

Review

Science review: Searching for gene candidates in acute lung injury

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Abstract

Acute lung injury (ALI) is a complex and devastating illness, often occurring within the setting of sepsis, and carries an annual mortality rate of 30–50%. Although the genetic basis of ALI has not been fully established, an increasing body of evidence suggests that genetic predisposition contributes to disease susceptibility and severity. Significant difficulty exists, however, in defining the exact nature of these genetic factors, including large phenotypic variance, incomplete penetrance, complex gene–environment interactions, and strong potential for locus heterogeneity. We utilized the candidate gene approach and an ortholog gene database to provide relevant gene ontologies and insights into the genetic basis of ALI. We employed a Medline search of selected basic and clinical studies in the English literature and studies sponsored by the HopGene National Institutes of Health sponsored Program in Genomic Applications. Extensive gene expression profiling studies in animal models of ALI (rat, murine, canine), as well as in humans, were performed to identify potential candidate genes (<http://www.hopkins-genomics.org/>). We identified a number of candidate genes for ALI, with blood coagulation and inflammation gene ontologies being the most highly represented. The candidate gene approach coupled with extensive gene profiling and novel bioinformatics approaches is a valuable way to identify genes that are involved in ALI.

Keywords acute lung injury, candidate genes, gene expression, gene ontology, microarrays, polymorphisms

Introduction

Acute lung injury (ALI) is a common and devastating illness that most often occurs in the setting of sepsis [1]. Despite impressive technologic advances in our ability to monitor this critically ill population, ALI continues to carry an annual mortality rate of 30–50%. Recently, advances in the management of patients with ALI with low tidal volume ventilation offered hope that combined mechanistic and physiologically sound approaches to ALI may further reduce mortality from this illness [2]. Our understanding of the pathogenesis of sepsis and ALI recently improved with the

appreciation that inflammation is a fundamental component of the pathophysiology and is exacerbated by conventional or high tidal volume ventilation [3]. Unfortunately, these insights have not fully been translated into novel and effective strategies designed to increase survival. Furthermore, our improved understanding of ALI at both the molecular and population levels has not provided an explanation for the heterogeneity in patient susceptibility and outcome. Clearly, both genetic and environmental factors must be involved. Although the genetic basis of ALI has not been fully established, an increasing body of evidence suggests that

genetic predisposition contributes to disease susceptibility and severity [4–7]. Why do some patients with Gram-negative sepsis develop ALI whereas others do not? Why is low tidal ventilation of great benefit in some patients and not in others? What are the genetic determinants that convey risk for progression to multiorgan failure in patients with ALI? A complete understanding of the genetic basis of ALI susceptibility and disease severity would address these important questions.

Because ALI is a complex illness, alterations in specific illness genes will probably not explain the physiologic derangements fully. Large phenotypic variance, incomplete penetrance, complex gene–environment interactions, and potential locus heterogeneity all make genetic evaluation of ALI difficult. Moreover, the sporadic nature of ALI makes a conventional genomic approach such as linkage mapping (or ‘positional cloning’) impossible. Briefly, linkage mapping involves scanning entire genomes of families affected by an illness using known regularly spaced variable DNA segments, thus identifying those genetic variants (alleles) that are shared by affected family members more frequently than would be expected based on chance [8]. These regions can then be isolated and cloned, and further evaluated as disease genes. One advantage of linkage mapping is that investigators need no prior knowledge of the biology underlying an illness; this is especially important in complex disorders such as sepsis and ALI. However, this approach has the disadvantage of requiring large families with both affected and unaffected individuals. This is a major limitation to the use of linkage mapping in the evaluation of ALI, given the sporadic nature, low incidence, and lack of affected families associated with this illness.

