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Genetic variants in the *angiopoietin-2* gene are associated with increased risk of ARDS

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Abstract *Objective:* Angiopoietin-2 (Ang-2) is a potent regulator of vascular permeability and inflammation in acute lung injury and acute respiratory distress syndrome (ARDS). Genetic variants in the *Ang-2* gene may lead to altered activities of *Ang-2* (or *ANGPT2*) gene. The aim of this study was to assess if genetic variants of *Ang-2* are associated with the risk of ARDS. *Design:* Unmatched, case-control study nested within a prospectively enrolled cohort. *Setting:* Intensive care units (ICU) of an academic medical center. *Patients:* About 1,529 critically ill patients with risk factors for ARDS consecutively admitted to the ICUs from 1999 to 2006. Cases were 449 patients who developed ARDS and controls were 1,080 subjects who did not developed ARDS. *Intervention:* None. *Measurements and results:* Nine tagging SNPs (tSNPs) spanning the entire *Ang-2* gene were genotyped in all patients. The results were analyzed using logistic regression models, adjusting for covariates. The variant *T* allele of one tSNP (rs2515475) was significantly associated with increased risk of ARDS ($OR_{adjusted} = 1.28$; $P = 0.042$). This association was stronger in subjects with extrapulmonary injuries ($OR_{adjusted} = 1.79$; $P = 0.004$). Haplotype *TT* in block 2 containing the *T* allele of the rs2515475 was also significantly associated with higher risk of ARDS ($OR_{adjusted} = 1.42$; $P = 0.009$), particularly in subjects with extrapulmonary injuries ($OR_{adjusted} = 1.90$; $P = 0.004$). *Conclusion:* Common genetic variation in the *Ang-2* gene may be associated with increased risk of ARDS, especially among patients with extrapulmonary injuries.

Keywords Angiopoietin-2 · Genetic susceptibility · ARDS · Tagging single nucleotide polymorphism · Haplotype · Molecular epidemiology

Introduction

Acute respiratory distress syndrome (ARDS) is characterized by non-cardiogenic pulmonary edema and acute respiratory failure in seriously ill patients [1]. The pathological alterations of ARDS include diffuse endothelial and epithelial damage, with neutrophils, macrophages, erythrocytes, hyaline membranes, and protein rich edema

fluid in the alveolar spaces, as well as capillary injury, and disruption of the alveolar epithelium [2, 3]. Endothelial activation and dysfunction has been shown to play a major role in the development of organ injuries thereby representing an independent parameter for worse clinical outcome in critically ill patients [4, 5].

Angiopoietin-2 (Ang-2) is a vascular growth factor that binds to the endothelial cell-specific receptor, Tie-2 [6, 7]. Ang-2 functions as an antagonist ligand of the Tie-2 [8]. Therefore, Ang-2 is able to destabilize blood vessels, to enhance vascular leakage, to induce vascular regression, and to prime the endothelium to respond to VEGF and other angiogenetic and inflammatory cytokines [9, 10]. Ang-2 is stored in endothelial cell-specific storage granules (Weibel-Palade bodies) and is rapidly released upon stimulation of the endothelium, serving as a potential marker of endothelial activation and dysfunction [11]. In hyperoxic acute lung injury, *Ang-2* gene is overexpressed in lung epithelial cells, where it has critical roles in oxidative injury, epithelial cell death, and inflammation [12]. Increased circulating Ang-2 levels have been observed in patients with acute lung injury [12] and sepsis [13, 14]. Circulating Ang-2 levels were correlated with mortality of ARDS and acute lung injury [15].

Human *Ang-2* (*ANGPT2*) gene is located on chromosome 8 and has been found to be highly polymorphic [16, 17]. Genetic variants in the *Ang-2* gene may affect *Ang-2* gene expression or vascular angiogenesis [18, 19]. Studies of a single nucleotide polymorphism (SNP) in angiogenesis-associated diseases suggest that the effects of individual SNPs of *Ang-2* on disease susceptibility are limited [20, 21]. However, no studies have been addressed the impact of multiple genetic variants of *Ang-2* on disease risk.

