Journal of Cellular Physiology

Biomarkers of Lung-Related Diseases: Current Knowledge by Proteomic Approaches

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The lung epithelial surface is one of the vital barriers or sensors in the body responding to the external atmosphere and thereby always subjecting to direct toxicological exposure, stress, stimulus, or infection. Due to its relatively higher sensitivity in response to toxicants, the use of lung epithelial cell culture and lung tissue from animal models or patients has facilitated our learning to lung physiopathology and toxicopharmacology. The recent advancement of proteomics has made it possible to investigate the cellular response at a global level. In this review, the potential applications of proteomic approach in studying lung-related diseases and biomarker discovery will be discussed.
J. Cell. Physiol. 221: 535–543, 2009.

The lungs are the vital organs for respiration. From the anatomical point of view, the lung exhibits a specialized architecture and quite not resembles to the other organs in the body, that is, reflecting its designated role for gaseous exchange (Weibel and Gomez, 1962). The substance of the lung is of a light and spongy texture. In general, the lungs have around 300 million alveoli; each individual alveolus is wrapped in a bundle of capillaries where the primary site of gaseous exchange with the blood takes place (Weibel and Gomez, 1962). The alveoli consist of an epithelial layer and extracellular matrix surrounded by capillaries. There are three main alveolar cell types in the alveolar wall, namely type I cells, type II cells, and macrophages. Type I cells are the cells that are made of the alveolar wall; whereas type II cells are secretory cells that secrete surfactants and form the aqueous protein-containing layer which facilitate gaseous exchange as well as preventing collapse of the alveoli (Penney, 1988). Needless to say, macrophages are critical mediators in cellular defense against foreign invaders like germs and viruses (Bowden, 1976).

As noted, the lung epithelial surface is one of the vital barriers or sensors in the body responding to the external atmosphere and thereby always subjecting to direct toxicological exposure, stress, stimulus, or infection. As a result, the respiratory system is inevitably an ideal location prone to various diseases and complications (Table 1) (World Health Organization, 2008).

Over the past decades, the use of lung epithelial cell culture and lung tissue from animal models or patients has facilitated our learning to lung physiopathology and toxicopharmacology. Even so, without the assistance of a comprehensive tool, our understanding in this field would be hampered. In light of this, the utilization of proteomic approach has become an indispensable tool and proven to be very useful for the elucidation of the mechanisms as well as finding cure of various emerging lung diseases. For the vast emerging literatures published in just a few years, in this review, the potential applications of proteomic approach in studying lung-related diseases and biomarker discovery will be discussed.

Studying Models for Lung-Related Diseases

In general, three major types of studying samples have been employed in lung cell proteomic studies, including in vitro lung cell cultures, bronchial fluids obtained from lung [bronchoalveolar lavage fluid (BALF)] (Wattiez and Falmagne, 2005) or sputum (secretions expectorated from the respiratory tract) (Kips et al., 1998) as well as the other body fluids (i.e., serum, plasma, blood cells, or urine for instance), and the most traditional method of using lung tissues from biopsies or autopsies (Fig. 1). Overall, each method has its own merit to the understanding of the etiology of the disease or the action mechanism of an agent to the lung cells. Usually, biomarker study by sampling of body fluids is a convenient way to obtain the starting material, compared with using biopsies or autopsies. The generation of gene-knockout mice (e.g., cystic fibrosis (CF) transmembrane receptor knockout mice) as a studying model of CF also tremendously facilitated the discovery of the biomarkers and the understanding of this severely genetic disease (Bensalem et al., 2005; Brouillard et al., 2005).

In order to facilitate the process of biomarker discovery, many enrichment methods and strategies have been developed. In recent years, the advancement of proteomics has pushed to the level of investigating the proteome in individual organelles so-called the "subcellular proteome." In this scenario, only a particular type of organelle inside the cell, for example, mitochondria or nuclear, is first separated from all the other cellular components and then being studied (Lau et al., 2003; Wang et al., 2008). This method reduces the complexity and also indirectly provides for an enrichment of the proteins being studied and as a corollary of this, it can facilitate the resolution

Contract grant sponsor: Hong Kong Research Grants Council; Contract grant numbers: HKU7218/02M, HKU7395/03M.

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Received 29 June 2009; Accepted 30 June 2009

Published online in Wiley InterScience (www.interscience.wiley.com.), 13 August 2009. DOI: 10.1002/jcp.21893

TABLE 1. Top 10 causes of death in the world in 2004 and the mortality figures [data were extracted from World Health Organization (WHO, 2008)]

Causes of death	Deaths in millions	% of deaths
Coronary heart disease	7.20	12.2
Stroke and other cerebrovascular diseases	5.71	9.7
Lower respiratory infections	*4.18*	*7.1*
Chronic obstructive pulmonary disease	*3.02*	*5.1*
Diarrhoeal diseases	2.16	3.7
HIV/AIDS	2.04	3.5
Tuberculosis	*1.46*	*2.5*
Trachea, bronchus, lung cancers	*1.32*	*2.3*
Road traffic accidents	1.27	2.2
Prematurity and low birth weight	1.18	2.0

Deaths by lung-related diseases are italicized.
During 2004, an estimated 59 million people died.

of protein separation and enhance the sensitivity of protein detection by mass spectrometry. Subcellular proteomics is particularly useful to delineate the functional significance of the subcellular localization of a protein where conventional proteomics using whole cell extract can never achieve this result. Further, the use of laser capture dissection on tissues can efficiently obtain more homogeneous cell type for research instead of contamination of mixtures of cancer and normal cells (Gutstein and Morris, 2007). Other methods like albumin depletion in serum samples before proteomic analysis also facilitated the identification of biomarkers in which otherwise masked by these albumin proteins on 2D gels (Lei et al., 2008). In addition, partial purification or concentration of substances secreted into lung fluids or released to the culture medium from cultured lung cells after being challenged by stimulatory agents also greatly provided a very good means for proteomics to study lung response and identify biomarkers for various diseases and complications.