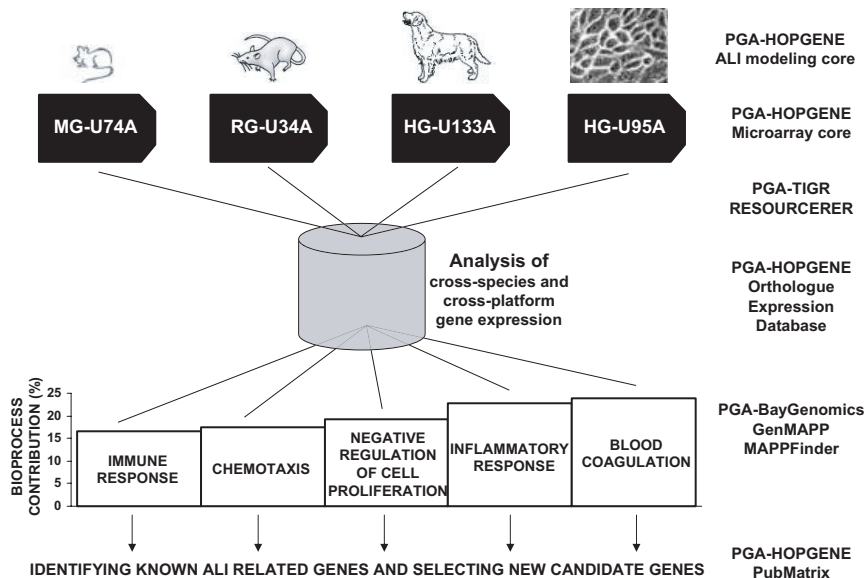
The candidate gene approach refers to a strategy for investigating the genetic basis of complex illnesses such as ALI, which can be performed using unrelated cases and controls. In the candidate gene approach, investigators study the association between variants (polymorphisms) in a certain gene, or allele and a specific disease by studying the frequency of the target variant allele in a population of affected patients and comparing this with the frequency in controls. Unlike linkage mapping, this approach requires an element of prior knowledge of disease pathogenesis so that candidate genes can be identified. Of particular interest in the candidate gene approach are publicly available databases of single nucleotide polymorphisms (SNPs; www.ncbi.nlm.nih.gov/SNP). Studying an association between one or more SNPs and a disease can help researchers to focus on certain areas of DNA and potentially identify candidate genes.

In the absence of significant insights into disease pathogenesis, comprehensive gene array analysis of tissues derived from animal models of disease is also often helpful in identifying candidate genes. However, this presents a difficult challenge in determining how best to analyze the

unprecedented quantities of data generated by these approaches. In September 2000, the US National Heart, Lung, and Blood Institute launched the Programs for Genomic Applications (PGAs), funding 11 centers to generate novel data and resources for the research community at large in order to advance functional genomic research related to heart, lung, blood, and sleep disorders. These resources include state of the art software programs for array analysis and normalization, SNP analysis, phenotyping of animal models of disease, and a rich array of analytical tools (summarized and updated on the PGA homepage: <http://www.nhlbi.nih.gov/resources/pga>). Several PGAs are working to discover and model the associations between single nucleotide sequence differences in the genes and pathways that underlie interindividual variation in inflammatory responses and their relationship to disease risk, outcome, and treatments in common human lung disorders, including ALI. For example, the HopGene PGA website (<http://www.hopkins-genomics.org/>) contains extensive array data for rat, murine, and canine models of ALI, ALI candidate genes with preliminary evaluation for relevant SNPs, and preliminary genotyping of these genes in controls and patients with sepsis and ALI. In this review we explain how the candidate gene approach has provided a menu of gene ontologies that may help to unravel the genetics and pathogenesis of ALI and, most importantly, to identify novel targets for therapy.

One approach to the identification of genes relevant to ALI susceptibility and severity is to examine general trends in the expression of common groups of genes in response to ALI in diverse species. This commonality might relate to unsuspected evolutionarily conserved responses to lung injury. At the same time, known biologic pathways and genes, either activated or suppressed in ALI, can be used as a validation of novel candidate genes that are implicated in the same pathway [9]. Figure 1 illustrates the HopGene approach to searching for candidate genes involved in ALI expression, as defined as the response to increased mechanical stress delivered by increased tidal volume ventilation or to cyclic stretching of human endothelium *in vitro*. Gene expression alterations in response to mechanical ventilation alone, in the absence of additional inflammatory stimuli, are easily detected by microarray techniques, and they may therefore provide a powerful framework on which to characterize normal lung responses to mechano-transduction stresses. Responses of four different biological systems (rat, mouse, dog, and human endothelial cell culture) to levels of mechanical stretch relevant to ALI were investigated (Fig. 1). The control and ventilated lung samples were collected at the time point at which the defining feature of ALI (i.e. vascular leakage) was detected in each animal model [9]. The total mRNA extracted from obtained tissues was hybridized to corresponding species-specific Affymetrix GeneChip (Santa Clara, CA, USA). Generated gene expression profiles from different species-specific platforms were linked using RESOURCERER – the PGA developed ortholog linking tool [10].