In this study, we evaluated common genetic variations across the entire *Ang-2* gene using a haplotype tagging SNP approach to test the hypothesis that variations in *Ang-2* gene are associated with ARDS risk in patients with clinical risk factors for ARDS. Since pulmonary and extrapulmonary acute lung injury are known to express different morphological and inflammatory patterns [22, 23], and *Ang-2* is selectively expressed by endothelial cells at sites of pathologic angiogenesis [24, 25], we further hypothesized that the associations between *Ang-2* polymorphisms and ARDS risk may be related to the sites of initial insult predisposing to pulmonary or extrapulmonary ARDS.

Methods

Study subjects

This study is part of an ongoing molecular epidemiology project investigating the influences of genetic factors on the development and outcomes of ARDS. Details of the study have been described previously [26]. Briefly, study subjects in the present study were selected from patients admitted to the intensive care units (ICU) at Massachusetts General Hospital (MGH, Boston, MA) from September 1999 to November 2006. Patients with clinical risk factors for ARDS such as sepsis, septic shock, trauma, pneumonia, aspiration, or multiple transfusions were eligible for inclusion (Supplemental Table 1). Exclusion criteria included age of <18, diffuse alveolar hemorrhage, chronic lung diseases other than COPD or asthma, directive to withhold intubation, immunosuppression not secondary to corticosteroid, and treatment with granulocyte colony-stimulating factor. Baseline characteristics and acute physiology and chronic health evaluation (APACHE) III scores were recorded on ICU admission. Baseline clinical and laboratory information were collected in the first 24 h of ICU admission. All patients were followed daily during their ICU stay for development of ARDS. Patients who were intubated and fulfilled the American–European Consensus Committee (AECC) criteria for ARDS [27] were considered as ARDS cases, whereas at-risk patients who did not meet the criteria for ARDS were considered as controls. The MGH Human Subjects Committee approved the study and informed written consent was obtained from all subjects or surrogates.

SNP selection and genotyping

Tagging SNPs were selected based on HapMap phase II release [17]. A genomic region of 66.6 kbp on chromosome 8p23.1 containing the entire *Ang-2* gene plus about 3 kbp each upstream and downstream was selected. Nine haplotype tagging SNPs were identified using the following tagging criteria: pairwise tagging of the HapMap CEU Population with $r^2 \geq 0.8$ and a minor allele frequency (MAF) $\geq$10% (Supplemental Table 2). Pairwise linkage disequilibrium (LD) between the nine tagging SNPs in *Ang-2* was measured by D' and r^2, and was calculated based on the genotypes of 1,529 study subjects using the Haploview software. Genomic DNA was extracted from whole blood using Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN) and/or the AutoPure LS® Workstation (Qiagen, Valencia, CA).

Genotyping was performed using TaqMan® SNP Genotyping Assay and ABI Prism® 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Laboratory personnel were blinded to case-control status and 10% of randomly selected samples were interspersed in the plates for quality controls. All genotyping results were reviewed by two investigators independently.

Statistical analysis

We compared baseline variables using χ^2 test, Fisher's exact test, Student's *t*-test, or Wilcoxon test, as appropriate. The Hardy–Weinberg equilibrium was evaluated using χ^2 test. The LD between SNPs, haplotypes and their frequencies were estimated using the expectation maximization algorithm [28]. Haplotypes were coded as an additive fashion. Logistic regression model was used to assess the associations of *Ang-2* polymorphisms with ARDS in both dominant and additive models, adjusting for potential confounding factors including age, gender, APACHE III score, diabetes, and history of steroid use.

To adjust for multiple comparisons in SNP association analyses, the false discovery rate (FDR) was assessed using SAS procedure PROC MULTTEST [29]. To account for multiple comparisons in estimating haplotype association, we conducted global tests of association by simultaneously including all of the haplotypes in the logistic regression model and then comparing it with a null model without any of the SNPs or haplotypes [30]. This multiloci global test automatically adjusts for multiple testing based on the *df* of the corresponding χ^2 test.

All statistical analyses were performed using the SAS statistical software package (version 9.1, SAS, Cary, NC). A P value < 0.05 was considered to be statistically significant.

Results

Study population

Although individuals of all races were screened for this study, we restricted our analysis to Caucasians since 92% of ICU admissions at MGH were Caucasians. A total of 1,529 consecutive ICU admissions meeting the study criteria and with no exclusion criteria were recruited. Among them 449 patients were diagnosed as ARDS cases and 1,080 subjects were classified as non-ARDS controls. The baseline characteristics of the study population on admission to the ICU are shown in Table 1. Compared with controls, patients who developed ARDS had higher APACHE III scores, higher serum bilirubin levels, and lower platelets counts.

