Proteomics as a Method for Early Detection of Cancer: A Review of Proteomics, Exhaled Breath Condensate, and Lung Cancer Screening

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The study of expressed proteins in neoplasia is undergoing a revolution with the advent of proteomic analysis. Unlike genomic studies where individual changes may have no functional significance, protein expression is closely aligned with cellular activity. This perspective will review proteomics as a method of detecting markers of neoplasia with a particular emphasis on lung cancer and the potential to sample the lung by exhaled breath condensate (EBC). EBC collection is a simple, new, and noninvasive technique, which allows sampling of lower respiratory tract fluid. EBC enables the study of a wide variety of biological markers from low molecular weight mediators to macromolecules, such as proteins, in a range of pulmonary diseases. EBC may be applied to the detection of lung cancer where it could be a tool in early diagnosis. This perspective will explore the potential of applying proteomics to the EBC from lung cancer patients as an example of detecting potential biomarkers of disease and progression.

KEY WORDS: exhaled breath condensate; proteomics; lung cancer; screening tests.
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BACKGROUND

Neoplasia is one of the major causes of premature morbidity and mortality in the developed world, accounting for up to 12% of all deaths.[1] Early detection is vital for the effective treatment of many neoplasms, providing evidence to substantiate the implementation of population-wide screening programs in the hope of reducing the mortality and morbidity in a number of these cancers. Lung cancer is an example of a neoplasm which presents late but which could be cured by surgery if detected early in the disease process. This review will therefore focus on this disorder as an example of how novel methods have the potential to facilitate early detection of cancers.

Lung cancer is a major cause of cancer-related death in industrialized countries worldwide,[2] affecting the lives of 1.2 million people who are diagnosed with the disease each year.[3] Nearly 170,000 men and women in the United States die each year from lung cancer, accounting for nearly 25% of all cancer deaths (Table 1).[4] The prognosis for lung cancer patients is poor with 5-year survival rates being less than 10%.[5] Curative surgery is efficacious only for those patients who are diagnosed sufficiently early in the disease process The poor prognosis is explained by the fact that 50% of patients already have distant metastases at diagnosis.[2] If lung cancer is localized at the time of diagnosis and treated promptly by surgery, the 5-year survival rate increases to 52%.[6]

The benefits of early diagnosis have prompted research into methods of screening for early stage lung cancer in high-risk or smoking populations. Screening for other cancers such as breast, colorectal, and cervical neoplasias have succeeded in reducing mortality rates through the benefits of early detection.[7] However, less certainty exists about screening for other neoplasms, including lung and prostate cancers.[8,9] There has been increasing interest in using exhaled breath as a simple tool for screening, diagnosing, and even monitoring diseases of the airway, including lung cancer.[10,11] Changes in the protein profile secreted into the lower respiratory tract may be detected in exhaled breath condensate (EBC) of lung cancer patients as an indication of the underlying neoplastic processes, presenting a potential screening tool for the early detection of lung carcinomas.

Alternative or complimentary screening tools with the potential to improve sensitivity are needed to demonstrate the clinical significance of screening for lung cancer. Lung cancer is an obvious neoplasm to target as the population at risk, i.e., smokers, is clearly defined. EBC may present a simple, noninvasive, and more accurate alternative to the current lung cancer screening technologies.

Proteins and Carcinogenesis

Understanding carcinogenesis, tumor progression, and metastasis requires a careful analysis of effector molecules such as proteins, which act as crucial components of the network of signalling pathways that drive neoplasia.[12,13] Whereas carcinogenesis is usually because of genomic mutations, the subsequent translational changes in the protein products indicate both molecular mechanisms and potential markers of neoplasia.[14] The interactions of proteins in an intricate network determines the function of the organism and are indicative of biological complexity downstream from the alterations within the genes of the neoplastic cell. The detection of protein patterns may be a method of interpreting signalling pathways and other cellular processes that contribute to cancer development and metastasis.[13] An increased under-