Figure 1



The approach to identify mechanical stress induced candidate genes using schematic representation of cross-species ortholog database and gene ontology processes. Total RNA from rat, mouse, and canine ventilator-injured lung tissues and human endothelial cell cultures exposed to injurious mechanical stretch was extracted, and gene expression data were generated by hybridization of corresponding total mRNAs to HG-U34A, HG-U74A, HG-U133A, and HG-U95A Affymetrix arrays, respectively. These steps were performed at the PGA-HopGene center. Gene expression profiles were analyzed by the HopGene PGA center using *in silico* multispecies cross-platform database applying the RESOURCERER tool designed by the TIGR PGA center. Gene ontology assignment and analysis was conducted by HopGene PGA center using the MAPPFinder tool designed by the BayGenomics PGA center. The acute lung injury (ALI)-related ontologic groups were selected using filtering conditions described in Table 1. The contribution of each bioprocess to ALI is represented as the percentage of values calculated by MAPPFinder and shown in the percentage changed genes column. The literature search was done by the HopGene PGA center using PubMatrix tool designed by PGA collaborators at the National Institute on Aging, National Institutes of Health.

Gene ontologies generated by the Gene Ontology™ Consortium (<http://www.geneontology.org>) were assigned to corresponding genes using PGA developed tools GenMAPP and MAPPFinder [11,12]. Genes with change in expression of 20% or higher and a false discovery rate of less than 10% were selected from the microarray data derived from the four mechanical stress-challenged species. This relaxed filtering approach was introduced by Munson and other speakers at the Symposium on the Functional Genomics of Critical Illness and Injury [13] and was successfully applied for selection of candidate genes [14]. This approach is especially suited to identification of genes with elevated basal expression levels, upregulation of which will not produce a high fold change ratio, meaning that these genes will be missed by more stringent conditions. The slight increase in false discovery rate will be applied equally throughout all gene ontologies and will not affect individual ontology selection.

Selected using this approach, stretch-affected genes were dynamically linked to an expansive menu of known gene ontologies using MAPPFinder tool developed by the PGA-BayGenomics Center (Fig. 1). The MAPPFinder analyzed total of 1329 bioprocesses and selected 32 related to ALI ($Z > 2.0$). Further filtering for bioprocesses that had five or

more involved genes (number of changed genes column in Table 1) and exclusion of broadly defined terms such as 'signal transduction' revealed five prominent mechanical stretch-related ALI biological processes with different degrees of contribution to this injury (Table 1; the unfiltered MAPPFinder output is provided in Additional file 1). A total of 49 genes were involved in these bioprocesses (Table 1, number of changed genes), of which 10 genes were involved in two bioprocesses and three genes in three bioprocesses simultaneously (Table 2). Thus, the actual candidate list comprised 33 individual genes.

Negative regulation of cell proliferation ontology

IL-1 β and IL-6 were the most commonly upregulated ALI-related genes that were cited as lung injury related in 287 and 173 references, respectively (Table 2). IL-6 is a differentiation factor cytokine with activity toward a wide variety of biologic systems [15–17] and IL-1 β is involved in regulating multiple biological pathways, including inflammatory and immune responses and immune cell differentiation [18]. Clinical studies showed that IL-1 β and IL-6 concentrations in bronchoalveolar lavage fluid from patients with established severe ALI (acute respiratory distress

Table 1**Biological processes identified by MAPPFinder based on gene expression**

GOID	GO Name	Number of changed genes	Number of measured genes	Number of genes in GO	Percentage changed genes	Percentage present genes	Z score
7596	Blood coagulation	8	26	74	30.77	35.14	3.56
6954	Inflammatory response	13	44	168	29.55	26.19	4.35
8285	Negative regulation of cell proliferation	8	32	129	25.00	24.81	2.88
6935	Chemotaxis	7	31	107	22.58	28.97	2.28
6955	Immune response	13	61	569	21.31	10.72	4.17

The bioprocesses affected by mechanical stretch were identified using MAPPFinder [12] software, designed by the BayGenomics PGA group for dynamic linkage of gene expression data to the Gene Ontology (GO; <http://www.geneontology.org>) hierarchy. Data were filtered by number of changed genes (>5) and Z score (>2.0) and sorted by percentage changed genes.

syndrome) were higher than in bronchoalveolar lavage fluid from normal volunteers [19,20]. Moreover, IL-1 β was self-sufficient in causing acute lung tissue injury when overexpressed in mouse lungs [21] and was directly related to ALI in another mouse model [22]. Consistent with current concepts of the role played by the ventilator in ALI, studies by Copland and coworkers [23] identified upregulation of IL-1 β , with cluster analysis confirming linked expression of these genes; this is again consistent with very early signal amplification, which begins to evolve a mechanically stimulated inflammatory phenotype.

