probes for the  $-460C/T$ ,  $+405C/G$  and the  $+936C/T$  polymorphism assays were ordered from Applied Biosystems. Genotyping was performed by laboratory personnel blinded to subject status, and a random 10% of the samples were repeated to validate genotyping procedures. Two authors reviewed independently all genotyping results.

### Analysis of VEGF levels in plasma

Plasma samples from day 2 after development of ARDS were available from 71 patients for the measurement of circulating

VEGF levels. Blood samples were collected in 10 ml vacuum tubes and centrifuged for 10 min. Plasma samples were stored at  $-80^{\circ}\text{C}$  until analysis. Plasma VEGF levels were quantified in duplicate according to the manufacturer's recommendations with a commercially available ELISA kit (R&D Systems, Minnesota, USA). No statistically significant differences were observed between patients with plasma samples and those without plasma samples in terms of age, sex, APACHE III and risk factors for ARDS.

### Statistical analysis

The demographic variables between different groups were compared by  $\chi^2$  tests for categorical variables and by the Student *t* test and/or non-parametric test for continuous variables. The Hardy-Weinberg equilibrium was evaluated using the  $\chi^2$  test. A logistic regression model was used to assess the effect of *VEGF* polymorphisms on the mortality of patients with ARDS, with adjustments for potential confounding factors such as age, sex, APACHE III score, diabetes, history of steroid use, number of units of red cells transfused and

**Table 1** Characteristics of survivors and non-survivors among patients with ARDS

Covariables	Non-survivors (n = 168)	Survivors (n = 226)	p Value
Mean (SD) age (years)	67.98 (14.75)	53.88 (18.62)	<0.01
Female, n (%)	72 (42)	92 (41)	0.67
Mean (SD) APACHE III score	89.60 (22.28)	68.95 (21.15)	<0.01
Diabetes, n (%)	31 (18)	38 (17)	0.70
Chronic liver disease, n (%)	8 (10)	6 (3)	<0.01
History of steroid use, n (%)	27 (16)	19 (8)	0.02
Bilirubin >2.0 mg/l, n (%)	41 (27)	27 (14)	<0.01
Creatinine >2.0 mg/l, n (%)	63 (38)	54 (24)	0.10
Haematological failure ( $\leq 80\,000$ platelets/ $\text{mm}^3$ ), n (%)	48 (29)	35 (16)	<0.01

chronic liver disease. Linkage disequilibrium between the SNPs, haplotypes and their frequencies was estimated using the expectation maximisation algorithm. Association between haplotypes and the risk of ARDS was assessed using an "expectation substitution" approach to account for unknown phase.<sup>21</sup> Haplotypes were coded as an additive fashion. Mortality from ARDS was regressed on haplotype counts by logistic regression, using the most common haplotype as the reference haplotype. The multiple Cox regression model was applied to test the effect of the *VEGF* polymorphisms on overall survival, adjusting for confounding factors such as age, sex, APACHE III score, diabetes, history of steroid use and chronic liver disease. The correlations between haplotype probabilities and plasma VEGF levels were estimated by the Spearman correlation test. All statistical analyses were performed using the SAS statistical software package Version 9.1 (SAS Inc, Cary, North Carolina, USA).

## RESULTS

### Patient population

Between September 1999 and March 2005, 1253 Caucasian patients with risk factors of ARDS were enrolled into the prospective cohort. In this study population, 394 (31.4%) were diagnosed as having ARDS and 859 did not develop ARDS (fig 1).

Clinical risk factors for ARDS and baseline characteristics between subjects with and without ARDS are shown in tables 1 and 2 in the online data supplement (available at <http://thorax.bmj.com/supplemental>). Among the 394 patients with ARDS studied, the 60 day mortality was 42.6% (168/394). Logistic analysis showed that older age, APACHE III, steroid use, haematological failure and liver disease were the major prognostic factors for survival (table 1).

### VEGF genotypes in relation to ARDS mortality

Genotyping of the  $-460C/T$ ,  $+405C/G$  and  $+936C/T$  polymorphisms was successfully achieved for all subjects and followed the Hardy-Weinberg equilibrium. The genotype frequencies of these three polymorphisms in the current study were broadly similar to those reported by others in Caucasian populations.<sup>22-28</sup> When overall cases of ARDS were compared with patients without ARDS, no significant difference was found in the distribution of genotypes for any polymorphisms studied ( $p > 0.05$ ).

We tested for an association between mortality from ARDS and any of the three SNPs among the 394 patients with ARDS (table 2). No significant association was found between the  $-460C/T$  or  $+405C/G$  polymorphisms and the risk of mortality in patients with ARDS ( $p > 0.05$ ), but the  $+936TT$  genotype was

significantly associated with a higher mortality from ARDS than the  $+936CC$  genotype (adjusted OR 4.29, 95% CI 1.12 to 16.40,  $p = 0.01$ ). A similar association was also observed for the  $+936CT$  genotype, but this association was not statistically significant (adjusted OR 1.60, 95% CI 0.90 to 2.80,  $p = 0.10$ ). When the mutant  $+936TT$  and  $+936CT$  were combined to form a united genotype, this combined  $+936CT+TT$  genotype was significantly associated with increased mortality compared with the  $+936CC$  genotype (adjusted OR 1.98, 95% CI 1.14 to 3.42,  $p = 0.01$ ). No significant interactions between any genotypes and age, sex or types of injury were detected.

