PROTEOMICS IN PLEURAL EFFUSIONS

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Abstract
Normal pleural fluid in humans is thought to have a low protein concentration, but in disease states where fluid accumulates into the pleural space, the protein profile may change dramatically. In the near future, research on such altered fluid proteome may give diagnostic and prognostic information or even predict therapeutic responses. One-dimensional, two-dimensional, differential gel electrophoresis as well as mass spectrometry have become the most important techniques for identifying disease-related proteins among various pleural pathologies. However, to date very few studies have applied proteomic technology to pleural fluids with the aim of discovering reliable and specific disease biomarkers.

Key words: Proteomics, pleural effusion, malignancy

Introduction

The mapping of the human genome may be considered one of the most important scientific advances. It is known that genes can generate different messenger (m)RNA, and that newly formed proteins undergo various post-translational modifications (eg., phosphorylation, glycosylation, acetylation, ubiquitination) that can alter their functions and characteristics. These facts are significant in transcriptome (whole cell mRNA that occurs in a given time) and proteome (set of expressed proteins by the complete genome of a cell along its entire life, or in a given time) studies.

Protein expression studies have more advantages as compared with gene expression studies for several reasons: a) proteins rather than genes determine how a cell works in a given time; b) a single gene or even a single mature mRNA may be associated with multiple proteins due to splicing, RNA editing, or posttranslational modifications; and c) plasma and pleural fluid have small quantities of DNA and RNA, but contain a tremendous array of different proteins that might be considered important disease markers in the future.

Biomarkers

An ideal biomarker should be sensitive, specific, and easily performed in a non-invasive way. In the search for the ideal biomarker, different types of biological samples for the identification of differentially expressed proteins may be used, but very few proteomic studies on pleural fluid specimens have been reported to date (1).

Under physiological conditions pleural fluid is a plasma ultrafiltrate with an estimated liquid amount of 0.15 mL/kg in each hemithorax. The development of pleural effusions in pathological conditions implies the enrichment of the fluid with secreted or membrane-shed proteins which may constitute molecular signatures or fingerprints for specific diseases (biomarkers). Therefore, understanding and utilizing the differential protein profile that exists between pleural effusions from different causes opens a new window of opportunity for discovering otherwise undetectable low-abundance biomarkers (2, 3).

Current Proteomic Techniques

Biospecimen Collection

Success in proteomics depends very much on careful specimen preparation. A standardized protocol for sample collection and storage is essential for reproducible experiments. From a proteomics perspective, pleural fluid samples are generally collected

in sterile tubes without anticoagulants or other additives when the primary goal is to catalog and quantitate proteins. Furthermore, some degree of degradation may occur over time in proteins of stored pleural fluid samples, even using -80ºC freezers. Therefore, careful validation and interpretation are essential when analyzing a large set of pleural fluid samples which have been stored in tissue banks over a period of time.

Protein separation

The first step in the identification of proteins of interest is the separation of protein complex mixtures into their individual components (fractionation).

Prefractionation

As for serum, pleural fluid processing for proteomic analysis requires the use of prior methodologies (eg., chromatography) which remove high-abundance proteins, thus increasing the proportion of less abundant ones in samples (4).

Gel electrophoresis

High-resolution one-, two-dimensional, and differential gel electrophoresis (1D GE, 2D GE, and DIGE, respectively) have traditionally been used as protein separation strategies in the field of proteomics.

In 1D GE, protein mixtures are separated in a gel on the basis of their molecular weights, whereas in 2D GE and DIGE, they are first separated (first dimension) according to their isoelectric points along a continuous pH gradient, and then a second dimension (molecular weight) is applied (Figure 1). After electrophoresis, protein spots in a gel can be visualized using a variety of radioactive, chemical stains or fluorescent markers and, depending on the type of staining, a range of 200 to 3000 proteins per gel can be visualized.

The DIGE technique is similar to the 2D GE, except that samples are initially labeled with fluorescent cyanine dyes (Cy2, Cy3 and Cy5 derivatives), and then run on a single 2D gel (5).