Table 1. Histopathological Classification of Lung Cancer and Approximate Proportions (adapted from Kumar et al.[2])

Classification of lung cancer	Approximate proportions (%)
Nonsmall cell lung carcinoma (NSCLC)	70–75
Squamous cell carcinoma	25–30
Adenocarcinoma, including bronchoalveolar carcinoma	30–35
Large cell carcinoma	10–15
Small cell lung carcinoma (SCLC)	20–25
Combined patterns	5–10
Mixed squamous cell carcinoma and adenocarcinoma	
Mixed squamous cell carcinoma and SCLC	

standing of the functional role of proteins regulating key cell processes will probably have a major impact on health outcomes. Clinical proteomics is emerging as a new way to explore those proteins regulating a variety of cellular activities within a given type of cancer and within a specific cancer cell.

Proteomic Technologies

Proteomics may be defined as the large-scale characterization of proteins expressed by the genome.[12] Unlike the study of a single protein or pathway, proteomic methods enable a systematic overview of expressed protein profiles, which, in the case of neoplasia, ultimately could improve the diagnosis, prognosis, and management of patients by revealing the protein interactions affecting overall tumor progression.[13] Furthermore, differential protein expression analysis can be used to compare neoplastic with normal tissue and is able to indicate a range of protein markers potentially indicative of disease.[4] It is highly likely that proteomics will signpost candidate markers and indicate mechanisms that are in need of greater analysis. Once identified, these individual markers may become mundane single protein assays in clinical laboratories, similar to examples such as the prostate specific antigen, carcinoembryonic antigen, and C-reactive protein.[14–17] Furthermore, individual cancer profiling could identify pathways which have been activated and which are suitable for tailored chemotherapeutic strategies to each individual neoplasm. The human genome is considerably smaller than previously thought, and in this post genome project era, many of the genes associated with tumorigenesis are now known. A smaller genome, however, does not reflect a simple proteome. It is becoming apparent that extensive posttranslational modifications such as phosphorylation, glycosylation, and proteolytic processing are common events, which present a challenge for protein analysis. These posttranslational modifications can significantly alter protein function and thus the characteristics of the cell or tissue in which it is expressed. Thus, in the post genome era, one of the challenges of proteomics is to understand the characteristics of tissue through knowledge of effector proteins and to apply this to clinical usage. Protein phenotypes are the determinants of the characteristics of a particular cancer, which would not necessarily be predicted by genomic analysis alone. Thus, proteomics has the potential to contribute to understanding protein messengers and to be applied to clinical usage.

Proteomics employs protein microarrays, electrophoresis, and mass spectrometry for the detection, identification, and characterization of proteins (Table 2).[4,13] These proteomic tools have their own individual advantages and limitations affecting their ability to assess the protein profile.

Protein microarrays use either multiple different capture antibodies dotted separately on a slide (forward microarrays) or multiple tissue/protein samples, again dotted and fixed on a single slide (reverse microarrays). Whereas these methods can detect the presence of numerous proteins or the level of expression in multiple tissue samples, respectively, the technique is limited by the availability of specific and sensitive antibodies, which, as an example, has proved to be an issue for known lung cancer markers such as the cytokeratins. Furthermore, antibody specificity must be validated by immunoblotting, and internal controls are required, particularly if the antibodies in the microarray do not bind with predictable affinity and specificity. Nevertheless, the continuing increase in the number of commercially available antibodies makes the clinical application of protein microarrays feasible. Detection of low abundance proteins also remains a problem, and methods for multiple protein amplifications, analogous to the polymerase chain reaction for DNA, are being developed as arrays but are not yet available.[18]

One of the main methods for separating proteins before mass spectrometry is gel electrophoresis. Gel electrophoresis separates denatured proteins according to their molecular weights. In addition, two-dimensional gels allow an extra degree of resolution or separation of proteins based upon the pH/p*K*a, which is particularly useful in separating proteins of a similar molecular weight. Individual protein spots can be stained and digested into peptides, which can be analyzed by mass spectrometry. The peptide mass 'fingerprint' is then matched with sequences from protein databases.