### Association between VEGF haplotypes and ARDS mortality

Haplotype analyses were conducted to evaluate the combined effect of the three polymorphisms on survival in patients with ARDS. Consistent with observations from early studies,<sup>29</sup> *VEGF*  $-460C/T$  and  $+405C/G$  polymorphisms in our study were in strong linkage disequilibrium (correlation coefficient ( $R$ ) = 0.68, Lewontin's  $D'$  ( $D'$ ) = 0.99), whereas their linkage with the  $+936C/T$  polymorphism was much weaker ( $R = 0.04$ ,  $D' = 0.1$  and  $R = 0.1$ ,  $D' = 0.20$ , respectively). Six haplotypes with frequencies  $> 1\%$  were estimated of the eight possible haplotypes (table 3). Interestingly, the  $-460T+405C+936T$  ( $TCT$ ) haplotype was significantly associated with an increased risk of mortality from ARDS (adjusted OR 2.89, 95% CI 1.30 to 6.43,  $p = 0.009$ ), and the  $-460C/+405G/+936T$  ( $CGT$ ) haplotype was marginally significantly associated with a higher risk of mortality from ARDS (adjusted OR 1.90, 95% CI 0.94 to 3.87,  $p = 0.07$ ). No associations were found between other haplotypes and mortality from ARDS in this study population.

### Relationship between VEGF polymorphisms and plasma VEGF levels

To investigate the relationship between *VEGF* polymorphisms and circulating VEGF levels, plasma VEGF levels were categorised according to *VEGF* polymorphisms. Since the plasma levels of VEGF were not normally distributed (Kolmogorov-Smirnov test,  $p = 0.01$ ), a non-parametric median two-sample test was used to compare the VEGF levels between different genotype carriers. The median (interquartile range) plasma VEGF level in individuals carrying the  $+936CT+TT$  genotype was significantly lower than that in individuals with the *VEGF* $+936CC$  genotype (49 (16-98) pg/ml vs 112 (47-162) pg/ml,  $p = 0.02$ ). Based on the sample sizes and median plasma VEGF levels between the  $+936CT+TT$  and the *VEGF* $+936CC$  carriers, the power to detect a difference at  $\alpha = 0.05$  level was calculated to be 99%. At haplotype levels, the

**Table 2** Associations of *VEGF* genotypes and mortality from ARDS in all patients (n = 394)

Genotypes	Genotype frequency (%)	OR* (95% CI)	p Value*
460TT	29.4	1.00	
460CT	46.7	1.33 (0.83 to 2.14)	0.24
460CC	23.9	0.77 (0.44 to 1.35)	0.37
405GG	43.9	1.00	
405CG	43.4	1.37 (0.84 to 2.24)	0.20
405CC	12.7	0.87 (0.43 to 1.74)	0.69
936CC	73.9	1.00	
936CT	22.8	1.60 (0.90 to 2.80)	0.10
936TT	3.3	4.29 (1.12 to 16.40)	0.03
936CT+TT	26.1	1.98 (1.14 to 3.42)	0.01
P <sub>trend</sub> †	1.93 (1.10-1.94)		0.005

\*Estimated by logistic regression models, adjusting for age, sex, history of alcohol abuse, diabetes, steroid use, APACHE III score and multiple ( $> 1$ ) risk factors for ARDS.

†The genotypes were coded as continuous variables (1, 2 and 3 for wild type, heterozygous and homozygous genotypes, respectively) and entered together into the logistic regression model.

**Table 3** Associations between *VEGF* haplotypes and ARDS mortality

Haplotype†	Haplotype frequency (%)	OR* (95% CI)	p Value*
Global test			0.026
CGC	39.6	1.0	
TCC	28.9	0.85 (0.59 to 1.24)	0.4
TGC	16.6	0.95 (0.58 to 1.55)	0.8
CGT	7.4	1.90 (0.94 to 3.83)	0.07
TCT	5.3	2.89 (1.30 to 6.43)	0.009
TGT	1.7	1.09 (0.20 to 5.84)	0.92

\*Estimated by logistic regression models, adjusting for age, sex, history of alcohol abuse, diabetes, steroid use, APACHE III and multiple risk of (>1) of ARDS.

†The order of the polymorphisms is as follows: -460C/T, +405C/G, +936C/T.

plasma VEGF levels were inversely correlated with haplotype *CGT* probability (Spearman coefficient  $-0.26$ ,  $p < 0.05$ ). A similar correlation was also observed between plasma VEGF levels and the haplotype *TCT* probability, but this correlation did not reach statistical significance (coefficient  $-0.14$ ,  $p = 0.26$ ). Thus, haplotypes containing the *936T* allele tended to correlate with lower plasma VEGF levels. There was no significant difference between survivors and non-survivors in plasma levels of VEGF ( $p > 0.05$ ).

### Association between *VEGF* polymorphisms and ARDS survival

The Cox proportional hazard model was used to analyse the associations between *VEGF* polymorphisms and survival in patients with ARDS. After adjusting for other predictors of survival (age, sex, APACHE III score, diabetes, history of steroid use, haematological failure and chronic liver disease), both the +936CT+TT genotype (hazard ratio (HR) 1.71, 95% CI 1.09 to 2.72,  $p = 0.02$ ) and haplotype *TCT* (HR 2.11, 95% CI 1.32 to 3.37,  $p < 0.01$ ) were significantly associated with lower survival probability, consistent with the results assessed by logistic regression models.