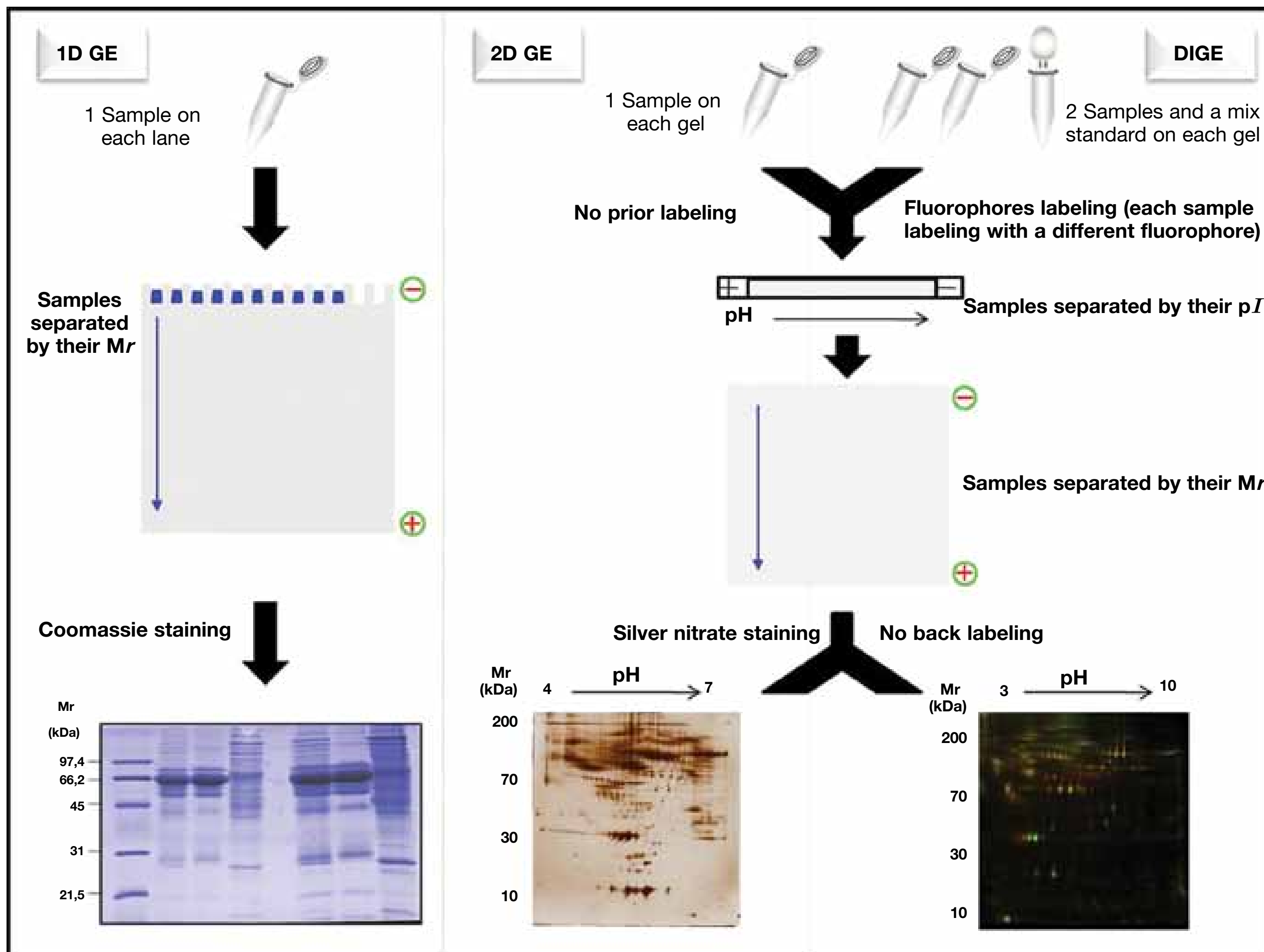

Figure 1. Electrophoretic methods in proteomics. Mr: Molecular weight; pI: isoelectric point

Protein Identification

Mass spectrometry

Mass spectrometry (MS) is one of the most common technologies used in proteomics for identifying those proteins previously separated on the basis of their physico-chemical properties (5).