Associations of *Ang-2* tagging SNPs with ARDS risk

The genotyping success rates ranged from 98 to 99% and did not deviate significantly from Hardy–Weinberg equilibrium. Genotype frequencies did not differ between cases and controls for all tagging SNPs. In overall analyses, variant genotypes of rs2515475 ($P = 0.042$) and rs2959811 ($P = 0.028$) in LD block 2 showed borderline significant associations with increased risk of ARDS development (Table 2).

Table 1 Baseline characteristics of the study population

Characteristics	All patients ($n = 1,529$)	Patients developed ARDS ($n = 449$)	Patients did not develop ARDS ($n = 1,080$)	*P* value
Age (year)	61.9 ± 17.3	58.1 ± 18.0	62.7 ± 16.9	0.101
Female	604 (39.5)	184 (41.0)	420 (38.9)	0.456
APACHE III score	70.1 ± 23.7	77.8 ± 23.7	66.9 ± 23.0	<0.0001
Pulmonary injury[a]	856 (56.0)	325 (72.4)	531 (49.2)	<0.0001
Pneumonia[b]	772 (50.5)	303 (67.5)	469 (43.4)	<0.0001
Aspiration	136 (8.9)	45 (10.0)	91 (8.4)	0.318
Pulmonary contusion	62 (4.1)	20 (4.5)	42 (3.9)	0.610
Extrapulmonary injury	673 (44.0)	124 (27.6)	549 (50.8)	<0.0001
Sepsis from extra-pulmanary sources	526 (34.4)	89 (19.8)	437 (43.0)	0.020
Trauma without pulmonary contusion	110 (7.2)	31 (6.9)	79 (7.3)	0.777
Multiple transfusion	168 (11.0)	46 (10.2)	122 (11.3)	0.549
Bilirubin (mg/dL)	1.6 ± 3.7	2.2 ± 5.0	1.3 ± 2.9	<0.0001
Creatinine (mg/dL)	1.9 ± 1.9	1.8 ± 1.4	1.9 ± 2.1	0.345
Diabetes	379 (24.8)	79 (17.6)	300 (27.8)	<0.0001
Platelets ($\times 1,000/mm^3$)	207.7 ± 131.1	188.2 ± 141.1	202.8 ± 126.8	0.006
Liver cirrhosis/failure	70 (4.6)	28 (6.2)	42 (3.9)	0.059
History of steroid use	151 (9.9)	52 (11.6)	99 (9.2)	0.158
History of alcohol abuse	170 (11.1)	62 (13.8)	108 (10.0)	0.040
PaO_2/FiO_2	230.3 ± 132.6	160.4 ± 96.0	261.2 ± 132.3	<0.0001
PEEP[c]	7.0 ± 3.0	8.2 ± 3.6	6.26 ± 2.3	<0.0001
Pre-ICU hospital stay (days)	3.6 ± 8.4	3.4 ± 7.8	3.5 ± 8.4	0.966

Data are presented as *n* (%) or mean ± SD

[a] Patients with both pulmonary and extrapulmonary injuries ($n = 83$) were classified into the subgroup of pulmonary injury

[b] Including sepsis with pneumonia source ($n = 733$)