### DISCUSSION

Our data have shown that the +936TT and +936CT+TT genotypes of the *VEGF* gene are significantly associated with an increased risk of mortality from ARDS. At the haplotype level, the *TCT* haplotype was significantly associated with a higher mortality from ARDS. In addition, we showed that *VEGF* polymorphisms contributing to increased mortality in patients with ARDS were correlated with lower plasma levels of VEGF.

This study has a number of strengths. First, the prospective determination of ARDS using the AECC definition minimised phenotype misclassification since there is no diagnostic gold standard for ARDS. Second, the *VEGF* polymorphisms and the circulating VEGF levels were determined in a parallel manner. Thus, the functional effect of *VEGF* polymorphisms can be assessed. Third, restricting analyses to Caucasian patients reduced the possibility of altered genotype frequencies in different ethnicities.

Among the three functional SNPs studied, none of the -460C/T and +405C/G polymorphisms was found to have a significant effect on mortality from ARDS. However, the +936TT and the combined+936CT+TT genotypes were significantly associated with ARDS mortality, suggesting that +936 polymorphisms may have a stronger role than either the -460C/T or +405C/G polymorphisms in *VEGF* gene functions. Recently, a small case-control study found that the frequency of the combined +936CT+TT genotype in patients with ARDS was significantly higher than in controls. This study also found that the +936CT and TT genotypes were associated with the APACHE III score in patients with ARDS, but the association between the +936 CT/TT genotype and increased mortality from ARDS did not reach

statistical significance (OR  $> 1.80$ ,  $p > 0.05$ ).<sup>26</sup> In the present larger study, a significant association between the +936CT+TT genotype and mortality was found in patients with ARDS. At the haplotype level, the haplotype containing the +936T allele was also associated with mortality from ARDS, further supporting the effects of +936C/T variants on outcomes in patients with ARDS.

Our data showed that the +936CT/TT genotype was significantly correlated with lower plasma levels of VEGF, consistent with the functions of this polymorphism reported in other studies.<sup>14 15</sup> Although the plasma VEGF levels were measured at only one time point and may not therefore fully reflect the temporal response of VEGF over the course of the disease, our results suggest that VEGF may be a protective factor in the severity of ARDS. Decreased levels of VEGF in the lungs have been correlated with a higher lung injury score and lower survival rates in patients with ARDS,<sup>10 11 30</sup> whereas increased VEGF production in the lungs resulted in less lung injury.<sup>31</sup> In animal models, administration of VEGF prevented lung injury-induced death.<sup>30 32</sup> In alveolar epithelial cells, VEGF mRNA expression increased during recovery from oxygen injury.<sup>33</sup> In neonatal rats exposed to high oxygen levels, intraocular injection of VEGF at the onset of experimental hyperoxia prevented apoptotic death of endothelial cells and rescued the retinal vasculature.<sup>34</sup> Taken together, these findings indicate that VEGF may function as a protective factor in ARDS.

Although the haplotypes predicted in the present study were identical to those previously reported, the overall distribution of haplotype frequencies in the present study was different from that in Chinese and Korean populations.<sup>29 35</sup> Different haplotype frequencies in different populations may be due to different ethnic backgrounds or different disease states. Interestingly, the frequencies of *TCT* (5.3%) and *CGT* (7.4%) haplotypes in the present study were similar to those reported in the Chinese population (6.0% and 6.4%, respectively).<sup>29</sup> In Asian populations, both the *TCT* and *CGT* haplotypes were significantly implicated in a reduced risk of lung cancer, and the *CGT* haplotype was associated with decreased survival in patients with breast cancer.<sup>29</sup> These observations suggested that *TCT* and *CGT* haplotypes may play an important role in disease processes. However, since these previous studies did not measure circulating VEGF levels, the mechanisms connecting the *VEGF* haplotypes with development or severity of the disease are unclear. Our study not only indicated that the *TCT* haplotype was associated with mortality from ARDS, but also showed that the *CGT* and *TCT* haplotypes correlated with lower circulating levels of VEGF protein, which suggests that these functional haplotypes may contribute to mortality from ARDS by affecting VEGF production.

We acknowledge some potential limitations to this study. First, the sample size was relatively small. Additional studies with larger populations are required to confirm the associations between the *VEGF* haplotypes and mortality in patients with

ARDS. Second, the *VEGF* gene is a polymorphic gene, and the present study only investigated three SNPs. Further studies on other functional *VEGF* SNPs are needed to define the role of the *VEGF* polymorphisms in prognosis in ARDS. Third, measurement of plasma VEGF levels was done at one time point so the role of the plasma VEGF level in the natural history of ARDS remains to be defined. Finally, since genetic polymorphisms often vary between ethnic groups, further studies are necessary to clarify the association between the *VEGF* polymorphisms and the outcome in patients with ARDS in diverse ethnic populations.