In an MS protein identification workflow, a protein obtained by a gel or liquid chomatography is first digested. Figure 2 outlines how the resulting peptides are ionized to produce charged molecules which travel through the analyzer to the mass detector. Two of the most commonly used ionization techniques are matrix-assisted laser-desorption ionization (MALDI) and electrospray ionization (ESI). In the first, proteins and peptides are mixed with matrix molecules and then ionized using a laser (Figure 2). In ESI, samples are passed through a fine metal needle to which high voltage is applied. The resulting spray of ionized peptides is delivered to the mass analyzer (5).

In MS techniques the type of mass analyzer employed influences the results. Some are based on the time-of-flight device (TOF), as in MALDI-TOF and SELDI-TOF. Other examples of MS analyzers include the quadrupole (Q), quadrupole 'ion trap' (IT), and Fourier transform ion cyclotron (FT-ICR-MS or FTMS). In addition, the technique referred to as liquid chromatography (LC)-MS combines the physical separation (LC) and the mass analysis (MS) capabilities of these procedures.

Isotope-coded affinity tag (ICAT)s and Surface-enhanced laser desorption/ionization (SELDI)

In ICAT, a prototype approach of chemical tagging, cysteine residues of protein samples are labeled with biotinylated tags of light and heavy formats. After labeling, the samples are pooled, digested, and analyzed by MS to quantify the signal intensity of the light and heavy versions of the same peptide.

In SELDI, protein biochips are stained with a protein capture bait (like receptors, ligands, antibodies, DNA oligonucleotides, etc.) which enrich the protein or peptide of interest. Subsequently, MS identifies them (6).

Protein Validation

Enzyme-linked immunosorbent assay (ELISA) and immunobloting

The differential proteins identified with the proteomic technologies described earlier can be quantified through ELISA or immunoblotting. These analytical procedures may confirm proteomic results in a large set of clinical samples.

Protein array systems

Microarray technology allows for the rapid detection of changes in protein expression without the use of the expensive specialty equipment required by MS-based proteomics. Protein "chips" or microarrays are first generated by the ordered immobilization of different affinity probes (eg., antibody, DNA aptamer, lectin, etc) onto a surface (eg., coated glass slides). The protein targets of affinity probes are usually, but not always, known. The arrays are then incubated with protein samples under conditions that allow affinity reactions to occur. Frequently, protein samples are prelabeled with fluorescent dyes that permit the presence of certain proteins to be measured with a high-resolution scanner. The best application of this methodology is the screening of samples when the proteins under study are known. Array size is typically restricted by the availability of antibodies. Although initially claimed as the proteomic technology of choice, it has some limitations such as the cross reactivity of multiplex sandwich immunoassays, and the lack of standardization and sensitivity of experiments. Notwithstanding, new formats of antibody microarrays are now being applied to achieve a high throughput and the parallel detection of low-abundance proteins in body fluids. Technologies used in the field of proteomics are depicted in Figure 3.

Proteomic Applications

As previously mentioned, one of the main goals of pleural fluid proteomics is to find protein fingerprints or biomarkers that reflect various disease states. The few studies which have made use of proteomic methodologies on pleural fluid specimens are summarized in Table 1. Findings are somewhat coincidental for some proteins as potential biomarkers in pleural effusion differentiation: pigment epithelium derived factor (PEDF) -probably the protein most consistently altered-, apolipoproteins, clusterin, fibrinogen β chain and S100 A9 (calprotectin) (4, 6-12). However, all these studies lacked adequate sample sizes, were heterogeneous in the comparison groups and the proteomic technologies applied, and most did not validate their results. Many of the claimed unique proteins are linked to inflammatory and immune responses, iron metabolism or angiogenesis, which may affect their specificity in the differential diagnosis between malignant and benign pleural conditions.