Proteomic Studies to Understand the Underlining Mechanisms of Human Diseases

Using various cutting-edge proteomic approaches, in recent years, lots of newly identified biomarkers of lung-related diseases have been reported. In this article, we are particularly interested in the study of (1) genetic diseases; (2) inflammatory and allergy diseases; (3) infectious diseases; and (4) lung cancer.

Genetic Diseases

Cystic fibrosis

CF, which is an autosomal recessive inherited disease, results from the mutations in the cystic fibrosis transmembrane conductance receptor (CFTR) gene (Tsui, 1995; Zielenski and Tsui, 1995). Mutation of this receptor results in defective anion secretion and excessive Na^+ reuptake across epithelia, leading to insufficient intraluminal hydration. This causes mucus accumulation and defective impaction states in intestine as well as impaired mucociliary clearance in airways (Stutts et al., 1995; Kunzelmann and Mall, 2003). Currently, there is no cure for individuals with CF. However, through more in-depth studies, this would allow scientists to understand the mechanisms governing the etiology of this disease, and more effective medications could be applied to patients which can prolong their lifespan resulting from this disease.

Several recent proteomic studies have provided some interesting candidate proteins as possible biomarkers of CF. It was shown that low abundance inflammatory-associated autoantigens like myeloperoxidase were identified by immunoproteomic approach from sputum of subjects with CF. This approach was also exploited for detecting proteins expressed by the *Pseudomonas aeruginosa* strain PA01, which is a frequent pathogenic organism invading lungs of CF patients. Capture of PA01 antigens using circulating antibodies from CF subjects implicated in vivo expression of *Pseudomonas* proteins. Of all the CF subjects tested, but not controls, were immunoreactive against immunocaptured *Pseudomonas* proteins, implicating their possibility as biomarkers of *P. aeruginosa* infection (Pedersen et al., 2005). In another study of saline-induced sputum from adults with CF with an exacerbation, extensive proteolytic IgG degradation and influx of inflammation-related proteins were observed, indicating these changes as predictors of CF lung exacerbation (Sloane et al., 2005). Also, while the main high-molecular mass proteins in the sputum from all subjects were the mucins MUC5B and MUC5AC, these appeared degraded in CF adults with an

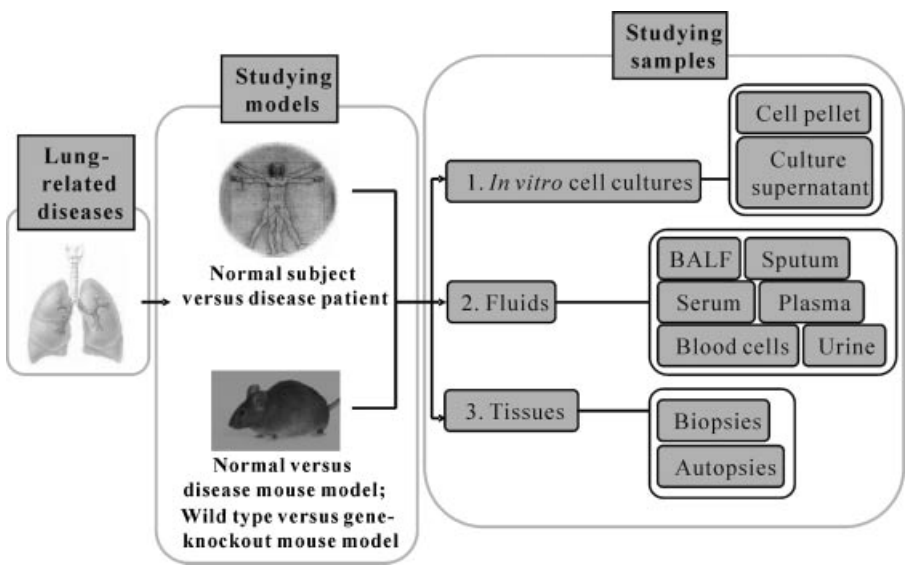

Fig. 1. Current studying models and methods employed in proteomics for biomarker discovery of lung-related diseases.

exacerbation. The glycosylation of these mucins also showed reduced sulfation, increased sialylation, and reduced fucosylation in CF adults compared with controls (Schulz et al., 2007).

Besides using sputum, serum samples from CF patients have been pooled and compared with equivalent pools of control sera in order to identify patterns of protein expression unique to CF by antibody microarrays. The set of significantly differentially expressed protein was enriched in protein mediators or inflammation from the NFκB signaling pathway (Srivastava et al., 2006). Also, in pediatric CF patients, SELDI-TOF-MS BALF profiling identified 53 unique, reliable proteins. They included the neutrophil proteins, α-defensin 1 and 2, calcium-binding proteins S100A8, S100A9, and S100A12, as well as novel forms of S100A8 and S100A12 with equivalent C-terminal deletions. Concentrations of these molecules are abnormally high in early CF patients. The data provide new insights into CF lung disease and identify novel proteins strongly associated with CF airway inflammation (McMorran et al., 2007). Using CF bronchial biopsies in normal and CF patients, the chaperone 75 kDa glucose-regulated protein and ubiquinol-cytochrome *c* reductase complex core protein I and one form of nidogen, a pseudogene of aconitase 2, were increased in CF. Aberrant chaperone and metabolic proteins in CF may reflect molecular changes of CF as well as CF-linked inflammation, infection and cellular stress response (Frischer et al., 2006).