The most interesting ALI related candidate genes in this ontological group were B-cell translocation (*BTG*) and type-2 phosphatidic acid phosphohydrolase (*PAP2*) genes. Microarray analysis of alveolar macrophages [24] showed that expression of *BTG-2* was stimulated by diesel exhaust particles, which are a known cause of adverse respiratory system reactions. The correlation between high expression of *BTG-2* transcript and cell death was reported for the alveolar epithelial A549 cell line [25]. Our microarray analysis revealed a trend toward upregulation of another candidate gene, *BTG-1*, in mechanical stretch challenged human pulmonary artery endothelial cells. These findings suggest that the *BTG* gene family is ubiquitously represented in lung tissues and could be a viable candidate for further investigation.

Another potential candidate for involvement in ALI is the *PAP2* gene, which encodes sphingosine-1-phosphate (S1P) and ceramide-1-phosphatases, which are degrading plasma membrane enzymes. It has been reported that pulmonary phosphatidic acid phosphohydrolases directly control surfactant secretion and indirectly regulate cell division, differentiation, apoptosis, and mobility through lowering S1P levels (see review by Nanjundan and Possmayer [26]). As we previously showed, S1P possess properties that are protective against inflammatory lung injury [27] and vascular leakage [28,29], and therefore the S1P regulating enzyme became a very attractive ALI-related candidate. Moreover, our

group also linked S1P effects to chemotaxis, which is yet another gene ontology identified by our candidate gene approach. We demonstrated that S1P regulates secretion of the potent chemoattractant IL-8 in human bronchial epithelial cells [30] and regulates endothelial cell chemotaxis [31].

Immune response ontology

The gene encoding the cytokine IL-13, similar to another ontology member, namely cytokine IL-1 β , described above, is involved in multiple biologic pathways (Table 1). It has been shown that this cytokine has protective properties and attenuates vascular leakage during lung injury inflicted by IgG immune complexes [32]. Further investigations linked this IL-13 protective effect to pulmonary vascular endothelium. Corne and coworkers [33] showed that IL-13 exerts its effects in part by stimulating pulmonary isoform-specific vascular endothelium growth factor accumulation.

Another gene assigned to this ontology, the *VNT* gene, which encodes vitronectin, is also involved in pulmonary vascular permeability regulation. Binding of the *VNT* gene product to $\alpha_v\beta_3$ integrin receptor increases vascular leak and activates an integrin-induced proinflammatory response [34]. Interestingly, the $\alpha_v\beta_3$ integrin receptor was found on the luminal and abluminal faces of the lung microvascular endothelium and could not be detected on the apical surface of the alveolar epithelium [35].

The same gene product distribution pattern was described for the candidate gene *IL1R2* (HopGene Candidate Gene List), which encodes IL-1 receptor type II; based on a cyclic stretch model in human pulmonary endothelial cells [36], this gene was previously selected by our group. Later, overexpression of this gene was confirmed in our *in vivo* ALI models. The lack of expression of *IL1R2* – the ligand of which, IL-1 β , is a central cytokine in ALI – by epithelial cells [37] suggests that this candidate gene also is integrated into the vascular component of ALI. These and other observations throughout the present review relate most of the selected