The following is an example of a study whose objective was to search for differentially expressed proteins in pleural effusions as potential biomarkers of

cancer. Rodriguez-Piñeiro et al. (4) identified candidate biomarkers in pleural fluid and serum by the application of proteomic methodologies which included prefractionation, protein separation using 1D GE,

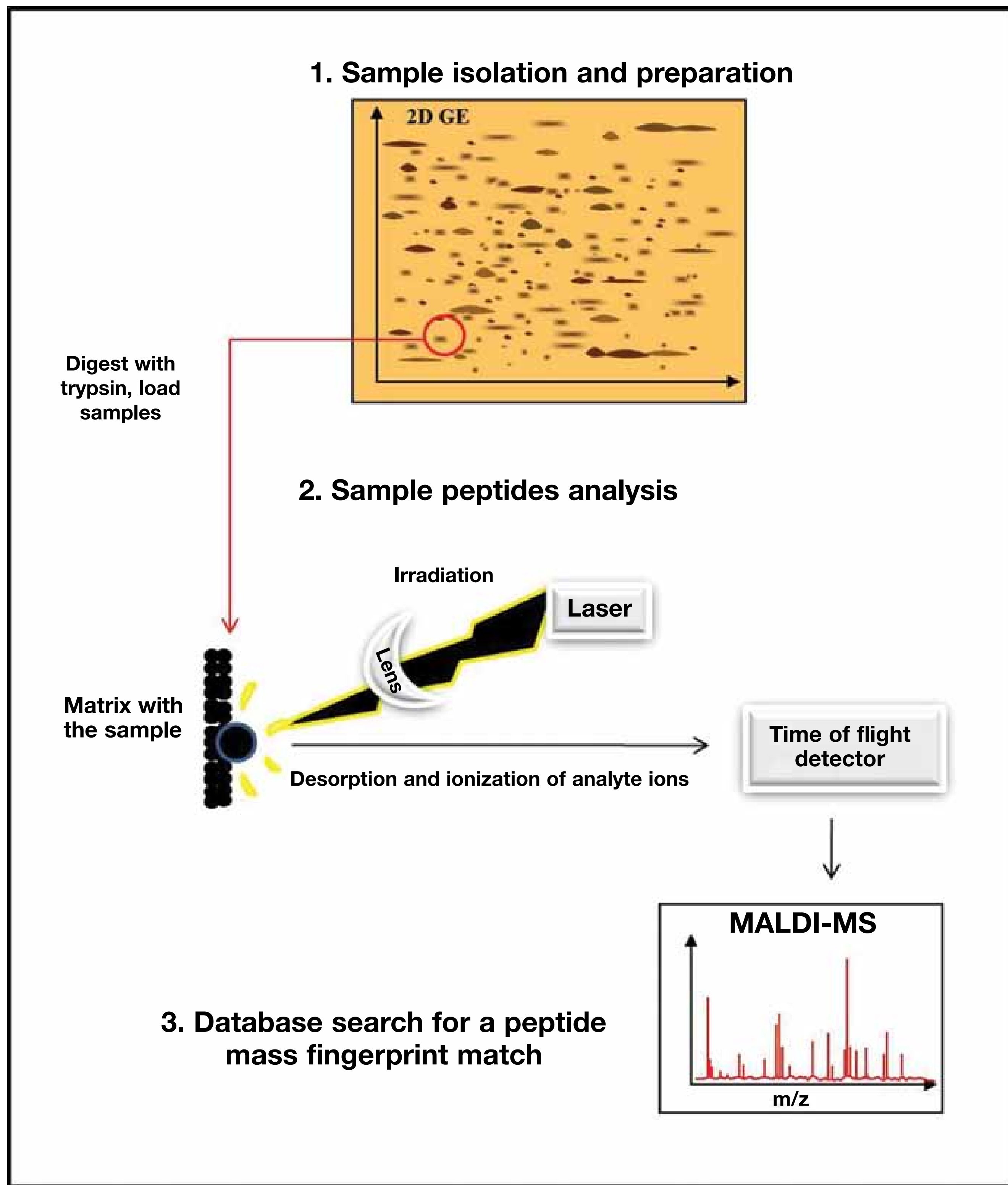

Figure 2. Diagram for mass spectrometry analysis (MALDI)