[c] PEEP: positive end-expiratory pressure

Table 2 Associations between *Ang-2* tagging SNPs and ARDS risk

Polymorphism	All subjects (n = 1,529)		Patients with pulmonary injury (n = 856)		Patients with extrapulmonary injury (n = 673)	
	OR (95% CI)[b]	P value[a]	OR (95% CI)[b]	P value[a]	OR (95% CI)[b]	P value[a]
rs2916702	0.88 (0.74–1.04)	0.129	0.85 (0.69–1.05)	0.125	0.97 (0.73–1.29)	0.831
rs2442468	1.01 (0.86–1.19)	0.894	1.16 (0.94–1.43)	0.159	0.78 (0.58–1.04)	0.090
rs2442635	0.93 (0.78–1.09)	0.353	0.87 (0.71–1.07)	0.174	1.07 (0.80–1.42)	0.648
rs2515435	0.90 (0.76–1.07)	0.233	0.83 (0.68–1.02)	0.079	1.09 (0.82–1.46)	0.557
rs2515466	1.12 (0.93–1.35)	0.223	1.18 (0.94–1.48)	0.156	1.02 (0.73–1.43)	0.903
rs2515475	**1.28 (1.01–1.63)**	**0.042**	1.03 (0.76–1.39)	0.865	**1.79 (1.21–2.65)**	**0.004**[c]
rs2959811	**1.21 (1.02–1.45)**	**0.028**	1.13 (0.92–1.39)	0.258	**1.35 (1.02–1.81)**	**0.040**[d]
rs2442599	0.95 (0.78–1.14)	0.550	0.94 (0.74–1.19)	0.590	0.96 (0.70–1.31)	0.792
rs2897911	0.98 (0.83–1.17)	0.852	1.03 (0.83–1.28)	0.766	0.86 (0.63–1.16)	0.317

Values in bold denote statistically significant

[a] Data were analyzed using additive model

[b] Adjustment for age, gender, APACHE III score, liver cirrhosis/failure, steroid use, and diabetes

[c] FDR $P = 0.0036$

[d] FDR $P = 0.180$

To further investigate whether genetic associations are related to the sites of injuries, subjects were divided into two subgroups based on the sources of clinical injuries: pulmonary injury ($n = 856$; including patients with pneumonia, aspiration or pulmonary contusions) and extra-pulmonary injury ($n = 673$, consisting of patients with sepsis from extra-pulmonary sources, trauma without pulmonary contusion and multiple transfusion). Patients with both pulmonary and extrapulmonary injuries ($n = 83$) were classified into the subgroup of pulmonary injury. Extrapulmonary injury and diabetes were less frequent in patients with ARDS. When patients were stratified by pulmonary and extrapulmonary injury, genotype frequencies of tSNP rs2515475 was significantly different between cases and controls among subjects with extrapulmonary injury ($P = 0.017$). Logistic regression analysis adjusting for covariates showed that associations of rs2515475 and rs2959811 with ARDS risk were stronger in patients with extrapulmonary injury (OR = 1.79; 95% CI, 1.21–2.65; $P = 0.004$ for rs2515475; OR = 1.35; 95% CI, 1.02–1.81; $P = 0.040$ for rs2959811). The association of rs2515475 with ARDS development remained significant even after adjusting for multiple comparisons (FDR $P = 0.004$). But the association between rs2959811 and ARDS risk became insignificant after adjusting for multiple comparison (FDR $P = 0.180$). No significant associations were observed for the other seven tagging SNPs.

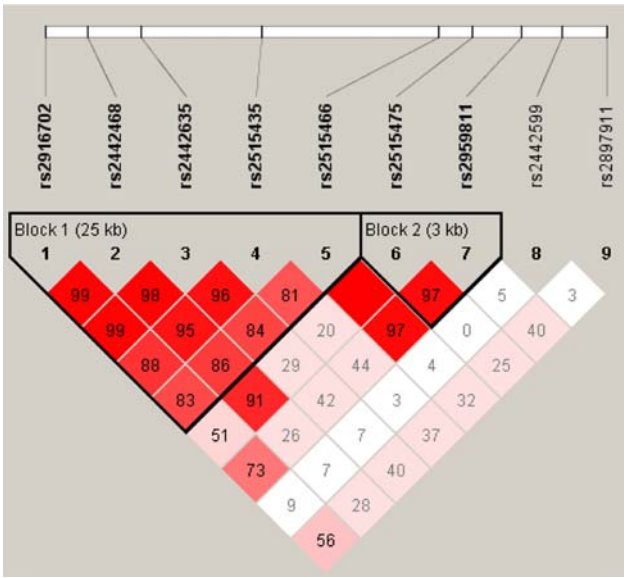

Fig. 1 Linkage disequilibrium (LD) plot. Coefficients (D') of pairwise LD between the nine tagging SNPs in the *Ang-2* gene arrayed by physical location. Blocks of high LD are outlined as triangles and numbered as indicated in the figure. Shading reflects differences in pairwise LD (*white* r^2 = low LD; *red* r^2 = near-perfect LD). Numbers in squires are estimates of pairwise coefficients (D'), expressed in percentages. Unreported values reflects D' of 1.0 (100%)