Further, an examination of protein profiles of cultured CF lung epithelial cells (IB3-1) (ΔF508/W1282X) by 2DE-MS analysis gave rise to 194 identified proteins. Among the high abundance proteins identified were sets of proteins associated with inflammation, including the classical NFκB, p65 (RelA) and NFκB, p65 (RelB) (Pollard et al., 2005). Following this study, by comparing the IB3-1 cells with IB3-1/S9 daughter cells repaired by gene transfer with AAV-(wild-type) CFTR, by simultaneous measurement of de novo biosynthetic rate with [^{35}S]methionine of all 194 proteins in both cell types resulted in the identification of an additional 31 CF-specific proteins. This kinetic portion of the high abundance CF proteome, hidden from direct analysis of abundance, included proteins from transcription and signaling pathways such as NFκB, chaperones such as HSC70, cytoskeletal proteins, and others. The conclusion of this study was that measurement of biosynthetic rates on a global scale could be used to identify disease-specific differences within the high abundance CF proteome (Pollard et al., 2006). Finally, using *cftr*$^{-/-}$ mice, annexin A1 was found to be absent in their colonic crypts. Concurrently, annexin A1 was also undetectable in lungs and pancreas of these mice. It was suggested that the absence of this inhibitory mediator of the host inflammatory response was associated with colonic up-regulation of the proinflammatory cytosolic phospholipase A2. More importantly, annexin A1 was also down-regulated in nasal epithelial cells from CF patients, suggesting that decreased expression of annexin A1 contributed to the worsening of the CF phenotype (Bensalem et al., 2005). Following this study, the same group also demonstrated that, mCICA3, a member of a family of calcium-activated chloride channels considered to be key molecules in mucus secretion by globlet cells, was significantly decreased in the colon and lung of the *cftr*$^{-/-}$ mice. These results substantiated the conclusion that a CICA-related function in the CF colon was dependent on CFTR expression and may be correlated with the impaired expression of mCICA3 (Brouillard et al., 2005).

Inflammatory and Allergy Diseases

Asthma

Asthma is a chronic disorder of airway inflammation characterized by variable airflow obstruction and bronchial hyperresponsiveness (Maddox and Schwartz, 2002). It is suggested that T-helper type 2 lymphocytes, and the cytokines that they produce, in conjunction with reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced by inflammatory and epithelial cells, contributed as the key mediators of the chronic airway inflammation of asthma (Calhoun et al., 1992; Horwitz and Busse, 1995; Renauld, 2001; Zhou et al., 2001). The airway surface is covered by a thin fluid layer, termed as the airway surface liquid and this acts as a barrier to protect the underlying epithelium (Widdicombe, 2002). The identification of secreted proteins in this fluid may be important in understanding the etiology of asthma.

By using ovalbumin (OVA) as an allergen in mouse model of asthma, several groups of researchers provided new insights into pathogenesis of asthma and they may be useful as surrogate biomarkers for asthma. It has been shown that OVA-induced oxidative stress resulted in proteolysis of annexin-1 and consequent up-regulation of cytosolic phospholipase A2 activity and leukotriene production in the lungs of these mice (Chung et al., 2004). Nitrotyrosine is a marker of cellular oxidative stress; over 30 different proteins were targets of nitration following OVA challenge, including the antioxidative enzyme catalase. Oxidative modification at Cys^{377} of catalase and loss of the enzyme activity was observed. Subsequent analysis of human BALF showed that catalase activity was reduced in asthma by up to 50% as compared with normal controls. This study provided insights that catalase inactivation might be the one of the culprits contributing to the chronic inflammatory state of the asthmatic airway (Ghosh et al., 2006). While other studies showed that lungkine, a recently described chemokine, a family of chitinases including Ym1, Ym2, FIZZ1, and acidic mammalian chitinase, gob-5, a protein that mediates mucus secretion, and surfactant protein-D, a C-type lectin capable of modulating inflammatory responses, were significantly increased in the BALFs of OVA-treated asthmatic mice (Zhao et al., 2005; Zhang et al., 2009). Finally, using plasma peptides from control and chronic asthma mice induced by OVA, the authors quantitatively identified the fragment f of complement 3 (C3f), which is important for inflammation, was significantly higher in controls than chronic asthma mice. This study suggested that native small peptides detected by non-2DE techniques may be useful and specific biomarkers of disease (Yeo et al., 2004). In human subjects, clinical study of patients by using peripheral T-lymphocytes from six normal individuals and six patients with asthma, increased expression of phosphodiesterase 4C and thioredoxin-2 and decreased expression of glutathione *S*-transferase M3 were confirmed in asthmatic patients, which may indicate as important biomarkers and therapeutic targets (Jeong et al., 2007). Finally, it has been shown that gelsolin level was elevated in the BALF of patients with asthma. The elevated level of gelsolin was proposed to improve the fluidity of airway surface liquid in asthma by breaking down filamentous actin that was released by dying cells during inflammation (Candiano et al., 2005).

Acute respiratory distress syndrome

Acute respiratory distress syndrome (ARDS) is an important cause of morbidity and mortality in critically ill patients (Ware and Matthay, 2000). It is characterized by an acute pulmonary inflammatory process with epithelial apoptosis and interstitial and intra-alveolar edema, and the subsequent fibroblast proliferation, migration, and fibrosis (Dechert, 2003).