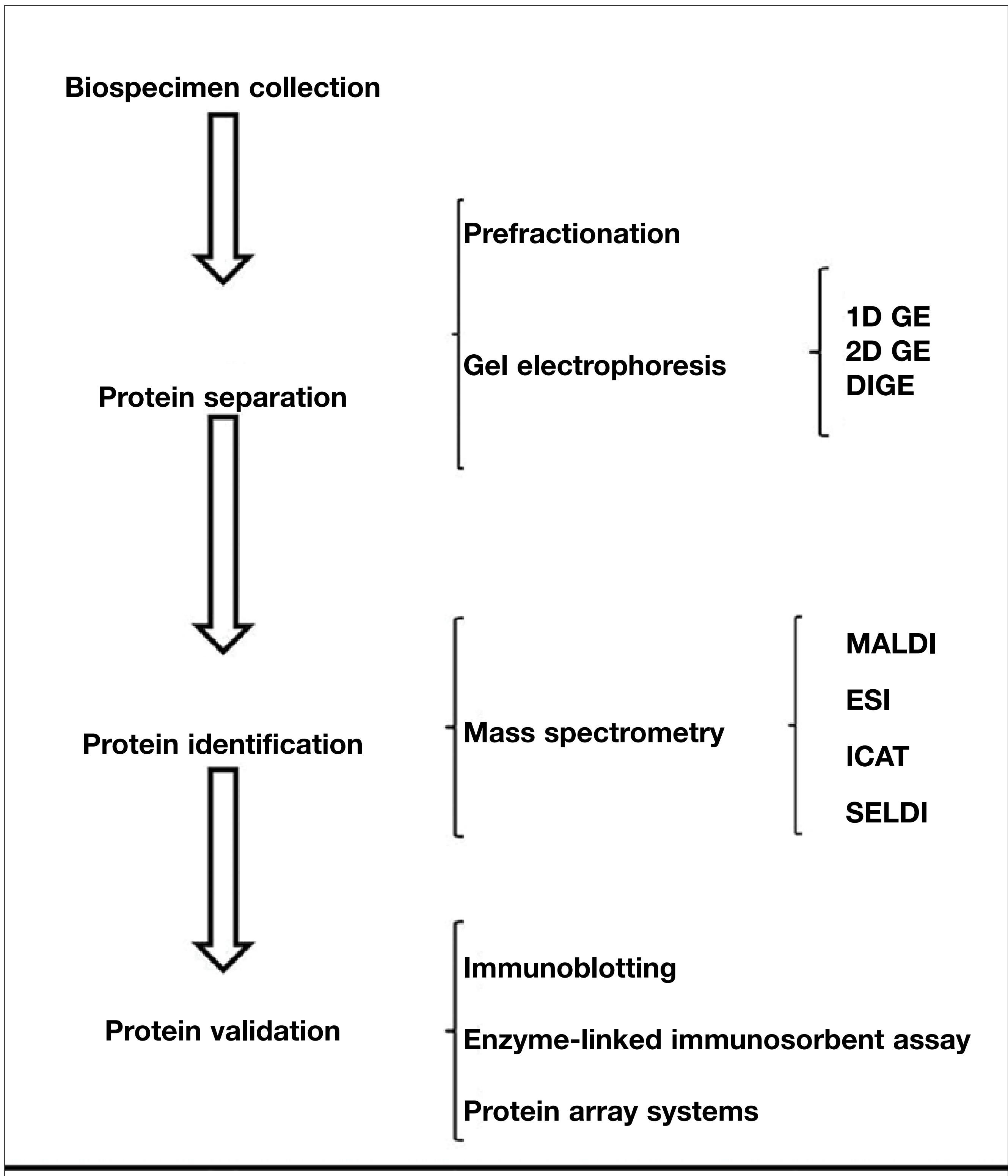

Figure 3. Technologies used in the field of proteomics

2D GE and DIGE, and identification and validation of altered spots by MALDI-TOF and immunologic techniques respectively. Authors compared 4 malignant pleural effusions from lung adenocarcinoma, and 4

tuberculous effusions. Forty-one differential protein spots between the groups were obtained, but only 35 were further identified. One of the most significant biomarker candidates was PEDF, a potent anti-angio-

