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Original Article

Plasma protein biomarkers of ALI and ARDS

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Abstract: Proteomics provides abundant information on the relationships between biological molecules and disease mechanisms. We selected 507 signaling proteins in the bloodstream using antibody microarray technology to identify the difference in protein expression between acute lung injury/acute respiratory distress syndrome (ALI/ARDS) patients and healthy subjects. Biological analysis of the 507 proteins revealed systemic dysregulation of inflammation, immune responses and apoptosis in ARDS. Protein-protein networks that were involved in ALI/ARDS are described. The most important signaling pathways related to ALI/ARDS are cytokine-cytokine receptor interactions and chemokine signaling pathways. IL-23, glypican 3, and leptin R (receptor) might be potential predictors for ALI/ARDS. This study identified novel potential biomarkers that might be involved in the inflammatory response, immune responses, and metabolism in ALI and ARDS.

Keywords: Acute lung injury, acute respiratory distress syndrome, biomarker, antibody array

Introduction

Acute lung injury (ALI) is a major clinical problem worldwide, accounting for one of the primary causes of in-hospital hypoxemic respiratory failure. Over the past several decades, severe sepsis and acute respiratory distress syndrome (ARDS) have been the most common causes of mortality in critically ill patients [1]. Although a growing number of advanced interventions and strategies have been applied to ALI/ARDS patients, the mortality rates still remain unacceptably high at more than 40% for ARDS [2]. Therefore, more recently, the focus has shifted from the treatment of ARDS to early identification and prevention in at-risk populations.

So far, research has focused on the identification of humoral or cellular biological markers of ARDS [3], but most studies focused on a single protein or a single inflammatory pathway, which might not completely account for the onset, perpetuation, or resolution of ALI/ARDS [4]. Therefore, a systematic and comprehensive understanding of proteomics and the signaling pathway networks of ALI/ARDS is needed. Enzyme-linked immunosorbent assay (ELISA)-based methods are considered among the most robust platforms for biomarker discovery and are known for their high degree of sensitivity. In contrast, proteomics-based methodologies analyze global biological changes and provide an opportunity to examine the complexity that is inherent in ALI and ARDS [5-7]. Recent advancements in protein array technology have created a high-throughput platform for biomarker screening by ELISA [8]. In this study, we employed the Raybiotech L-Series 507 antibody array platform, a novel antibody array that simultaneously detects 507 serum proteins, to identify potential predictive markers for ALI/ARDS.

In this study, we quantitatively detected 507 proteins in the plasma of patients with ALI or ARDS at the 3rd day and the 7th day after the diagnosis of ALI/ARDS. Then, we analyzed the protein network and identified new candidate biomarkers of ALI/ARDS.

Material and methods

Subjects

Twelve patients, including five ALI patients and seven ARDS patients, as defined by the American-European Consensus Conference [9, 10], were recruited at the Department of Re-

Table 1. Clinical characteristics of patients with ALI/ARDS

	Control		ARDS				ALI			
Sample number	1	2	3	4	5	6	7	8	9	10
Date			Day 3	Day 7	Day 3	Day 7	Day 3	Day 7	Day 3	Day 7
Age, yr	35	36	20	20	53	53	56	56	57	57
Male/Female	M	F	M	M	F	F	F	F	M	M
PaO_2/FiO_2			166	180	154	196	234	266	224	286

spiratory Medicine, Zhongshan Hospital, Fudan University. Two healthy and nonsmoking volunteers were enrolled in the control group. The study was approved by the Ethics Committee of Zhongshan Hospital, Fudan University, and informed consent was obtained from all subjects. With the progression of disease, the PaO_2/FiO_2 ratio was evaluated every day, and blood samples were collected at day 3 and day 7 if the patients still met the ALI/ARDS criteria. Finally, two patients with ALI and two with ARDS were involved in the analysis (**Table 1**).

Sample collection and preparation

Blood samples were obtained at day 3 and day 7 after the diagnosis of ALI/ARDS. Plasma was then frozen and stored in aliquots at -80°C.

Cytokine antibody array detection

An antibody-based membrane array for measuring inflammatory factors (A Custom Raybio Human Inflammation Antibody Array kit) was purchased from Raybiotech (Norcross, Ga., USA), including our request of 507 inflammatory mediators. As we described previously, each antibody was spotted in duplicate onto one membrane [11]. According to the protocol from the manufacture, the positive signals identify membrane orientation and compare the relative expression levels among the different membranes. Horseradish peroxidase-conjugated antibody served as a positive control and was also used to identify the orientation. The positive control for density in the microarray was a known protein at a controlled concentration to control the quality of the sample load, the density of spots, and the operation.