In summary, the findings of this study suggest that the +936TT and +936CT+TT genotypes and the TCT haplotype of the *VEGF* gene contribute to increased mortality and to inter-individual variations in plasma VEGF levels in patients with ARDS. The associations found in this study are biologically plausible and may have significant implications for the treatment of ARDS.

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Clinical risk factors for ARDS and baseline characteristics between subjects with and without ARDS are shown in tables 1 and 2 in the online data supplement available at <http://thorax.bmj.com/supplemental>.

## Authors' affiliations

**R Zhai, W Zhou, L Su, D C Christiani**, Department of Environmental Health, Harvard School of Public Health, Boston, Massachusetts, USA

**M N Gong**, Division of Pulmonary and Critical Care Medicine, Mount Sinai School of Medicine, New York, USA

**T B Thompson, D C Christiani**, Pulmonary and Critical Care Unit, Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA

**P Kraft**, Department of Biostatistics, Harvard School of Public Health, Boston, Massachusetts, USA

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# **Genotypes and haplotypes of *VEGF* gene are associated with higher ARDS Mortality and lower VEGF plasma levels**

**R Zhai, M N Gong, W Zhou, T B Thompson, P Kraft, L Su, D C Christiani**

## **Authors' affiliations**

**R Zhai, W Zhou, L Su, D C Christiani**, Department of Environmental Health, Harvard School of Public Health, 665 Huntington Ave, Boston, MA, USA

**M N Gong**, Division of Pulmonary and Critical Care Medicine, Mount Sinai School of Medicine, 1190 Fifth Ave, New York, New York, USA

**T B Thompson, D C Christiani**, Pulmonary and Critical Care Unit, Department of Medicine, Massachusetts General Hospital, Harvard Medical School, 55 Fruit Street, Boston, MA, USA

**P Kraft**, Department of Biostatistics, Harvard School of Public Health, 665 Huntington Ave, Boston, MA, USA

Correspondence to: David C. Christiani, M.D., Harvard School of Public Health, 665

Huntington Avenue, Boston, MA 02115, USA. E-mail: [dchristi@hsph.harvard.edu](mailto:dchristi@hsph.harvard.edu); Tel: 617

432 3323; Fax: 617 432 6981.

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**Background:** Endothelial injury is an important prognostic factor in acute respiratory distress syndrome (ARDS). Vascular endothelial growth factor (VEGF) plays a critical role in endothelial destruction and angiogenesis. Genetic variations of *VEGF* have been associated with VEGF production. A study was undertaken to investigate the impact of *VEGF* gene polymorphisms on ARDS development and clinical outcomes.

**Methods:** Three *VEGF* polymorphisms (*-460C/T*, *+405C/G*, and *+936C/T*) were determined in 1253 ICU patients with risk factors for ARDS. Among them 394 patients developed ARDS. Patients were followed for 60-day survival. Plasma VEGF levels were measured in 71 patients with ARDS.

**Results:** The *+936TT* (OR=4.29; 95%CI, 1.12-16.40; p=0.03) and *+936CT+TT* (OR=1.98; 95%CI, 1.14-3.42; p=0.01) genotypes were significantly associated with increased ARDS mortality. Plasma VEGF levels in ARDS patients with the *+936CT+TT* genotype (median 49pg/ml, IQR 16-98pg/ml) were significantly (p=0.02) lower than that in subjects with the *+936CC* genotype (median 112pg/ml, IQR 47-162pg/ml). At haplotype levels, haplotype *TCT* (*-460T+405C+936T*) was significantly associated with higher ARDS mortality (OR=2.89; 95%CI, 1.30-6.43; p=0.009). Haplotype *CGT* (*-460C+405G+936T*) was associated less strongly with increased ARDS mortality (OR=1.90; 95%CI, 0.94-3.84; p=0.07). Lower plasma VEGF levels were correlated to the probability of haplotype *CGT* (coefficient=-0.26, p<0.05) but the same trend of correlation was not significant to haplotype *TCT*.

**Conclusions:** *VEGF* polymorphisms may contribute to ARDS prognosis and inter-individual variations in circulating VEGF levels.

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Acute respiratory distress syndrome (ARDS) is lung injury characterized by alveolar injury and increased pulmonary vascular permeability (1-2). The pathogenetic basis of ARDS is incompletely understood; however, emerging evidence has suggested that severity and outcome of ARDS depend significantly on the balance between alveolar epithelial and/or vascular endothelial injuries and their repair mechanisms (2-6).

Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen that promotes angiogenesis and mediates vascular permeability (6-7). Over-expression of VEGF in the lungs induces an increased pulmonary vascular permeability, resulting in pulmonary edema (8). However, VEGF expression in human lung epithelial cells can also increase neovascularization, thereby contributing to the repair of endothelium injuries (6, 9). In human lungs, low VEGF levels were associated with the severity of ARDS, while elevated VEGF levels were associated with ARDS recovery, indicating a role of VEGF in the repair process of lung injury (10-13).

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The *VEGF* gene is located on chromosome 6p31.3. Several functional single nucleotide polymorphisms (SNPs) in this gene have been described in the literature. The +936C/T polymorphisms (rs3025039) have been related to lower levels of plasma VEGF (14-15), whereas the -460C/T (rs833061) and +405C/G (rs2010963) polymorphisms have been shown to significantly increase VEGF production (16). Recent studies have indicated that these three SNPs are implicated in the risk of several disorders in which vascular injury and acceleration of inflammation are critical in disease development (17-18). However, the combined effect of these polymorphisms on ARDS risk and prognosis has not been evaluated. Based on the biological function of these three *VEGF* polymorphisms on VEGF production, and the pathologic significance of VEGF in ARDS, we hypothesized that *VEGF* polymorphisms may

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contribute to the development of and poor outcomes in ARDS and correlate to circulating VEGF levels in patients with ARDS.