Table 1. Some relevant proteomic studies in pleural effusions

Study	Proteomic technologies		Identified proteins			Patient population	Comments
	Separation	Identification and validation	Number	Validated	Others (non validated)		
Bard et al., 2004 (7)	1D GE Prefraction-ation	MALDI-TOF Immunoblot	53	MHC class II, HSP-90, Immunoglobulins G, M, A, E.	Sorting-nexin (SNX25), B-cell translocation gene 1(BTG1), PEDF, Bamacan, Thrombospondin-2	4 mesotheliomas and 5 metastatic pleural effusions (2 non–small cell lung carcinoma, 2 breast and 1 ovarian adenocarcinomas)	Identification of protein composition of exosomes. The most abundant proteins were immunoglobulins and comple ment components
Tyan et al., 2005 (8)	2D GE	LC-MS	124		Fibrinogen β-chain, Complement C3, PEDF, Apolipoprotein A1, Clusterin, Gelsolin, Hemopexin	43 malignant pleural effusions from lung adenocarcinoma	Descriptive proteomic profile of malignant pleural effusions
Hsieh et al., 2006 (9)	Prefraction-ation 2D GE	MALDI-Q -TOF Inmunoblot ELISA	7	PEDF	Fibrinogen γ- and β-chain precursors	14 malignant pleural effusions and 13 transudative effusions	PEDF levels were significantly lower in malignant than in transudative pleural effusions
Heg-mans et al., 2009 (6)	Prefraction-ation	SELDI-TOF Protein arrays	5	Apolipo-protein C1	Isoforms of Apolipoprotein C1	41 mesotheliomas vs48 patients with other causes (including 40 metastatic effusions)	Apolipoprotein C1levels discrimi nated pleural mesothelioma from other causes of exudates with moderate accuracy (AUC=0.755)
Rod-ríguez-Piñeiro et al., 2010 (4)	Prefraction-ation 1D GE, 2D GE, DIGE	MALDI-TOF Immunoblot	35	PEDF	Complement C3, Gelsolin Apolipoprotein A1, Hemopexin, Fibrinogen β-chain, S100 A9, S100 A8	4 malignant pleural effusions from lung adenocarcinoma and 4 tuberculous effusions	PEDF was significantly overexpressed in malignant pleural effusions

Yu et al., 2011 (10)	Prefraction ation 1D GE	LC-MS Immunoblot ELISA	482	Alpha-2-HS-glycoprotein (AHSG), Insulin-like growth factor-binding protein 2 (IGFBP2), Angiogenin, Cystatin-C	PEDF, S100 A9 and A8, Apolipoprotein E, Clusterin	68 malignant pleural effusions vs 119 non-malignant (59 paramalignant and 60 benign)	AHSG and IGFBP2 levels were increased in malignant pleural effusions
Hosako et al., 2012 (11)	DIGE	LC-MS Immunoblot Protein arrays	282	14-3-3 protein sigma, Heat shock protein 90, Annexin A1, Cathepsin D, Ceruloplasmin, Mimecan, Pyruvate dehydrogenase	Apolipoprotein E, Fibrinogen β-chain, Gelsolin, PEDF, S100A9, Calretinin	10 mesotheliomas vs 33 metastatic effusions (11 lung adenocarcinoma, 13 squamous cell,3 pleomorphic carcinoma of the lung,6 synovial sarcoma)	Expression of cathepsin D was lower in mesotheliomas than in lung adenocarcinomas
Wang et al., 2012 (12)	DIGE	MALDI-TOF Immunoblot	28	Serum amyloid P (SAP), Jumonji C-domain-containing protein (JMJD5), Fibrinogen γ, Transthyretin (TTR), Hemopexin	Complement C3, PEDF, Clusterin, Fibrinogen β-chain, S100 A9	10 malignant pleural effusions from lung adenocarcinoma vs 10 benign effusions (6 tuberculosis, 4 pneumonia)	Hemopexin, fibrinogen γ and TTR were up-regulated, whereas SAP and JMJD5 were down -regulated in malignant effusions