Functional profiling of the predictors

We analyzed the regulatory interaction network, and regulatory network analysis in the mammalian system was performed using Ingenuity Pathways Analysis (Ingenuity Systems, Redwood City, CA). A data set containing gene identifiers (Entrez gene ID) and corresponding expression values was uploaded into the application. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base (IPKB).

Biological pathways analysis

To obtain an overview of the human metabolic and regulatory pathways affected by ARDS, Web Gestalt was used to query for enriched pathways in the open source databases Kyoto Encyclopedia of Genes and Genomes (KEGG) and BioCarta. From our filter array, 507 mediators were present in the pathways and markers, including predictors in the BioCarta pathways. We identified overrepresented pathways by stratifying for at least three significantly enriched markers in each pathway.

Statistical analysis

Significance testing was performed using a Student's t test, one-way ANOVA, and Pearson's chi-squared test, as appropriate (SPSS Inc., Armonk, NY, USA). Statistical significance was set at $P<0.05$. The levels of markers that were statistically significant with a 10% increase or decrease at day 3 and day 7 between the ALI/ARDS and control group are shown in the tables.

Results

Antibody array

Membranes were developed with streptavidin-conjugated peroxidase and ECL chemiluminescence reagent and exposed to autoradiographic film. Each membrane represents one sample (**Figure 1**). No signal appeared on the negative control or the blank dot.

Different proteins associated with ALI and ARDS

Because WebGestalt and DAVID do not allow for a specific search for ARDS-specific func-