## **METHODS**

### **Study subjects**

The present study was part of an ongoing research investigating the effects of multiple genetic variants on the risks or prognosis of ARDS. Recruitment of subjects was started in September 1999 and will continue until 2010. Details of the study have been described previously (19-20). Briefly, all admissions to the intensive care units (ICU) at Massachusetts General Hospital (MGH, Boston, MA) were screened daily for study-defined clinical risk factors for ARDS such as sepsis, trauma, aspiration or massive transfusions and were followed prospectively during their ICU stay for the development of ARDS (Figure 1.). Subjects were classified as ARDS cases if they were intubated on positive ventilation and met the American European Consensus Committee (AECC) criteria for ARDS: (1) Pao<sub>2</sub>/fraction of inspired oxygen ratio of  $\leq 200$ mmHg; (2) bilateral infiltrates seen on chest radiographs; and (3) pulmonary arterial occlusion pressure  $\leq 18$  mm Hg or no clinical evidence of left atrial hypertension. All cases of ARDS were followed for all causes 60-day mortality. This study was approved by The Human Subjects Committees of MGH and the Harvard School of Public Health (Boston, MA), and informed written consent was obtained from all subjects or their appropriate surrogates.

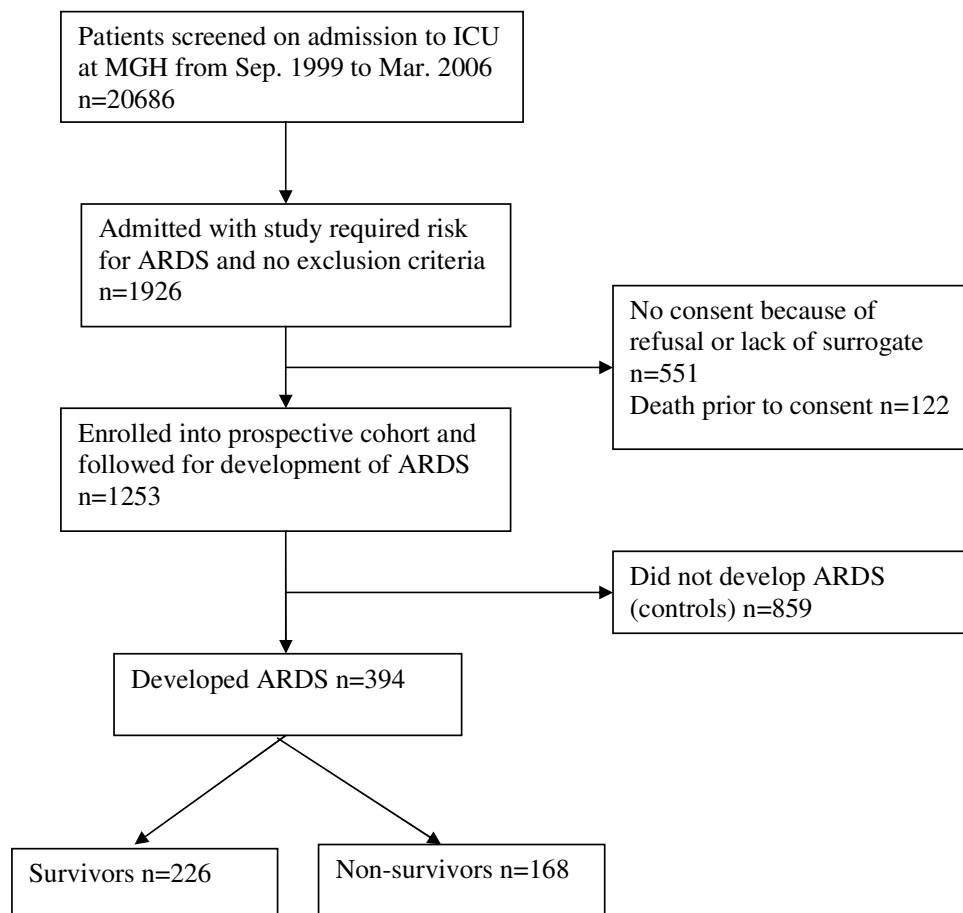
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### **DNA isolation and genotyping assays**

Genome DNA was extracted from peripheral blood samples using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN) following the manufacturer's protocol. The

allelic discrimination of the *VEGF* gene was assessed with the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster city, CA), using the fluorogenic 5' nuclease



**Figure 1.** Flow diagram of study design and patient selection for the case-control study. ICU: Intensive care unit. MGH: Massachusetts General Hospital, Boston, MA, USA. Patients who had previous history of ARDS, who were previously admitted to the ICU were excluded from being controls. Subjects listed in this Figure were all Caucasians. Non-caucasians were also enrolled but they were excluded from the analysis because they accounted for only 4% of all enrollment.