One of the key tools of proteomics is mass spectrometry (MS). Mass spectrometers analyze proteins after their conversion to gaseous ions, which can then be identified based on mass to charge ratio. Desorption and ionization techniques such as matrix-assisted laser desorption ionization (MALDI)-MS (Table 2) offer very high levels of sensitivity and mass accuracy for the detection and identification of proteins. The sensitivity and ease of sample preparation makes this technique convenient but it is not without limitations. These include difficulties detecting proteins larger than about 50 kDa and analysing complex samples such as serum. The type of MS technique used can affect the interpretation of the data retrieved, which should be analyzed by an experienced operator, which can lead to an element of subjectivity.

Other limitations are intrinsic to biological samples undergoing analysis. Differences in protein expression between tumors of different subtypes and stages (Table 1) may make interpretation difficult, unlike genomic analysis, which tends to be more constant. It is possible to use proteomics to distinguish between subtypes and stages of lung neoplasia,[19,20] but for a screening test to be robust, it must distinguish a neoplastic process not only from normal individuals but also from other nonmalignant diseases, each of which are likely to have a unique protein profile set. Furthermore, changes in protein expression and immunological capabilities occur during aging, which means that appropriate age-matched control subjects need to be included. Despite these inherent limitations, proteomics has been successful in detecting significant changes in protein profiles in a number of biological samples associated with the development of a range of neoplasia.

Table 2. Analytical Tools in Proteomics

Method		Principle	Advantages	Disadvantages
Electrophoresis				
Electrophoresis		When an electric field is applied to a solution containing a protein that has a net positive or negative charge, the protein migrates at a rate that depends on its net charge, size, and shape.		Gels must be stained before proteins can be visualized. Rarely useful by itself as proteins cannot be accurately identified without the use of another detection technique such as immunoblotting or mass spectroscopy.
	SDS-PAGE	Proteins migrate through inert matrix gel of polyacrylamide. Pore size is adjustable to retard protein of interest. SDS is a negatively charged detergent that unfolds proteins and frees them from other molecules. Proteins migrate at different rates toward positive electrode.	Separates all types of proteins, even those insoluble in water.	One-dimensional separation method has limited resolution. Closely spaced bands or peaks tend to overlap. Can only resolve a small number of proteins.
	Two-dimensional gel electrophoresis	Combines 2 separation procedures. First dimension: the solubilized, denatured proteins are separated by their isoelectric point (pH where net charge is 0) in a polyacrylamide gel. Second dimension: the narrow gel containing proteins separated by isoelectric focusing undergoes electrophoresis at a right angle in SDS-PAGE to separate by size.	Good resolution of mixture. Comparison of multiple gels facilitated by image analysis software. Posttranslational modifications can be discerned. Resolution of protein approximately 1 ng/mL.	Presence of high abundance proteins (i.e., albumin, immunoglobulins) may obscure low abundance proteins. Low throughput. Final identification of protein requires spot removal from gel, digestion, and analysis of peptides by mass spectrometry. Unable to resolve low molecular weight proteins (<10,000 Da). Not easily amenable to multivariate analysis.
	Two-dimensional fluorescence difference gel electrophoresis	Labels complex mixtures with fluorescent dyes before conventional two-dimensional electrophoresis. Different cyanine dyes are used to label protein from different samples and will be excited and emit at different light wavelengths. Up to three different samples can be labeled and mixed together (test, control, reference).	Analysis of differences between mixtures is simplified. Ratio of protein expression can be obtained in a single gel, and an internal standard can be used in each gel to reduce gel-to-gel variation. Very sensitive.	Presence of high abundance proteins (i.e., albumin, immunoglobulins) may obscure low abundance proteins. Low throughput. Final identification of protein requires spot removal from gel, digestion, and analysis of peptides by mass spectrometry. Many spots cannot be identified because of lack of material. Unable to resolve low molecular weight proteins (<10,000 Da).
Protein array				
Protein arrays	Multiplex protein arrays, cytokine arrays, tissue microarrays	In most common form, antibodies to known proteins are tethered to a surface (beads, nitrocellulose, etc.) and then detected using principles of immunoassays.	High sensitivity and throughput. Multiple analytes can be measured simultaneously. Identification of potential targets already known.	Limited antibody availability and specificity. Required some prior knowledge of expressed proteins. May not detect isoforms of analyte. Cost per sample may be prohibitive.
Mass spectroscopy (MS)				
MS	Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS)	Determines the precise mass of protein or peptide fragment from protein. Protein/peptide samples are mixed with organic acid matrix, dried on metal slide, and blasted by laser ionizing the peptide, which is accelerated in an electric field toward a detector. The time it takes to reach the detector is determined by the charge and mass. Peptide sequence information can be obtained with tandem mass spectrometers (MS–MS).		Highest resolution is for molecules <3,000 Da in size.