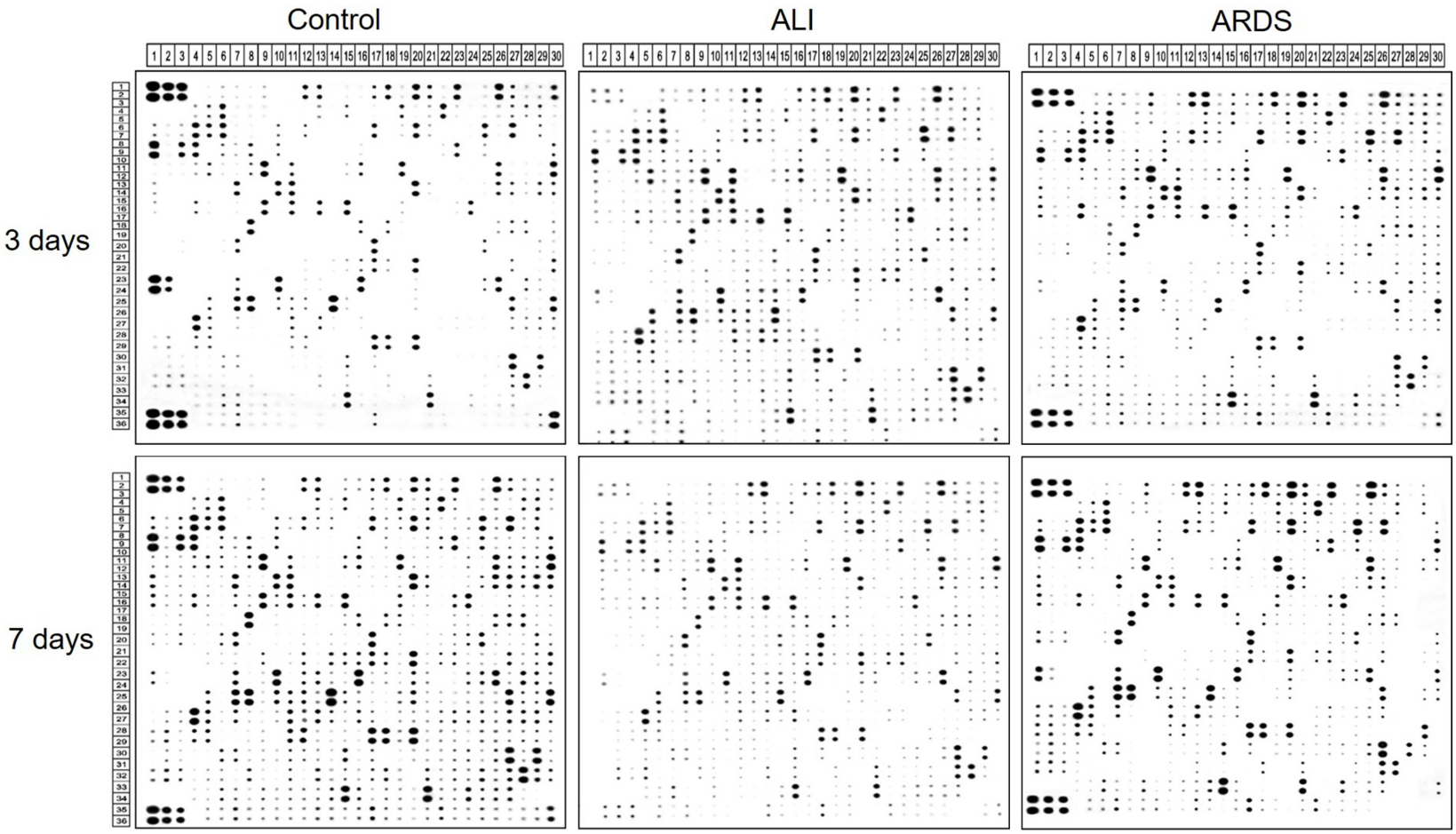

Figure 1. Scan patterns of proteins in the control, ALI and ARDS groups. For each plasma sample, two nitrocellulose membranes are included.

Table 2. Proteins identified in the plasma of ALI patients compared with controls at the 3rd day and 7th day

Protein Names	ALI 3rd day	ALI 7th day	Up (+) Down (-)
Activin RIIA	1.36	1.54	+
Adiponectin/Acrp30	1.54	1.95	+
Angiogenin	2.22	2.25	+
Chem R23	1.11	1.37	+
EN-RAGE	1.49	1.45	+
Epiregulin	1.91	2.19	+
Erythropoietin	1.16	1.15	+
Endothelin	2.13	2.58	+
Glypican 3	2.45	2.53	+
IGFBP-2	2.04	2.18	+
IL-3	2.07	2.73	+
IL-17	1.37	1.45	+
IL-23	1.17	1.24	+
IL-27	2.34	1.56	+
Leptin R	1.14	1.16	+
Lipocalin-1	1.11	1.11	+
MMP-9	1.67	1.99	+
SAA	1.36	1.18	+
Smad 7	1.68	1.57	+
Thrombospondin-2	1.26	1.15	+

Protein fold changes (>1.0) in the ALI group compared to the control group.

Table 3. Proteins identified in the plasma of ARDS patients compared with controls at the 3rd day and 7th day

Protein Names	ARDS 3rd day	ARDS 7th day	Up (+) Down (-)
Activin RIA/ALK-2	1.72	1.14	+
Activin RIIA/B	1.35	1.17	+
Chem R23	1.40	1.24	+
CXCR6	1.58	1.17	+
Glypican 3	2.94	3.44	+
ICAM-5	1.12	1.14	+
IL-1 sRII	1.20	1.27	+
IL-3	1.15	1.40	+
IL-3R alpha	1.18	1.16	+
IL-12 p40	1.16	1.22	+
IL-17	1.16	1.28	+
IL-23	1.33	1.45	+
IL-29	1.14	1.10	+
Leptin R	1.37	1.41	+
MFG-E8	1.23	1.12	+

Protein fold changes (>1.0) in the ARDS group compared to the control group.

tions of the predictors, and to further confirm the findings of the two online tools used above, we performed our own investigation on PubMed with the following keywords: inflammation, phagocytosis, hematopoiesis, or energy metabolism. In addition, we searched for reports on ARDS or its mouse models that described changes of expression levels (RNA and protein) or the abnormal presence of predictive markers in the plasma. First, we compared the significantly different proteins between the control group and the ALI group (**Table 2**). Some inflammatory cytokines and hormones were significantly increased, such as IL-27 and erythropoietin. Next, we compared the different proteins between the ARDS group and the control group (**Table 3**). Inflammatory cytokines and chemokines were increased.

Network analysis

To understand the co-expression relationships between these different proteins at a systemic level, we performed weighted gene co-expression network analysis. The results indicated that the most important signaling pathways related to ALI/ARDS are cytokine-cytokine receptor interactions and chemokine signaling pathways (**Figure 2**).

Discussion

In previous studies, some pro-inflammatory cytokines, such as TNF-a, IL-1 and IL-6, were detected in the bronchoalveolar lavage fluid (BALF) of patients with ARDS. These cytokines further contribute to the inflammatory milieu [3, 12, 13]. In the current study, we investigated the temporal proteomics changes in the plasma during ARDS using quantitative proteomics-based methods. We described protein-protein networks that are involved in ALI/ARDS. From the network, we identified some novel biomarkers for ARDS. The main and valuable proteins that we are interested in are IL-23, glypican 3, and leptin R (receptor).