Using shotgun proteomics, several interesting proteins were identified in the BALF of patients with ARDS, including insulin-like growth factor-binding protein-3 (IGFBP-3). Normal controls had low levels of IGFBP-3, whereas patients with early ARDS had a significant increase in IGFBP-3. These results exhibited that the IGF pathway regulated apoptosis of lung

fibroblasts but not lung epithelial cells, and IGF pathway may contribute to the fibroproliferative response in ARDS (Schnapp et al., 2006). In another study, using SELDI-TOF and electrophoresis-based proteomics, it was found that increased levels of apolipoprotein A1, S100A8, and S100A9 proteins, in the BALF of subjects challenged with bronchial lung endotoxin and patients with ARDS (de Torre et al., 2006). Additionally, in a more recent study, in order to address the complex and dynamic changes that occurred during the course of ARDS, a quantitative proteomic approach was employed to profile proteins in the BALF of patients with ARDS at days 1, 3, and 7 after the onset of illness and compared with those of healthy control subjects. Results indicated that there were an enrichment of proteins involved in inflammation, infection, and injury in ARDS patients. The protein network analysis signified that the protein interactions in ARDS were complex and redundant, and revealed unexpected central components in the protein networks. Nevertheless, the data in this study demonstrated the new relationships among proteins and identified new groups of mediators that could be targets for novel treatments (Chang et al., 2008).

Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is a lung disease in which the airways become narrowed and lead to a limitation of airflow, causing shortness of breathe (Barnes et al., 2003). In contrast to asthma, this limitation of airflow is poorly reversible and the syndrome worsens progressively over time. Cigarette smoking, which exposes the lung to a high concentration of ROS, is the major risk factor for COPD (Hogg, 2004).

One goal in COPD research is the identification of biomarkers for early diagnosis of the disease. Increased levels of surfactant protein-A (SP-A) were found in COPD but not in the normal or fibrotic lung. Also, elevated SP-A protein levels were detected from the induced sputum supernatants of COPD patients while the levels of other surfactant proteins (SP-B, SP-C, SP-D) were not altered. These data revealed that SP-A was linked to the pathogenesis of COPD and could be considered as a potential COPD biomarker (Ohlmeier et al., 2008). In a recent study, serum amyloid A (SAA) was identified as a novel blood biomarker of acute exacerbations of COPD (AECOPD) that was more sensitive than C-reactive protein alone or in combination with dyspnea. SAA may offer new insights into the pathogenesis of AECOPD (Bozinovski et al., 2008). By combining SELDI- and MALDI-TOF-MS analysis, results signified that the concentrations of neutrophil defensins 1 and 2, S100A8, and S100A9 were elevated in BALFs of smokers with COPD when compared with asymptomatic smokers. Increased concentrations of S100A8, salivary proline-rich peptide P-C, and lysozyme C were detected in BALFs of asymptomatic smokers when compared with nonsmokers, whereas salivary proline-rich P-D and Clara cell phospholipids-binding protein (CC10) were reduced in their concentration. These identified proteins and peptides might be useful in the future as diagnostic markers for smoke-induced lung irritations and COPD (Merkel et al., 2005). Several unfold protein response (UPR) proteins have been shown to be up-regulated in chronic smokers compared with nonsmokers and ex-smokers, including the chaperones, glucose-regulated protein 78 (GRP78), and calreticulin; a foldase, protein disulfide isomerase (PDI); and enzymes involved in antioxidant defense. The clinical relevance of this study was that chronic smoking induced an endoplasmic reticulum stress response in human lung. Failure of this compensatory system may contribute to the development of COPD (Kelsen et al., 2008). Lastly, lung tissues obtained from nonsmokers, smokers without COPD, and smokers with COPD found that MMP-13 and thioredoxin-like 2 were increased in patients with COPD. These two proteins increased in the healthy smoker group and then increased further in the COPD group, suggesting that these both might be more closely associated with the development of airflow limitation (Lee et al., 2009).

Air pollutant-induced allergy

Particulates, also known as ambient particulate matter (PM), are tiny airborne particles. There is a correlation between inhalation of PM and increases in the morbidity and mortality of respiratory diseases (Laden et al., 2000; Samet et al., 2000).