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Table 2. (continued)

Method	Principle	Advantages	Disadvantages
Surface-enhanced laser desorption/ ionization time-of-flight mass spectrometry (SELDI-TOF-MS)	Comparable to MALDI, the difference being that SELDI uses chromatographic chip arrays to selectively bind subsets of proteins from complex samples. The surfaces can be washed to remove nonspecifically bound proteins and substances that can interfere with the ionization process (salt, detergents, etc.).	High throughput via automation. Requires minimal sample preparation. Can be combined with prefractionation of material to enhance the detection of lower abundant proteins.	No direct identification of proteins. Less sensitive to high molecular weight protein (>20 kDa). May have instrument-to-instrument variation.
Stable isotope labeling	Biological samples are labeled with different stable isotopes using modifying agents targeting a specific amino acid (e.g., ICAT). After separation and mass spectrometry, peptides from the 2 samples differing in mass units specific for the isotope used (e.g., 8-Da mass shift for ICAT) can then be used to provide relative quantification.	Wider proteome coverage than other methods. Can obtain quantitative information on a large number of proteins; Usually yields IDs of relevant proteins.	Technically demanding; very low throughput capability; samples need to be trypsinized before analysis; reliable quantitative measurements likely on most abundant proteins.

Adapted from Hoehn and Suffredini.[12]

Proteomics and Lung Cancer

Using protein microarrays, it has been possible to demonstrate significant differences in the serum protein profile of lung cancer patients when compared with healthy controls and subjects with chronic obstructive pulmonary disease (COPD).[21] Two-dimensional gel electrophoresis in conjunction with MS detected a greater than twofold difference in specific serum proteomic profiles between lung cancer patients and healthy volunteers.[22] Proteomics has been used to define tumor subsets in resected lung specimens and has been demonstrated to distinguish primary adenocarcinomas from primary squamous cell carcinomas with 98% accuracy.[20] In addition to lung cancer, proteomics has been applied to the early detection and treatment of cancers of the ovary, pancreas, prostate, esophagus, breast, liver, and rectum.[23,24] For example, plasma surface-enhanced laser desorption/ionization (SELDI)-MS discriminated pancreatic cancer patients from healthy controls with a sensitivity of 97.2% and a specificity of 94.4%.[25] In addition, SELDI-MS identified significant differences in protein peaks in the plasma of controls and women with ovarian cancer.[26]

Thus, proteomic techniques could play a role in the early detection of neoplasia, by the analysis of samples such as plasma, but could also play a role in identifying particular tumor pathways, which could be targeted for chemotherapy, leading to a unique profile for each person's neoplasm. In addition, single markers could be identified for monitoring clinical response to treatment.