Associations of *Ang-2* haplotypes with ARDS risk

We used Haploview to assess pairwise LD and define haplotype blocks. Two haplotype blocks were constructed based on correlation coefficient (γ^2) between pairs of loci. (Fig. 1). Block 1 contained five tSNPs (rs2916702, rs2442468, rs2442635, rs2515435, and rs2515466) spanning 25 kbp on the upstream region. Block 2 included two tSNPs (rs2515475 and rs2959811) spanning 3 kbp on the down stream region. We further investigated whether combinations of alleles at multiple loci (haplotypes) were associated with ARDS risk. Reconstruction of haplotypes in LD block 1 generated five common haplotypes (≥5%) in our study population. In global test, haplotypes in LD block 1 were not significantly associated with ARDS risk ($P = 0.115$), although the individual

Table 3 Associations between *Ang-2* haplotypes and ARDS risk

LD block	Haplotype[c]	Frequencies (%)		All subjects (n = 1,529)		Patients with pulmonary injury (n = 856)		Patients with extrapulmonary injury (n = 673)	
		Cases	Controls	OR[d] (95% CI)	P value	OR[d] (95% CI)	P value	OR[d] (95% CI)	P value
Block 1[a]	Global test				0.115		0.388		0.082
	TCCCG	32.4	34.8	1.0		1.0		1.0	
	CGTGG	23.4	24.5	1.02 (0.80–1.29)	0.882	1.18 (0.88–1.57)	0.280	0.77 (0.51–1.15)	0.206
	CGTGA	24.3	22.1	1.26 (1.00–1.59)	0.049	1.43 (1.07–1.91)	0.014	1.01 (0.68–1.52)	0.956
	CCCCG	7.5	7.0	1.36 (0.95–1.95)	0.095	1.32 (0.84–2.25)	0.225	1.40 (0.77–2.56)	0.267
	CCTGG	7.1	5.0	**1.69 (1.15–2.48)**	**0.008**	1.37 (0.84–2.08)	0.209	**2.12 (1.13–3.99)**	**0.019**
Block 2[b]	Global test				**0.040**		0.440		**0.029**
	CG	58.5	61.8	1.0		1.0		1.0	
	CT	25.7	24.8	1.03 (0.92–1.39)	0.232	1.20 (0.94–1.54)	0.153	1.04 (0.72–1.49)	0.848
	TT	15.7	13.2	**1.42 (1.09–1.85)**	**0.009**	1.16 (0.83–1.63)	0.377	**1.90 (1.23–2.92)**	**0.004**

Values in bold denote statistically significant

[a] The order of polymorphisms is: rs2916702–rs2442468–rs2442635–rs2515435–rs2515466

[b] The order of polymorphisms is: rs2515475–rs2959811

[c] Haplotype frequencies <5% were not included in the analyses

[d] Adjustment for age, gender, APACHE III score, liver cirrhosis/failure, steroid use, and diabetes

haplotype *CCTGG* was significantly associated with increased risk of ARDS (OR = 1.69, 95% CI, 1.15–2.48; $P = 0.008$). In LD block 2, haplotypes were globally associated with ARDS risk ($P = 0.040$). Specifically, haplotype *TT* was significantly associated with ARDS risk in overall analysis (OR = 1.42; 95% CI, 1.09–1.85; $P = 0.009$). Similar with the results from individual tSNP analysis, haplotype association with ARDS risk was stronger in patients with extrapulmonary injury (OR = 1.90; 95% CI, 1.23–2.92; $P = 0.004$) (Table 3).

Discussion

In this study, we investigated the associations between genotypes and haplotypes of nine tagging SNPs in the *Ang-2* gene and the risk of ARDS. The variant *T* allele of one tSNP (rs2515475) was identified to be significantly associated with increased risk of ARDS, particularly in patients with extrapulmonary injury. These associations remained significant after adjusting for variables that were associated with ARDS development. Consistent with findings in genotype analyses, a haplotype harboring the *T* allele of rs2515475 was also associated with higher risk of ARDS, particularly among subjects with extrapulmonary injury. Our results suggest that ARDS of different origins may have distinctive pathogenic pathways and require different clinical management strategies.