Table 2

Candidate genes in acute lung injury

Encoded protein	Gene symbol	Gene Ontology					PubMatrix terms	
		NR	IM	CT	IN	BC	Lung	Lung injury
Interleukin-1 β	<i>IL1B</i>	x	x		x		1536	287
Interleukin-6	<i>IL6</i>	x					963	173
Tissue factor precursor	<i>TF</i>		x			x	411	54
Plasminogen activator inhibitor-1	<i>PAI1</i>					x	201	31
Cyclo-oxygenase-2	<i>COX2</i>				x		257	28
Interleukin-13	<i>IL13</i>		x		x		327	21
Vitronectin	\downarrow <i>VNT</i>		x				138	11
Macrophage stimulatory protein	\downarrow <i>MSP</i>					x	102	11
Plasminogen activator, urokinase receptor	<i>PLAUR</i>			x		x	83	8
Tissue-type plasminogen activator	<i>PLAT</i>					x	101	7
Fibrinogen α	<i>FGA</i>					x	22	4
CCAAT/enhancer binding protein beta	<i>CEBPB</i>				x		27	3
Proteinase activated receptor 2	<i>PAR-2</i>					x	35	2
Plasma prekallikrein	\downarrow <i>KLKB1</i>				x	x	22	2
Cell chemokine 2 (MCP-1)	<i>CCL2</i>			x	x		11	1
Interleukin-8 receptor	<i>IL8R</i>			x	x		11	1
Chemokine CXC ligand 2 (MIP-2 alpha)	<i>CXCL2</i>		x	x	x		4	1
CC chemokine receptor 5 (CD 195)	<i>CCR5</i>			x	x		3	1
Annexin I	<i>ANXA1</i>				x		56	0
Eosinophil granule major basic protein	<i>EBMP</i>		x		x		56	0
Guanine nucleotide-binding protein 2	<i>GBP2</i>		x				38	0
Cathepsin C	<i>CTSC</i>		x				17	0
Interleukin-1 receptor type II	<i>IL1R2</i>		x				11	0
Cyclin-dependent kinase inhibitor 1 (p21)	<i>CDKN1A</i>	x					8	0
B-cell translocation gene 2	<i>BTG2</i>	x					3	0
B-cell translocation gene 1	<i>BTG1</i>	x					2	0
Type-2 phosphatidic acid phosphohydrolase	<i>PAP2</i>	x					1	0
Insulin-like growth factor binding protein 6	\downarrow <i>IGFBP6</i>	x					1	0
CXC chemokine receptor type 4 (LESTR)	<i>CXCR4</i>		x	x	x		0	0
XC chemokine ligand 1 (lymphotactin)	<i>XCL1</i>		x	x			0	0
Allograft inflammatory factor-1	<i>AIF1</i>	x			x		0	0
Linker for activation of T cells	<i>LAT</i>		x				0	0
B-cell antigen receptor complex protein	\downarrow <i>CD79B</i>		x				0	0

Abbreviation in parenthesis represents the old cytokine nomenclature. The 'x' symbol designates gene ontology bioprocesses in which a given gene is involved. Numbers in PubMatrix terms columns represent citations containing the terms 'lung' and 'lung injury' terms. The ' \downarrow ' symbol indicates downregulated genes; all genes with unmarked gene names are upregulated. BC, blood coagulation; CT, chemotaxis; IM, immune response; IN, inflammatory response; NR, negative regulation of cell proliferation.

genes to the pulmonary vasculature, which seems crucial to the development of ALI.

Inflammatory response and chemotaxis ontologies

These ontologies were heavily represented by diverse cytokines and cytokine receptors (Table 2). The CC chemokine-2 and the CC chemokine receptor-5 genes were reported to be involved in several lung inflammatory disorders [38] and in amplifying inflammation in lung [39], respectively.

The gene encoding CXC chemokine ligand (CXCL2) was recently linked to ventilator-induced ALI [40] and hyperoxia-induced ALI [41], and it contributes to three out of five identified ALI-related bioprocesses. CXCL2 is involved in the inflammatory response as a potent neutrophil chemo-attractant, and inhibition of its receptor (CXC chemokine receptor [CXCR2]) leads to a marked reduction in neutrophil sequestration and lung injury [40]. A similar expression and ontology pattern to CXCL2 is noted for another chemokine receptor, namely CXCR4, which is expressed by human bronchial epithelial cells [42]. It has been shown that this receptor is heavily involved in allergic airway processes [43] and promotes small cell lung cancer cell migration by altering cytoskeletal regulation [44]. The role of this potential candidate gene in ALI is yet to be identified, but the similarity in expression, bioprocess involvement, and comparable upregulation of CXCL2 and CXCR4 by IL-1 β [42,45] warrant further investigation of the involvement of CXCR4 in ALI.

Another candidate gene regulated by IL-1 β , namely that which encodes annexin1 (*ANXA1*), is involved in these ontologies (Table 2) and limits neutrophil infiltration and reduces production of inflammatory mediators *in vivo* [46] by mimicking the effects of steroids at inflammatory sites [47]. This suggests a tightly IL-1 β regulated mechanism of neutrophil migration out of the bloodstream and into lung tissues during development of ALI.

The enzyme gene family was represented by the prostaglandin-endoperoxide synthase 2/cyclo-oxygenase-2 gene, the product of which is involved in eicosanoid synthesis and appears to be important in both edemagenesis and the pattern of pulmonary perfusion in experimental ALI. Gust and coworkers [48] showed that the effect of endotoxin on pulmonary perfusion in ALI could be in part the result of activation of inducible cyclo-oxygenase-2. Upregulation of the cyclo-oxygenase-2 gene is also linked to increased pulmonary microvascular permeability during combined burn and smoke inhalation injury in a sheep model [49].