A note of caution needs to be made to temper the enthusiasm for applying these techniques. The reproducibility of SELDI-MS both within and between studies may be suboptimal from which to draw conclusions in terms of biomarkers and can be somewhat insensitive, particularly when established tumor markers are not identified.[27,28] These constraints demonstrate the need for studies of reproducibility and applicability to be undertaken. In addition, problems of overfitting (an apparent discriminatory pattern, which occurs by chance) and poor validation of the proposed markers that have been identified can be an issue also.[29]

Screening for Lung Cancer

Sputum cytology, interval chest x-rays, and to date, computed tomography (CT) scans as population screening tools in smokers and ex-smokers have failed to show improved lung cancer mortality rates.[30] For a screening tool to be successful, the intervention should reduce mortality, and furthermore, the sensitivity, specificity, availability, and cost together with the associated morbidity of the screening tests must be reasonable. Whereas chest x-rays combined with sputum cytology can detect early stage lung cancers, disappointingly no overall improvement in mortality was observed.[27–29,31–33] Recently, interval thoracic CT as a screening tool for lung cancer has been shown to detect nodules of much smaller diameter compared to those detected by plain chest x-ray (3 vs <0.5 cm) and refinements in helical CT have reduced scanning time and radiation dose. Although preliminary results suggest 80–90% of CT screening-detected bronchial carcinomas are diagnosed as stage 1 tumors,[31,34] up to 60% of all lesions detected are found to be benign with morbidity and mortality associated with the subsequent biopsies. CT may create lead-time bias where early diagnosis appears to prolong survival falsely, and clearly leads to overdiagnosis and unnecessary surgical procedures.[35] These are important issues that will only be resolved by large-scale trials and are discussed in more detail for the interested reader at http://www.ahrq.gov/clinic/uspstf/uspslung.htm.

EXHALED BREATH CONDENSATE

EBC collection is a simple, safe, comfortable, and completely noninvasive method of sampling the lower respiratory tract in humans.[36,37] Exhaled breath enters a collection system, is

cooled by ice or a refrigeration system, allowing exhaled water vapor to condense.[38] The condensate contains a range of nonvolatile substances,[39] including macromolecules such as proteins.[40–42] Its potential as a diagnostic tool for a range of lung diseases has provoked research into applying this method as a possible screening tool for the early detection of lung cancer.[43] Unlike traditional methods of sampling secretions from the lower respiratory tract such sputum induction and bronchoalveolar lavage, EBC is noninvasive, does not disturb the underlying disease process, and can be repeated within a short period of time, even in asthmatic patients (Fig. 1).[38]

Collection of EBC

Standardization of EBC collection has yet to occur and leads to differences when comparing reports, as many studies have used a variety of condenser designs.[11,36,38,44] A degree of variability in repeated samples, even when using a commercial breath condenser,[45] has led to published recommendations for collection methods and equipment.[36] More research is required before a particular device can be recommended for protein detection. The optimum cooling temperature remains unclear, and although there is an expectation that colder conditions would be more efficient, this is unproved.[36] Dry ice, liquid nitrogen, and refrigerated units attain lower collection temperatures (−20°C),[46] however, devices using wet ice have been able to obtain samples of proteins with condensing chambers reaching temperatures of 1–2°C[47–50] (Conrad 2006, unpublished observations).