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assay with Taqman Minor Groove Binder (MGB) probes. The wide-type Taqman MGB probes were FAM labeled and the mutant probes were VIC labeled. The primers and probes

for the *-460C/T*, *+405C/G*, and the *+936C/T* polymorphism assays were ordered from Applied Biosystems. Genotyping was performed by laboratory personnel blinded to subject status, and a random 10% of the samples were repeated to validate genotyping procedures. Two authors reviewed independently all genotyping results.

### **Analysis of VEGF levels in plasma**

Plasma samples from day 2 after development of ARDS were available for 71 ARDS patients for the measurement of circulating VEGF levels. Blood samples were collected in 10ml vacuum tubes and were centrifuged for 10 minutes. Plasma samples were stored at  $-80^{\circ}\text{C}$  until analysis. Plasma VEGF levels were quantified in duplicate according to the manufacturer's recommendations with commercially available ELISA kit (R&D Systems, MN, USA). No statistically significant differences were observed between patients with plasma samples and those without plasma samples in terms of age, gender, APACHE III, and risk factors for ARDS.

### **Statistical analysis**

The demographic variables between different groups were compared by chi-square tests for categorical variables and by Student *t* test and or nonparametric test for continuous variables. The Hardy-Weinberg-equilibrium was evaluated using Chi-square test. Logistic regression model was used to assess the effect of *VEGF* polymorphisms on the mortality of ARDS with adjustments for potential confounding factors such as age, gender, APACHE III score, diabetes, history of steroid use, number of units of red cells transfused, and chronic liver disease. Linkage disequilibrium between the SNPs, haplotypes and their frequencies was estimated using the expectation maximization algorithm. Association between haplotypes and

risk of ARDS was assessed using an "expectation substitution" approach to account for unknown phase (21). Haplotypes were coded as an additive fashion. ARDS mortality was regressed on haplotype counts by logistic regression, using the most common haplotype as reference haplotype. The multiple Cox regression model was applied to test the effect of the *VEGF* polymorphisms on overall survival, adjusting for confounding factors such as age, gender, APACHE III score, diabetes, history of steroid use, and chronic liver disease. The correlations between haplotype probabilities and plasma VEGF levels were estimated by Spearman correlation test. All statistical analysis were performed using the SAS statistical software package (version 9.1, SAS Inc., Cary, NC).

## RESULTS

### Patient population

Between September 1999 and March 2005, 1253 Caucasian patients with risk factors of ARDS were enrolled into the prospective cohort. In this study population, 394 (31.4%) were diagnosed as ARDS cases and 859 did not develop ARDS (Figure 1).

**Table 1. Characteristics between survivals and non-survivors in patients with ARDS**

Covariables	Non-survivors (n=168)	Survivors (n=226)	p
Age (mean±SD)	67.98 (±14.75)	53.88 (±18.62)	<0.01
Female No (%)	72 (42)	92 (41)	0.67
APACHE III score (mean±SD)	89.60 (±22.28)	68.95 (±21.15)	<0.01
Diabetes N (%)	31 (18)	38 (17)	0.70
Chronic liver disease N (%)	8 (10)	6 (3)	<0.01
History of steroid use N (%)	27 (16)	19 (8)	0.02
Bilirubin>2.0mg/L N (%)	41 (27)	27 (14)	<0.01
Creatinine>2.0mg/L N (%)	63 (38)	54 (24)	0.10
Haematological failure N (%) (platelets $\leq 80000\text{mm}^{-3}$ )	48 (29)	35 (16)	<0.01

Clinical risk factors for ARDS and baseline characteristics between subjects with and without ARDS are shown in Table 1 and Table 2 in the online data supplement. Among 394

ARDS cases studied, the 60-day mortality was 42.6% (168/394). Logistic analysis showed that older age, APACHE III, steroid use, hematological failure, and liver disease were the major prognostic factors for survival (Table 1).

### ***VEGF* genotypes in relation to and ARDS mortality**

Genotyping of the *-460C/T*, *+405 C/G*, and *+936 C/T* polymorphisms was successfully achieved for all subjects and followed the Hardy-Weinberg equilibrium. The genotype frequencies of these three polymorphisms in the current study were broadly similar to those reported by others in Caucasian populations (22-28). When overall ARDS cases were compared with non-ARDS patients, no significant difference was found in the distribution of genotypes for any polymorphisms studied ( $P>0.05$ ).

We tested for association between ARDS mortality and any of the three SNPs among the 394 patients with ARDS (Table 2). No significant association was found between the *-460C/T* or *+405C/G* polymorphisms and the risk of ARDS mortality ( $P>0.05$ ). However, the *+936TT* genotype was significantly associated with higher ARDS mortality as compared with the *+936CC* genotype (adjusted OR=4.29; 95%CI, 1.12-16.40;  $p=0.01$ ). Similar association was also observed for the *+936CT* genotype, but this association was not statistically significant (adjusted OR=1.60; 95%CI, 0.90-2.80;  $p=0.10$ ). When the mutant *+936TT* and *+936CT* were combined to form a united genotype, this combined *+936CT+TT* genotype was significantly associated with increased ARDS mortality compared with *+936CC* genotype (adjusted OR=1.98; 95%CI, 1.14-3.42;  $p=0.01$ ). No significant interactions between any genotypes and age, gender, or types of injury were detected in the present study.