There is evidence of epithelial involvement in ALI, such as upregulation of the CCAAT enhancer binding protein gene (*C/EBP*), which regulates [50] expression of surfactant proteins A and D; these are heavily involved in pulmonary host defense and innate immunity [51] during ALI [52,53].

However, the number of candidate genes related to vascular endothelium, as was mentioned above, is striking. The following gene ontology was completely represented by vasculature related genes.

Blood coagulation ontology

That involvement of the blood coagulation pathway was identified in ALI-related bioprocesses is not unexpected. There are several reports of increased levels of coagulation factor III (tissue factor) and plasminogen activator inhibitor type 1 in patients with ALI [54–56] and ventilator-induced lung injury [57,58]. Fibrinogen A and plasminogen activator, urokinase receptor are involved in IL-1 β signaling and regulation, respectively. It has been shown that fibrinogen indirectly activates transcription of IL-1 β [59], which in turn increases expression of urokinase receptor [60]. Urokinase-type plasminogen activated receptor (uPAR) was assigned to blood coagulation and chemotaxis pathways by our approach (Table 2), and represents direct linkage between these two biological processes. It has been shown that uPAR not only promotes degradation of fibrin but also confers adhesive properties to cells by binding vitronectin. Staining of lung biopsy specimens from patients with ALI indicated that fibrin and vitronectin colocalize at exudative sites where macrophages bearing uPAR accumulate [61]. Opposite to expression of the *PLAUR* gene (which encodes plasminogen activator, urokinase receptor), downregulation of the *VNT* gene (Table 2) suggests dual regulatory mechanisms of macrophage sequestration at the injury site. Another downregulated gene was that which encodes plasma prekallikrein (*KLKB1*). It has been shown that prekallikrein not only participates in blood coagulation in tandem with factor XII [62] but also is a major source of bradykinin (potent stimulus of vascular permeability) during the inflammatory response [63].

This interconnection of coagulation and inflammation is well recognized in that inflammation leads to increased coagulation, and the two are linked by the vascular endothelium, which is particularly relevant to ALI (see review by Russell [64]). The cytokines IL-1 β and IL-6 activate neutrophils and monocytes, which in turn alter endothelial integrity. Furthermore, platelets bind to the injured endothelial surface and trigger a procoagulant and inflammatory response. These cytokines also directly activate tissue factor and plasminogen activator inhibitor type 1, which lead to activation of the extrinsic pathway of coagulation and inhibition of fibrinolysis, respectively. There is some evidence that the ‘crosstalk’ between coagulation and inflammation can be reversed. It has been shown that blood coagulation *in vitro* stimulates release of inflammatory mediators from neutrophils and endothelial cells [65,66]. Based on these reports and data generated by our cross-species analysis of ALI, we speculated that mechanical stretch causes the initial injury to the pulmonary endothelium, which is followed by platelet aggregation at the damaged site and activation of the coagulation cascade. Therefore, procoagulation genes

become major players in early stages of the onset of ALI, and then proinflammatory genes become upregulated and promote further ALI development. In order to prove or disprove this hypothesis, further studies of ALI are needed, especially time course analysis of the expression patterns of selected candidate genes in response to ALI.

Conclusion

Our data suggest that combined gene expression and gene ontology analyses of ortholog-linked multiple ALI models can be useful tools in selecting candidate genes that are involved in this patho-biologic process. We showed that 18 out of 33 genes selected by our procedure were previously linked to ALI. These results strongly implicate the other 15 selected genes as potential ALI-related candidates. Further analysis of these candidate genes may provide insight into the mechanisms of ALI and uncover unsuspected evolutionarily conserved targets that may lead to therapeutic strategies in this illness. The genetic determinants that render patients susceptible to the adverse effects of mechanical ventilation in the setting of ALI are unknown. The identification of novel therapeutic targets is essential if progress is to be made in the treatment of this condition. New molecular targets will be deduced from genetic susceptibility loci for ventilator-associated lung injury and evaluated. This approach will help to unravel the pathophysiologic mechanisms of ventilator-associated lung injury and will accelerate the development of therapies for this devastating disease.

Additional file

The following Additional file is available online:

Additional file 1

An Excel file containing unfiltered results of GOs related to ALI. See <http://ccforum.com/content/supplementary/cc2901-s1.xls>

Competing interests

The author(s) declare that they have no competing interests.

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