The association of common genetic variation in *Ang-2* with ARDS risk is biologically plausible for several reasons: (1) Ang-2 has been identified as a critical factor in pulmonary vascular leak required for the development of ARDS [13]; (2) Ang-2 levels in plasma and alveolar edema fluid are increased in patients with acute lung injury and pulmonary edema [12, 31, 32]; (3) Serum Ang-2 levels are associated with disease severity and inflammatory mediators in sepsis [9, 14]; (4) In vitro study has suggested that variations in the *Ang-2* gene alter gene expression [18]. It has been shown that genetic variations in the *Ang-2* gene affect vascular development and angiogenesis [19].

Although only one tagging SNP (rs2515475) was associated with increased risk of ARDS, it is worth noting that the associations of rs2515475 with ARDS development are more likely to depend on LD between rs2515475 and rs2959811 than on individual SNPs. In support of this, haplotype *TT* that contains the variant allele of rs2515475 and rs2959811 is significantly associated with increased risk of ARDS in the same direction as that of rs2515475 or rs2959811 variants. Consistent with the effect of rs2515475, the association between haplotype *TT* and ARDS was also stronger in patients with extrapulmonary injury, suggesting that the effects of rs2515475 and *TT* haplotype were similar. Moreover, these two tagging SNPs are probably representative genetic markers rather than causative genetic polymorphisms in ARDS development. Both the rs2515475 and rs2959811 are located in LD block 2 that capture other 15 tagging SNPs across a genomic region of about 2 kbp (Fig. 1; Supplemental Table 2). Since these 15 tSNPs are in highly LD with each other, the associations of rs2515475 and rs2959811 with ARDS development may reflect the combined contributions of genetic variants in the LD block 2 to ARDS development.

The associations between the *Ang-2* polymorphisms and ARDS susceptibility are more evident in subjects with extrapulmonary injury. Such effect modification by the type of lung injury may suggest a possible gene-environment interaction in ARDS. Similar heterogeneous effects on ARDS risk have been seen with candidate genes in inflammatory pathways, such as Inhibitor kappa B-alpha (*IκB*) gene and tumor necrosis factor (*TNF*) gene [26, 33]. The mechanisms underlining the different

response to genetic associations are not clear. However, it has been observed that pulmonary insult primarily affects the alveolar epithelium with a local alveolar inflammatory response, while the extrapulmonary insult affects the vascular endothelium by inflammatory mediators through blood stream [34]. The inflammatory response, histological and mechanical properties of the lung differ depending on whether the etiology of acute lung injury or ARDS is pulmonary or extrapulmonary [22, 23, 35, 36]. Acute lung injury resulting from distinct insults may lead to different gene expression profiles [37]. Since extrapulmonary injury is characterized with systematic vascular endothelium damage [34], and Ang-2 is mainly stored in endothelial cell-specific storage granules [11] and contributes to pulmonary endothelial barrier disruption [13], it is possible that the *Ang-2* variants affects ARDS development by regulating endothelial cell *Ang-2* expression and vascular stability [18].

The strengths of our study include a comprehensive approach toward characterizing the *Ang-2* gene by leveraging the LD that exists at this locus, carefully adjusting for potential confounders, as well as stringent laboratory quality-control procedures. Despite these strengths, we acknowledge some limitations of our study. First, although our results have been adjusted for multiple comparisons, we underscore the need for replication of our findings given the large number of false positive generated in genetic association studies [38]. Second, we did not re-sequence the gene and instead used publicly available SNP databases. Thus, some variation could have been missed due to incompleteness of these databases. Third, we did not examine the functions of *Ang-2* genotypes and haplotypes and Ang-2 levels, thus the functional significance of *Ang-2* variations in ARDS risk remains to be defined. Fourth, due to the study design, the results may not be generalized to the community setting, to patients with different risk factors for ARDS. In addition, the analyses were restricted to Caucasians, which reduced the possibility of confounding from different genetic make-up, but the extrapolation of the results to other ethnic groups might not be applicable.

In summary, we observed significant associations of genetic variants in *Ang-2* with increased risk of ARDS. We also found that the contributions of *Ang-2* polymorphisms to ARDS risk were associated with the etiology of lung injury. However, as this was the first study to comprehensively analyze the genetic variation in *Ang-2* and ARDS risk, further studies are needed to replicate these interesting findings.

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