The collection of EBC involves normal, relaxed tidal breathing.[39] Most studies suggest 10 minutes as the minimum duration of collection, although some used longer time periods.[11,36,48] The volume of condensate collected is largely dependant on the breath volume and hydration status of individual patients, as well as the different temperatures and materials used by the different condensing systems.[46,49,50] In order for the EBC to reflect the composition of the lining of the lower airways accurately, upper airway contaminants need to be kept to a minimum. Oral breathing, mouth rinsing, voluntary swallowing of saliva, and a saliva trap may decrease contamination.[11,36,39] Samples should be immediately frozen at −70°C to inactivate any proteases.[36] The addition of protease inhibitors may further prevent degradation of proteins, however, protease inhibitors can interfere with protein analysis. Storage under argon will avoid oxidization of proteins,[51] and storage in polyethylene tubes will minimize protein adhesion. The measurement and identification of proteins in EBC remains an area where increasing standardization will be required as the amount of protein varies even in healthy individuals (between 4 and 1.4 mg/mL).[52] The time of day of collection, type and amount of food and drink consumed before collection, tobacco smoking, medications, and presence of systemic diseases are some of the unknown factors that may affect EBC protein analysis.[36] As the effects are unknown, researchers should detail these variables to control for these potential sources of error, and ideally, samples should be collected after a period of fasting.

Application of Proteomics to EBC and Lung Cancer

Specific proteins have been identified in EBC that may act as significant markers of lung cancer. For example, endothelin-1 and interleukin-6 were both found to be increased in the EBC of nonsmall cell lung cancer patients when compared with healthy controls.[42,53] Studies on serum or tumor samples from lung cancer patients have demonstrated that distinct mass profile signatures can be detected using SELDI-MS technology but the identity of the proteins requires further processing and the results need further confirmation in other studies.[54,55]

Despite the ability to detect proteins in EBC, there has been no published research documenting the use of proteomics to identify differences between the EBC protein profiles of lung cancer patients and healthy controls. Biological samples relating to lung cancer to which proteomics has been applied include plasma, serum, bronchoalveolar lavage, and surgically resected specimens.[4] Analysis of EBC proteins has been restricted to specific proteins,[42,53] or measuring total protein concentration.[40,41,45]

CONCLUSIONS

Proteomics has provided a significant body of evidence supporting the concept of protein profiling to pinpoint biomarkers

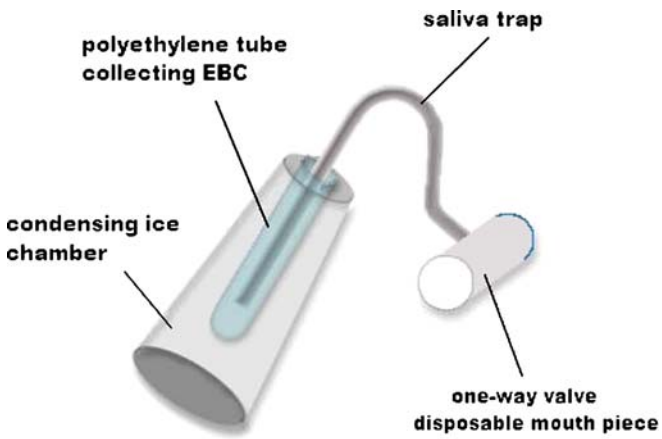

Figure 1. An example of a simple exhaled breath condensing device. A one-way valve allows air to be inspired and the exhaled breath to be cooled using ice or another cooling system, e.g., dry ice or a refrigerated circuit. The conditions (e.g., temperature) and types of materials used in EBC collection can influence the amounts and types of markers captured.

of carcinogenesis in a range both neoplasia and types of biological samples. Currently, these proteomic techniques are commonly used in the research laboratory, but have yet to become commonplace in the clinical laboratory. Their application to EBC could provide clinicians with a panel of markers as a simple, noninvasive, specific, and sensitive screening tool for the early detection of lung carcinomas. Despite this promising line of inquiry, a number of obstacles remain. It is not yet known if EBC contains enough proteins to be able to create the profile that will significantly differ from those of normal controls or other pulmonary diseases. EBC collection and the tools of proteomics in detecting proteins have still not been perfected, and unknown confounding variables may interfere with protein analysis. Whereas more research is needed before this technique can be applied in the clinical setting for the early detection of lung carcinomas or as a way of identifying tumor subtypes, it is a rapidly growing subject which will be producing interesting results for years to come.

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