It was shown that in the dose range of 10–100 μg/ml, organic diesel exhaust particles (DEP) extracts induced a progressive oxidative stress in the macrophage cell line RAW264.7. MALDI-TOF-MS analysis showed that 32 newly induced/NAC-suppressed proteins were identified. These included antioxidant enzymes (heme oxygenase-1 and catalase), proinflammatory components ($p38^{MAPK}$ and Rel A), and products of intermediary metabolism that were regulated by oxidative stress (Xiao et al., 2003). Later, the same group of researchers showed that treatment of BEAS-2B cells with organic DEP chemicals caused the induction of at least 14 proteins, among which heat shock protein (HSP70), HSP40, TPR2, and T-complex protein 1 (zeta-subunit) were known to play a role in the UPR. DEP extracts also induced the expression of IL-6 and IL-8 in the culture supernatant. These responses could be abrogated in the presence of NAC (Jung et al., 2007). These data exhibited that DEP induced oxidative stress response, UPR, and proinflammatory effects and that some of these proteins may serve as markers for PM exposures. Other airborne PM, with an aerodynamic diameter less than 10 μm (PM10), is a complex mixture of organic and inorganic compounds containing sulfates and various metals such as titanium (Pagan et al., 2003). Because acute and chronic exposures to TiO_2 particles have been shown to induce inflammatory responses in the airways and alveolar spaces of rats (Schapira et al., 1995; Warheit et al., 1997; Ahn et al., 2005; Kang et al., 2005), TiO_2-treated rats are tested as a good model for the study of the human epithelial response to PM10 particles. Treatment of epithelial cells with BSA-coated TiO_2 particles altered expression of 20 proteins. These included defense-related, cell activating, and cytoskeletal proteins implicated in the response to oxidative stress. Among one of these proteins was macrophage migration-inhibitory factor (MIF). MIF was expressed primarily in epithelium and was elevated in lung tissues and BALFs of TiO_2-treated rats. Interestingly, carbon black and DEP also induced expression of MIF protein in the epithelial cells (Cha et al., 2007). Last but not the least, ultrafine carbon black (ufCB), which is carbon-centered ultrafine particles ($\leq$0.1 μm in diameter), comprises the majority of fine particulate matters (Oberdörster and Utell, 2002). Significantly greater increases in neutrophils influx and total proteins in BALF have been observed after intratracheal instillation of ufCB, compared with carbon black (250 nm in diameter) (Li et al., 1996). UfCB also acts through a ROS-dependent pathway to cause vascular endothelial growth factor (VEGF) production and lung injury in mice (Chang et al., 2005). Further proteomic study was carried out with BALF from mice after treatment with ufCB. Results showed that ufCB exposure caused the increase of epidermal growth factor receptor (EGFR) and leukemia inhibitory factor receptor (LIFR) in BALF and decrease of both receptors in lung tissues, suggesting the acceleration of epithelial shedding from the lungs and increase of cell debris with membrane proteins EGFR and LIFR in BALF. These data revealed the mechanisms involved in the pathogenesis of ufCB-induced lung injury (Chang et al., 2007).

Infectious Diseases

SARS

Severe acute respiratory syndrome (SARS), caused by coronavirus (CoV), also known as atypical pneumonia, had emerged as a pandemic threat in 2003 (Drosten et al., 2003; Ksiazek et al., 2003; Peiris et al., 2003). Understanding its pathogenesis and developing specific diagnostic methods for its early diagnosis are crucial for the effective management and control of this disease.

Although the genome sequencing and comparative analysis provided some abundant information to predict the structures and functions of the proteins comprising this virus, the possible post-translational modifications and cleavage products are difficult to be obtained from the genome sequence. Using SARS-CoV-infected Vero E6 cells, several studies provided new perspectives in the functions of the SARS-CoV viral proteins and the cellular responses. In He's group, they identified and characterized the structural proteins of SARS-CoV isolated from Vero E6 cells infected with the BJ-01 strain of the virus (Ying et al., 2004). In another study, the cytosol and nucleus fractions of SARS-CoV-infected cells as well as the crude virions were analyzed by one-dimensional electrophoresis followed by ESI-MS/MS identification or by shotgun strategy with two-dimensional liquid chromatography-ESI-MS/MS. Of all the four predicted structural proteins of SARS-CoV, including S (spike), M (membrane), N (nucleocapsid) and E (envelope) proteins, were identified. This study indicated that 2D-LC-MS/MS made contribution to rapidly and accurately characterize whether the cells contained virus and may be used for rapid screening the virus, virus-infected cells or even body fluids (Zeng et al., 2004a). Further, characterization of the 3a protein of SARS-CoV in infected Vero E6 cells and sera from SARS patients showed that there was a tendency existed for co-mutation between the 3a protein and the spike protein of SARS-CoV isolates. The conclusion was that the 3a protein might be tightly correlated to the spike protein in the SARS-CoV functions and it may serve as a new clinical marker or drug target for SARS treatment (Zeng et al., 2004b). To analyze the differential cellular response to SARS-CoV, the proteome of Vero E6 cells with and without infection of SARS-CoV were resolved and quantitated with two-dimensional gel electrophoresis followed by ESI-MS/MS identification. Also, ICAT technology coupled with two-dimensional LC-MS/MS were applied to the differential proteins of infected cells. By combining these two methods, 355 unique proteins were identified and quantitated with 186 of them differentially expressed. This has been perhaps by far the most comprehensive proteomic index for SARS-CoV-infected cells and the identified differential profile furnished a valuable resource for diagnosis, drug development, and treatment for SARS (Jiang et al., 2005).

Using serum from SARS patients, several groups, including ours, provided useful biomarkers for the diagnosis of SARS. Our study in 2004, by using proteomic technology, showed that truncated forms of α_1-antitrypsin (TF-α_1-AT) were increased significantly and consistently in sera of SARS patients compared with control subjects. The dramatic increase in TF-α_1-AT may be the results of degradation of α_1-AT. As α_1-AT plays an important role in the protection of lung function, its degradation may be an important factor in the pathogenesis of SARS and also may be a useful biomarker for the diagnosis of SARS (Ren et al., 2004). Using SELDI-TOF-MS analysis on 89 sera collected from 28 SARS patients and 72 sera from 51 control patients with various viral or bacterial infections, and 10 sera from apparently healthy individuals, 9 significantly increased and 3 significantly decreased serum biomarkers were discovered in the SARS patients compared with the controls. Among these up-regulated markers, SAA protein was confirmed by tandem mass spectrometric analysis and there was a good correlation between SAA concentration and the extent of pneumonia. SAA could be used to monitor disease activity and response to treatment in SARS patients (Yip et al., 2005). In another study, using serum of 39 patients with early-stage SARS infection and 39 suspected SARS patients during the SARS outbreak period, the authors reported that 2 proteomic features having the highest diagnostic value were the N-terminal fragment of complement C3c α-chain and an internal fragment of fibrinogen α-E chain (Pang et al., 2006). In a recent study, a coronavirus proteome microarray that contained the entire proteomes of the human SARS-CoV and five additional coronaviruses was constructed. These microarrays were used to screen $\sim$400 Canadian sera from the SARS outbreak, including samples from confirmed SARS-CoV cases, respiratory illness patients, and healthcare professionals. These arrays can serve as a rapid, sensitive, and simple tool for large-scale identification of viral-specific antibodies in sera (Zhu et al., 2006).