IL-23 is secreted by dendritic cells and mononuclear macrophages, and it not only plays roles in eosinophilic airway inflammation [14] but also makes important contribu-

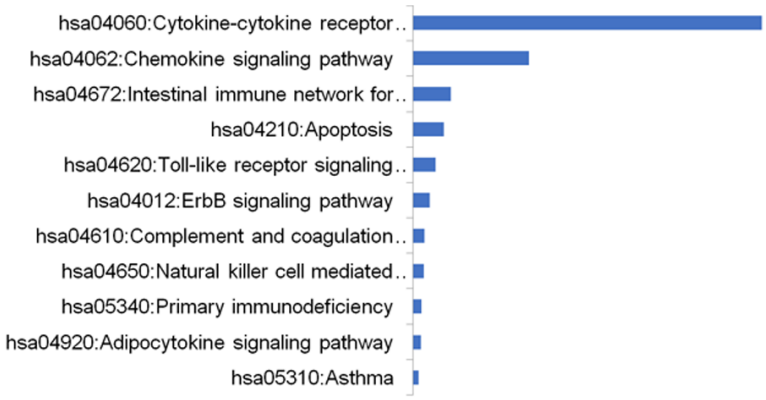

Figure 2. Pathway analysis related to ALI/ARDS. Cytokine-cytokine receptor interactions were the most important signaling pathway.

tions to the development of elastase-induced pulmonary inflammation and emphysema [15]. In our study, the level of IL-23 increased in both the ARDS and ALI groups compared with the control group. In addition, the expression of matrix metalloprotease (MMP)-9 as an inflammatory cytokine stimulated by IL-23 was shown in the comparison between the ALI group and control group. The level of IL-17 also significantly increased in the ARDS group compared to the ALI group. The upregulation of IL-17 might be regulated through the IL-23/IL-17 pathway, inducing the inflammatory cascade and further lung injury.

Glypican 3 is a member of the glypican family, participating in the control of cell differentiation and growth regulation [16]. Recently, glypican 3-redirected chimeric antigen receptor-engineered T lymphocytes (CARgpc 3 T cells) were considered potential immunotherapeutic agents for lung squamous cell carcinoma (LSCC) due to their high expression in LSCC but low expression in normal lung tissues [17]. Compared to control group patients, the expression of glypican 3 significantly increased in both ARDS and ALI patients. The results suggested that activated cell differentiation and migration occurred during the entire period of lung injury. Therefore, glypican 3 might be a potential biomarker for acute lung injury at an early stage.

The leptin receptor is considered a single-transmembrane-domain receptor of the cytokine receptor family associated with obesity [18]. Metabolic disorders have gained widespread attention because of chronic low-grade inflammation and glucose tolerance damage, which can induce airway hyperactivity through pulmonary adipocytokine/insulin signaling [19]. It was reported that the activities of p42/p44 MAPK, p38 MAPK, JNK1/2, and p65 NF-κB were enhanced by leptin in a time-dependent manner [20]. Intriguingly, leptin administration matures aspects of the lung structure and upregulates pulmonary expression of the SP-B gene [21]. The leptin receptor protein has been localized to type II pneumocytes in fetal ovine lungs, and leptin is considered to participate in the activation of surfactant production [22]. It was reported that leptin, as a signaling molecule, played an important role in paracrine interactions between type II pneumocytes and lipofibroblasts for surfactant synthesis [23]. Our data showed that the expression of the leptin receptor increased in both the ARDS group and ALI group compared to the control group, suggesting that the leptin receptormight be a potential protein to identify the occurrence of acute lung injury.

One limitation of this study is that the number of ALI/ARDS patients included is small. Based on these screening markers, however, we would like to expand the number of patients for further observation in the clinic and explore the relationship between various cytokine and protein pathways at the molecular and cellular levels.

In conclusion, we quantitatively detected 507 proteins in the plasma of patients at the 3rd and 7th day after the diagnosis of ALI/ARDS and found several potential biomarkers for ARDS. These biomarkers might be involved in the inflammatory response, immune responses, and metabolism in ALI and ARDS. The results also indicated that the most important signaling pathways related to ALI/ARDS are cytokine-cytokine receptor interaction and chemokine signaling pathways. A diagnostic or evaluation model of ALI/ARDS might be established in future studies.

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Disclosure of conflict of interest

None.

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