**Table 2. Associations of VEGF genotypes and ARDS mortality**

Genotypes	Genotype frequency (%)	OR* (95% CI)	p*	value
<b>All patients (n=394)</b>				
<i>460TT</i>	29.4	1.00		
<i>460CT</i>	46.7	1.33 (0.83-2.14)		0.24
<i>460CC</i>	23.9	0.77 (0.44-1.35)		0.37
<i>405GG</i>	43.9	1.00		
<i>405CG</i>	43.4	1.37 (0.84-2.24)		0.20
<i>405CC</i>	12.7	0.87 (0.43-1.74)		0.69
<i>936CC</i>	73.9	1.00		
<i>936CT</i>	22.8	1.60 (0.90-2.80)		0.10
<i>936TT</i>	3.3	<b>4.29</b> (1.12-16.40)		<b>0.03</b>
<i>936CT+TT</i>	26.1	<b>1.98</b> (1.14-3.42)		<b>0.01</b>
Ptrend#		<b>1.93</b> (1.10-1.94)		<b>0.005</b>

\*Estimated by Logistic regression models, adjusting for age, gender, history of alcohol abuse, diabetes, steroid use, APACHE III score, and multiple (>1) risk factors for ARDS.

# The genotypes were coded as continuous variables (1, 2, and 3 for wild type, heterozygous, and homozygous genotype respectively) and entered together into the logistic regression model.

#### Association between VEGF haplotypes and ARDS mortality

Haplotype analyses were conducted to evaluate the combined effect of the three polymorphisms on ARDS survival. Consistent with observations from early studies (29), VEGF *-460C/T* and *+405C/G* polymorphisms in our study were in strong linkage disequilibrium (correlation coefficient,  $R=0.68$ ; Lewontin's  $D'$ ,  $D'=0.99$ ), whereas their linkage with the *+936C/T* polymorphism was much weaker ( $R=0.04$ ;  $D'=0.1$  and  $R=0.1$ ;  $D'=0.20$ , respectively). Six haplotypes with frequencies greater than 1% were estimated of the eight possible haplotypes (Table 3). No association was detected between any haplotype and risk of ARDS development ( $P>0.05$ ). Interestingly, the *-460T+405C+936T (TCT)* haplotype

was significantly associated with increased risk of ARDS mortality (adjusted OR=2.89; 95%CI, 1.30-6.43; p=0.009), and the *-460C/+405G/+936T (CGT)* haplotype was marginal significantly associated with higher risk of ARDS mortality (adjusted OR=1.90; 95%CI, 0.94-3.87; p=0.07). No associations were demonstrated between other haplotypes and ARDS mortality in this study population.

**Table 3. Associations of VEGF haplotypes and ARDS mortality**

Haplotypes <sup>#</sup>	Haplotype frequency (%)	OR* (95% CI)	p value
Global test			0.026
<i>CGC</i>	39.6	1.0	
<i>TCC</i>	28.9	0.85 (0.59-1.24)	0.4
<i>TGC</i>	16.6	0.95 (0.58-1.55)	0.8
<i>CGT</i>	7.4	<b>1.90 (0.94-3.83)</b>	<b>0.07</b>
<i>TCT</i>	5.3	<b>2.89 (1.30-6.43)</b>	<b>0.009</b>
<i>TGT</i>	1.7	1.09 (0.20-5.84)	0.92

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<sup>#</sup> The order of the polymorphisms is as follows: *-460C/T, +405C/G, +936C/T*.

\* Estimated by Logistic regression models, adjusting for age, gender, history of alcohol abuse, diabetes, steroid use, APACHE III, multiple risk of (>1) of ARDS.

### Relationship between VEGF polymorphisms and plasma VEGF levels

To investigate the relationship between *VEGF* polymorphisms and circulating VEGF levels, plasma VEGF levels were categorized according to *VEGF* polymorphisms. Since the plasma VEGF values were not normally distributed (Kolmogorov-Smirnov test, p=0.01), a nonparametric median two-sample test was used to compare the VEGF levels between different genotype carriers. Median plasma VEGF level in individuals carrying the *+936CT+TT* genotype (median=49pg/ml; IQR, 16-98) was significantly (p=0.02; power>0.99) lower than that in individuals with the *VEGF+936CC* genotype (median=112pg/ml; IQR, 47-162). Based on the sample sizes and median plasma VEGF levels between the *+936CT+TT* and the *VEGF+936CC* carriers, the power to detect a difference at  $\alpha=0.05$  level was



calculated to be 99%. At haplotype levels, the plasma VEGF levels were inversely correlated with haplotype *CGT* probability (Spearman coefficient, -0.26,  $p < 0.05$ ). A similar correlation was also observed between plasma VEGF levels and the haplotype *TCT* probability, but this correlation did not reach statistical significance (coefficient, -0.14,  $p = 0.26$ ). Thus, haplotypes containing the *936T* allele tended to correlate to lower plasma VEGF levels. There was no significant difference between survivors and non-survivors in plasma VEGF levels ( $p > 0.05$ ).