Besides serum, the proteome of plasma from SARS patients were investigated. A complete proteomic analysis was performed on four patients with SARS in different time courses, and a total of 38 differential spots were selected for protein identification. Most of the proteins identified were acute phase proteins and their presence represented the consequence of serial cascades initiated by SARS-CoV infection. There were several proteins that have never been identified in plasma before using 2D gel electrophoresis, among which was peroxiredoxin II (Prx II). The high level of plasma Prx II in patients with SARS could be secreted by T cells, which indicated that active innate immune responses, along with the oxidation-associated injuries, may play a role in the pathogenesis of SARS (Chen et al., 2004). In another recent study, plasma proteins of SARS patients with those of a normal control group were compared. To enrich the low-abundance proteins in human plasma, two highly abundant proteins, albumin and IgG, were first depleted. The up-regulated proteins were identified as alpha-1 acid glycoprotein, haptoglobin, α_1-antichymotrypsin, and fetuin. The down-regulated proteins were apolipoprotein A1, transferrin, and transthyretin. Since all these up-regulated proteins identified above are well-known inflammation inhibitors, there is some mechanism that sustains the inflammation balance in SARS patients which is helpful for the proper treatment and therapy of this disease (Wan et al., 2006).

Tuberculosis

Tuberculosis (TB) is a disease caused by lower respiratory tract infection of *Mycobacterium tuberculosis*. Approximately one third of the world's population is currently infected with this pathogen and nearly 2 million people died of TB annually (Clark-Curtiss and Haydel, 2003). *M. tuberculosis* is a facultative intracellular bacterium, which persists and replicates within host phagocytes, predominantly macrophages, and dendritic cells (Clark-Curtiss and Haydel, 2003). Upon phagocytosis, *M. tuberculosis* establish themselves in an early endosomal compartment and arrest phagosome maturation, then replicate and colonize. The persistence of its colonization in phagosomes of host phagocytes and development of drug resistance makes TB one of the top 10 diseases that cause people to die from this disease in the world (World Health Organization, 2008). Therefore, proteomic technology becomes an indispensable tool as to identify early diagnostic markers for TB.

To identify proteins of *M. tuberculosis* unique to the intraphagosomal phase, mycobacteria were purified from phagosomes of infected murine bone marrow-derived macrophages and analyzed by 2-DE and MS. Results indicated that 11 mycobacterial proteins were exclusively detected in

intraphagosomal mycobacteria, when compared with those of broth-cultured mycobacteria. Six of the identified proteins were only observed in phagosomal mycobacteria, namely phosphoglycerate mutase 1 (Gpm; Rv0489), a lipid carrier protein (Rv1627c), a putative potassium uptake protein (TrkA; Rv2691), and the conserved hypothetical proteins Rv0428c, Rv1130, and Rv1191 have not been identified previously in broth-cultured mycobacteria, suggesting that these proteins were indeed unique to phagosomal *M. tuberculosis*. The identification of the roles of these proteins and targeted drug design would allow direct interference of their functions to control intracellular growth of mycobacteria (Mattow et al., 2006). In another study, proteins secreted in the culture medium from the *M. tuberculosis* K-strain, which is the most prevalent among the clinical isolates in Korea and belongs to the Beijing family, were analyzed by 2D PAGE and compared with those from the *M. tuberculosis* H37Rv and CDC1551 strains. Eleven proteins, Rv0652, Rv1636, Rv2818c, Rv3369, Rv3865, Rv0566c, MT3304, Rv3160, Rv3874, Rv0560c, and Rv3648c, were identified by MALDI-TOF-MS or LC-ESI-MS. All these proteins were cloned and expressed in *E. coli* and affinity purified. By using three of these recombinant proteins in an enzyme-linked immunosorbent assay (ELISA), when 100 sera from TB patients and 100 sera from the healthy controls were analyzed, rRv3369, rRv3874, and rRv0566c showed a sensitivity of 60%, 74%, and 43%, and a specificity of 96%, 97%, and 84%, respectively. These results displayed that the rRv3369 and rRv3874 proteins, which were expressed more abundantly in the more recently obtained clinical isolates of *M. tuberculosis* than in the laboratory-adapted H37Rv strain, were promising for use in the serodiagnosis of TB (Bahk et al., 2004). A 32-kDa putative glyoxalase in the culture filtrate of growing *M. tuberculosis* (originally annotated as Rv0577, which is now designed as CFP32) was identified, cloned, and characterized. Expression of CFP32 was provided by the serum recognition of recombinant CFP32 in 32% of TB patients by ELISA as well as the direct detection of CFP32 by ELISA in the induced sputum samples from 56% of pulmonary TB patients. Interestingly, per sample, sputum CFP32 levels (a potential indicator of increasing bacterial burden) correlated with levels of expression in sputum of interleukin-10 (an immunosuppressive cytokine and a putative contributing factor to disease progression) but not levels of gamma interferon (a key cytokine in the protective immune response in TB), as measured by ELISA. Together, these data suggested that CFP32 may be useful as a diagnostic, drug, and/or vaccine target for TB (Huard et al., 2003).