AUC: area under the curve; 1D GE: one dimensional gel electrophoresis; 2DGE: two dimensional gel electrophoresis; DIGE: differential gel electrophoresis; MALDI-TOF: matrix-assisted laser-desorption ionization time of-flight; SELDI-TOF: surface-enhanced laser desorption/ionization time of-flight; LC-MS: liquid chromatography mass spectrometry; ELISA: enzyme-linked immunosorbent-assay

genic factor which was significantly overexpressed in serum and pleural effusions from lung cancer patients. However, validation of this molecule by immunoblot was inconclusive due to the existence of more than 12 PEDF isoforms. An earlier proteomic study, based on 2D GE and MALDI-TOF techniques, also found PEDF to have a differential expression in 14 malignant and 13 transudative effusions (9). Yet, PEDF was significantly lower in the former than the latter, as determined by Western blot analysis (9). Neither study is comparable in the selected population and methodology, but they highlight that PEDF in pleural fluid is worth measuring, due to its relationship with angiogenesis and tumorigenesis (4, 9).

Future Perspectives

Contrary to traditional reductionist investigations, in which researchers have to decide beforehand which proteins are to be tested, proteomics permit the separation of proteins from a complex mixture possibly resulting in the most suitable markers being selected. At present, proteomics cannot replace invasive standardized diagnostic procedures such as pleural biopsy for labeling malignant effusions. Nevertheless, the use of newly discovered biomarkers holds great promise and opens the possibility of either selecting patients for more invasive procedures, or ruling out malignancy in poor candidates, with sufficient confidence as to avoid invasive diagnostic methods.

The ongoing rapid development of proteomic methodologies will give rise to new discoveries and generate new insights into the mechanisms of diseases, which may have a major impact on the way they are diagnosed and treated. Interactions between researchers, clinicians and statisticians are paramount in achieving these goals in the near future.

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References

1. Hu S, Loo JA, Wong DT. Human body fluid proteome analysis. Proteomics 2006;6:6326-53. [CrossRef]
2. Porcel-Pérez JM. ABC del líquido pleural. Semin Fund Esp Reumatol 2010;11:77-82. [CrossRef]
3. Porcel JM, Salud A, Vives M, et al. Soluble oncoprotein 185HER-2 in pleural fluid has limited usefulness for the diagnostic evaluation of malignant effusions. Clin Biochem 2005;38:1031-3. [CrossRef]
4. Rodríguez-Piñeiro AM, Blanco-Prieto S, Sánchez-Otero N, et al. On the identification of biomarkers for non-small cell lung cancer in serum and pleural effusion. J Proteomics 2010;73:1511-22. [CrossRef]
5. Bowler RP, Ellison MC, Reisdorph N. Proteomics in pulmonary medicine. Chest 2006;130:567-74. [CrossRef]
6. Hegmans JP, Veltman JD, Fung ET, et al. Protein profiling of pleural effusions to identify malignant pleural mesothelioma using SELDI-TOF MS. Technol Cancer Res Treat 2009;8:323-32.
7. Bard MP, Hegmans JP, Hemmes A, et al. Proteomic analysis of exosomes isolated from human malignant pleural effusions. Am J Respir Cell Mol Biol 2004;31:114-21. [CrossRef]
8. Tiang YC, Wu HY, Lai WW, et al. Proteomic profiling of human pleural effusion using two-dimensional nano liquid chromatography tandem mass spectrometry. J Proteome Res 2005;4:1274-86. [CrossRef]
9. Hsieh WY, Chen MW, Ho HT, et al. Identification of differentially expressed proteins in human malignant pleural effusions. Eur Respir J 2006;28:1178-85. [CrossRef]
10. Yu CJ, Wang CL, Wang CI, et al. Comprehensive proteome analysis of malignant pleural effusion for lung cancer biomarker discovery by using multidimensional protein identification technology. J Proteome Res 2011;10:4671-82. [CrossRef]
11. Hosako M, Muto T, Nakamura Y, et al. Proteomic study of malignant pleural mesothelioma by laser microdissection and two-dimensional difference gel electrophoresis identified cathepsin D as a novel candidate for a differential diagnosis biomarker. J Proteomics 2012;75:833-44. [CrossRef]
12. Wang Z, Wang C, Xiaobing H, et al. Differential proteome profiling of effusions from lung cancer and bening inflammatory disease patients. Biochim Biophys Acta 2012;1824:692-700.