#### **Association between *VEGF* polymorphisms and ARDS Survival**

Cox proportional hazard model was used to analyze the associations between *VEGF* polymorphisms and ARDS survival. After adjusting for other predictors of survival (age, gender, APACHE III score, diabetes, history of steroid use, hematological failure, and chronic liver disease), both the *+936CT+TT* genotype (HR=1.71; 95% CI, 1.09-2.72;  $p = 0.02$ ) and haplotype *TCT* (HR=2.11; 95%CI, 1.32-3.37;  $p < 0.01$ ) were significantly associated with lower ARDS survival, consistent with the results assessed by Logistic regression models.

## DISCUSSION

Our data showed that the +936TT and +936CT+TT genotypes of VEGF gene were significantly associated with increased risk of ARDS mortality. At haplotype levels, the TCT haplotype was significantly associated with higher ARDS mortality. In addition, we demonstrated that VEGF polymorphisms contributing to increased ARDS mortality were correlated with lower plasma VEGF levels.

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This study has a number of strengths. Firstly, the prospective determination of ARDS using the AECC definition minimized phenotype misclassification, since there is no diagnostic gold standard for ARDS. Secondly, the VEGF polymorphisms and the circulating VEGF levels were determined in a parallel manner. Thus, the functional effect of VEGF polymorphisms can be assessed. Thirdly, restricting analyses to patients of Caucasian reduced the possibility of altered genotype frequencies in different ethnicities.

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Among these three functionally SNPs, none of the -460C/T and +405C/G polymorphisms was found to significantly influence ARDS mortality. However, the +936TT and the combined +936CT+TT genotypes were significantly associated with ARDS mortality, suggesting that +936 polymorphisms may play a stronger role than either the -460C/T or +405C/G polymorphisms in VEGF gene functions. Recently, a smaller case-control study found that the frequency of the combined +936CT+TT genotype in ARDS cases was significantly higher than that in controls. This study also found that the +936CT and TT genotypes were associated with APACHE III score among the ARDS patients, but association between the +936 CT/TT genotype and increased ARDS mortality did not reach statistical significance (OR>1.80, p>0.05) (26). In the present, larger study, although +936CT+TT genotype was not associated with ARDS susceptibility, a significant association

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between the +936CT+TT genotype and ARDS mortality was found. At haplotype level, haplotype containing the +936T allele was also associated with ARDS mortality, further supporting the effects of +936C/T variants on ARDS outcomes.

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Our data showed that the +936 CT/TT genotype was significantly correlated to lower plasma VEGF levels, consistent with the functions of this polymorphism reported by other studies (14-15). Although the plasma VEGF levels were measured at only one point that may not reflect fully the temporal VEGF response over the course of ARDS, our results suggested that VEGF may be a protective molecule in ARDS severity. Decreased VEGF concentrations in the lungs have been correlated to higher lung injury score and lower ARDS survival rates (10, 11, 31). whereas increased VEGF production in the lungs resulted in less lung injury (33).

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In animal models, administration of VEGF could prevent lung injury-induced death (30, 32, 33). In alveolar epithelial cells VEGF mRNA expression increased during recovery from oxygen injury (34). In neonatal rats exposed to high oxygen, intraocular injection of VEGF at the onset of experimental hyperoxia prevents apoptotic death of endothelial cells and rescues the retinal vasculature (35). Taken together, these findings indicate that the VEGF may function as a protective factor in ARDS.

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Although the haplotypes predicted in the present study were identical to those previously reported, the overall distribution of haplotype frequencies in the present study was different from that in Chinese and Korean populations (29, 36). Different haplotype frequencies in different populations may be due to different ethnic backgrounds or different disease states.

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Interestingly the frequencies of TCT (5.3%) and CGT (7.4%) haplotypes in the present study were similar to those reported in the Chinese population (6.0% and 6.4% for TCT and CGT, respectively) (29). In Asian populations, both the TCT and CGT haplotypes were significantly

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implicated in reduced risk of lung cancer, and the *CGT* was associated with decreased breast survival (29, 36). These observations suggested that *TCT* and *CGT* haplotypes may play important roles in disease processes. However, since these previous studies did not measure circulating VEGF levels, the mechanisms connecting the *VEGF* haplotypes and disease development or severity were unclear. Our study not only indicated that the *TCT* haplotype was associated with ARDS mortality, but also demonstrated that the *CGT* and *TCT* haplotypes correlated with lower circulating VEGF protein levels, suggesting that these functional haplotypes may contribute to ARDS mortality by affecting VEGF production.

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We acknowledge some potential limitations to this study. Firstly, sample size in this study was relatively small. Additional studies with larger populations are required to confirm the associations between the *VEGF* haplotypes and ARDS mortality. Secondly, the *VEGF* gene is a polymorphic gene, and the present study only investigated three SNPs. Further studies on other functional *VEGF* SNPs are needed to define the role of the *VEGF* polymorphisms in ARDS prognosis. Thirdly, plasma VEGF measurement was done at one time point so the role of plasma VEGF level in the natural history of ARDS remained to be defined. Finally, since genetic polymorphisms often vary between ethnic groups, further studies are necessary to clarify the association between the *VEGF* polymorphisms and ARDS outcome in diverse ethnic populations.

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In summary, our study suggested that +936TT, +936CT+TT genotypes and *TCT* haplotype of *VEGF* gene are attributable to increased ARDS mortality and to inter-individual variations in plasma VEGF levels. The associations found in this study are biologically plausible and may have significant implications for ARDS treatment.

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