Mtb81, a novel serological marker for TB identified by serological proteome analysis, is a promising antigen for the serodiagnosis of TB. It is suggested that the combination of Mtb81 and the previously identified 38-kDa antigen, in conjunction with other novel *M. tuberculosis* antigens, would lead to optimal sensitivity for the serodiagnosis of TB and potentially improve clinical sensitivity for TB-positive individuals co-infected with HIV (Hendrickson et al., 2000). Following this study, the same group reported the use of multiepitope polyproteins in serodiagnosis of active TB. This time, three more TB antigens, Mtb11 (also known as CFP-10), Mtb8, and Mtb48, were tested together with the previously reported 38-kDa protein as well as their previously identified antigen Mtb81. Improved reactivity was observed, which indicated that the use of multiple antigens with some of which in a single polyprotein, can be used to facilitate the development of a highly sensitive test for *M. tuberculosis* antibody detection (Houghton et al., 2002). Finally, in a recent study, serum proteomic profiles were obtained from patients with active TB and controls by SELDI-TOF-MS. A supervised machine-learning approach based on the support vector machine (SVM) was used to obtain a classifier that distinguished between the groups in two independent test sets. A classifier trained on the 20 most informative peaks achieved diagnostic accuracy of 90%. From these, two peptides (SAA protein and transthyretin) were identified and quantitated by immunoassay. Since these peptides reflected inflammatory states, the authors also quantitated neopterin and C-reactive protein. Application of an SVM classifier using combinations of these values gave diagnostic accuracies of up to 84% for TB. The potential biomarkers for TB that was identified in this study through proteomic fingerprinting and pattern recognition have a plausible biological connection with the disease and could be used to develop new diagnostic tests (Agranoff et al., 2006).

Lung Cancer

Lung cancer is a malignant disease of heterogeneous histologies and has been divided into two major groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC accounts for 75% of lung cancers, and falls into three major types: squamous cell carcinoma (SCC), adenocarcinoma, large cell lung cancer (LCLC), and also less frequently found carinoids (Srivastava et al., 1994). Cigarette smoking is a leading cause of lung cancers, resulting in a death toll of 1.3 million annually (Ezzati et al., 2005). Worldwide mortality rate is increasing probably due to early smoking trend in teenage group. Cigarette, made from tobacco and contained more than thousands of known and unknown toxic compounds and carcinogens, strongly promote lung cancer development. Lung cancer may be seen on chest X-ray and computed tomography (CT scan) (Swensen et al., 2005). The diagnosis is confirmed with a biopsy. Possible treatments include surgery, chemotherapy, and radiotherapy. With treatment administrated to patients, the 5-year survival rate is just around 14% (Jemal et al., 2005). Although early treatment seems promising in higher survival rate, it is virtually impossible to cure advanced stage lung cancer. No matter what, there is an urgent need to set up biomarkers for early lung cancer detection as well as proper drug development for treating this disease.

In Beer's group, a series of 93 lung adenocarcinomas and 10 uninvolved lung samples were examined by 2D-PAGE. Results indicated that antioxidant enzyme AOE372, ATP synthase subunit d (ATP5D), β1,4-galactosyltransferase, cytosolic inoraganic pyrophosphatase, glucose-regulated Mr 58,000 protein, glutathione-S-transferase M4, prolyl 4-hydroxylase β subunit, triosephosphate isomerase, and ubiquitin thiolesterase (UCHL1) were identified being significantly over-expressed in lung adenocarcinomas (Chen et al., 2002). Using the same batch of samples, Beer's group also demonstrated that 14 of 21 isoforms of cytokeratin 7 (CK7), CK8, CK18, and CK19 occurred at significantly higher levels in tumors compared with those of uninvolved adjacent tissue (Gharib et al., 2002). Similarly, our recent study also demonstrated that increased expressions of CK8 and CK14 in cadmium-induced lung cell transformation (Lau and Chiu, 2007). It is suggested that increased expressions of specific cytokeratins can act as a scaffold to bind to apoptotic factors, preventing them to do their functions, which can provide additional survival advantages to evade apoptosis. Therefore, specific isoforms of individual CK may have utility as diagnostic or predictive markers in lung tumorigenesis. Using laser capture microdissection, differential proteins between primary lung adenocarcinoma with (LNM AdC) and without lymph node metastasis (non-LNM AdC) were identified by 2D-DIGE-MS. It was found that annexin A3, was significantly up-regulated in LNM AdC compared with non-LNM AdC. Immunohistochemistry also showed that annexin A3 over-expression was frequently observed in LNM AdCs and matched lymph node metastases compared with that of non-LNM AdCs. These data indicated that annexin A3 might serve as a novel biomarker for lymph node metastasis and prognosis in lung

