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

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.
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°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 χ² 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 χ² 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>
<thead>
<tr>
<th>Covariables</th>
<th>Non-survivors (n = 168)</th>
<th>Survivors (n = 226)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (SD) age (years)</td>
<td>67.98 (14.75)</td>
<td>53.88 (18.62)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>72 (42)</td>
<td>92 (41)</td>
<td>0.67</td>
</tr>
<tr>
<td>Mean (SD) APACHE III score</td>
<td>89.60 (22.28)</td>
<td>68.95 (21.15)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>31 (18)</td>
<td>38 (17)</td>
<td>0.70</td>
</tr>
<tr>
<td>Chronic liver disease, n (%)</td>
<td>8 (10)</td>
<td>6 (3)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>History of steroid use, n (%)</td>
<td>27 (16)</td>
<td>19 (8)</td>
<td>0.02</td>
</tr>
<tr>
<td>Bilirubin &gt;2.0 mg/l, n (%)</td>
<td>41 (27)</td>
<td>27 (14)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Creatinine &gt;2.0 mg/l, n (%)</td>
<td>63 (38)</td>
<td>54 (24)</td>
<td>0.10</td>
</tr>
<tr>
<td>Haematological failure (&lt;80 000 platelets/mm³), n (%)</td>
<td>48 (29)</td>
<td>35 (16)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
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.21 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 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, +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 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 +936CT and +936CC 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.

Table 2

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

*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.

Association between VEGF haplotypes and ARDS mortality
Haplotypes were combined to form a united genotype, this combined +936CT+TT genotype 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 α = 0.05 level was calculated to be 99%. At haplotype levels, the
Association between VEGF polymorphisms and ARDS mortality

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 VEGF levels. There was no significant difference between survivors and non-survivors in plasma levels of VEGF (p>0.05).

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).

### Table 3

<table>
<thead>
<tr>
<th>Haplotypes†</th>
<th>Haplotype frequency (%)</th>
<th>OR (95% CI)</th>
<th>p Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global test</td>
<td></td>
<td>1.0</td>
<td>0.026</td>
</tr>
<tr>
<td>CCG</td>
<td>39.6</td>
<td>0.85 (0.59 to 1.24)</td>
<td>0.4</td>
</tr>
<tr>
<td>TCC</td>
<td>28.9</td>
<td>0.95 (0.58 to 1.55)</td>
<td>0.8</td>
</tr>
<tr>
<td>TGG</td>
<td>16.6</td>
<td>1.90 (0.94 to 3.83)</td>
<td>0.07</td>
</tr>
<tr>
<td>CTG</td>
<td>7.4</td>
<td>2.89 (1.30 to 6.43)</td>
<td>0.009</td>
</tr>
<tr>
<td>TCT</td>
<td>5.3</td>
<td>1.09 (0.20 to 5.84)</td>
<td>0.92</td>
</tr>
<tr>
<td>TGT</td>
<td>1.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*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.

In the present 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 +936CT 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. 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, whereas increased VEGF production in the lungs resulted in less lung injury. In animal models, administration of VEGF prevented lung injury-induced death. In alveolar epithelial cells, VEGF mRNA expression increased during recovery from oxygen injury. In neonatal rats exposed to high oxygen levels, intravenous injection of VEGF at the onset of experimental hyperoxia prevented apoptotic death of endothelial cells and rescued the retinal vasculature. 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. 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). 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. 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 interindividual 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.

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Competing interests: Dr Christiani is a paid scientific advisor to Gentra Corporation.

REFERENCES