adenocarcinoma (Liu et al., 2009). Comparative proteome analysis on 20 human lung squamous carcinoma (hLSC) tissues and paired normal bronchial epithelial tissues revealed 76 differential proteins, among which 68 proteins were identified by PMF. Serological proteome analysis of 10 hLSC tissues was performed to identify the tumor-associated antigens. The results revealed 36 ± 8 differential proteins reactive with patients' autologous sera, of which 14 proteins were identified. Six of the 14 proteins, alpha enolase, pre-B cell-enhancing factor precursor, triosephosphate isomerase, phosphoglycerate mutase 1, fructose-bisphosphate aldolase A, and guanine nucleotide-binding protein beta subunit-like protein, were up-regulated in hLSCs, indicating potential application of these 6 hLSC-associated antigens in diagnosis and therapy of hLSC (Li et al., 2006). In another study, the authors investigated the distribution and expression of various Prx isoforms in lung cancer and compared this with normal lung from human and mouse. Among the Prx isoforms, immunohistochemical staining showed the isoforms Prx I, II, III, and V were predominantly expressed in bronchial and alveolar epithelium as well as in alveolar macrophages of the normal mouse lung. The isoforms I and III were over-expressed in lung cancer tissues compared with normal lungs. The up-regulation of Prx I and Prx III in lung cancer tissue may be advantageous for tumor cells to survive in this oxidative stress microenvironment (Park et al., 2006). Using tobacco carcinogen nicotine-derived nitrosamine 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) hamster pulmonary adenocarinoma (PAC) model and human PAC tissue arrays with matched and unmatched normal lung tissues, it was shown that Raf-1, an effector of the EGFR, and phosphorylated CREB, an effector of the β-adrenoreceptor, were over-expressed in a significant subset of human PACs and could be served as early biomarkers of PAC development (Cekanova et al., 2007). By proteomic analysis of urine samples from 10 healthy donors and 5 lung cancer patients with NSCLC (adenocarcinoma cell type), some interesting biomarkers such as CD59 glycoprotein, transthyretin, G(M2) activator protein (GM2AP), and Ig-free light chain were found to be differentially expressed (Tantipaiboonwong et al., 2005). Using a panel of serum biomarkers for the diagnosis of lung cancer, it was found that carcinoembryonic antigen, retinol binding protein, α_1-antitrypsin, and SCC antigen were collectively found to correctly classify the majority of lung cancer and control patients (Patz et al., 2007).

Besides biopsies, several proteomic studies on various lung cancer cell lines were performed. Comparative expression proteomic analysis on human normal (BEAS-2B) and malignant (A549) lung epithelial cells found that aldehyde dehydrogenase, Prx I, fatty acid binding protein, aldoketoreductase, and destrin were up-regulated; whereas galectin-1, transgelin, and stathmin were down-regulated. An augmented expression of Prx I was confirmed in cancer tissues compared with normal tissues from lung cancer patients, suggesting that it could be a potential biomarker of lung cancer (Chang et al., 2001). M-BE, a SV40T-transformed human bronchial epithelial cell line with the phenotypic features of early tumorigenesis at high passage, was cultured in the conditioned media to collect its secretory proteins. Cathepsin D was found to be increased either in culture media or in cells during passaging. Tissue microarray and sandwich ELISA analyses also validated significantly higher levels of cathepsin D in SCC tissues as well as in plasma of lung squamous cell carcinoma patients, respectively, as compared with normal donors, indicating a potential role as a biomarker for lung cancer (Lou et al., 2007). Similarly, using NSCLC cell line A549, 14 proteins were found to be secreted in the conditioned medium, including peptidyl-prolyl *cis-trans* isomerase A, manganese superoxide dismutase, Prx I, phosphatidylethanolamine-binding protein, glutathione S-transferase P, ubiquitin carboxyl-terminal hydrolase isozyme L1 (PGP9.5), alpha enolase, phosphoglycerate mutase 1, galectin-1, and dihydrodiol dehydrogenase (DDH). Compared with normal lung tissues, higher DDH mRNA and protein expression levels were found in 15 NSCLC tissues. Concurrently, serum level of DDH was significantly higher in NSCLC patients than nonmalignant lung tumor and healthy controls. These data suggested that DDH could be served as a tissue marker and a novel serological marker of NSCLC (Huang et al., 2006). Further, protein profiles of four different human NSCLC cell lines were compared with normal human bronchial epithelial cells using 2-DE and MALDI-TOF-MS. Among the protein identified, tumor suppressor gene 14-3-3σ was down-regulated whereas the proto-oncogene translation elongation factor 1δ was up-regulated in NSCLC cell lines. These proteins may be shared among these NSCLC cell lines and closely tied in lung carcinogenesis (Liu et al., 2004). Finally, to determine the metastasis-associated proteins in NSCLC, comparative proteomic analysis was performed of two NSCLC cell lines with different metastatic potentials, the non-metastatic CL1-0 and highly metastatic CL1-5 cell lines. Results indicated that the up-regulation of S100A11 expression in NSCLC tissues was significantly associated with higher tumor-node-metastasis stage and positive lymph node status, indicating that S100A11 might be an important regulatory molecule in promoting invasion and metastasis of NSCLC (Tian et al., 2007).

Conclusion

From this review, we summarized panels of biomarkers among most commonly contracted lung-related diseases in the world. We forecast that the emergence of further state-of-the-art and cutting-edge technologies from proteomics will reveal the molecular basis and novel insights into various lung-related diseases and complications. However, for the betterment of human life, much effort are needed to validate whether these biomarkers and drug targets are really useful in early detection, prediction, and so are prevention and cure of lung diseases. Thus, only through the successful production of reliable diagnostic kits and targeted drugs could prove the real value of all these studies, that is, the era of lung proteomics is on its way.

Acknowledgments

We would like to acknowledge our debt to the authors whose works are cited in this article. Their works are illuminating and insightful. We would also like to express our sincere apologies to other hundreds of authors whose works are not listed herewith on the ground of time and space constraints. Last but not least, we gratefully thank Celia N. L. Lau for her proof-reading of the manuscript.

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