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Translational Research and Plasma Proteomic in Cancer

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ABSTRACT

Proteomics is a recent field of research in molecular biology that can help in the fight against cancer through the search for biomarkers that can detect this disease in the early stages of its development. Proteomic is a speedily growing technology, also thanks to the development of even more sensitive and fast mass spectrometry analysis. Although this technique is the most widespread for the discovery of new cancer biomarkers, it still suffers of a poor sensitivity and insufficient reproducibility, essentially due to the tumor heterogeneity. Common technical shortcomings include limitations in the sensitivity of detecting low abundant biomarkers and possible systematic biases in the observed data. Current research attempts are trying to develop high-resolution proteomic instrumentation for high-throughput monitoring of protein changes that occur in cancer. In this review, we describe the basic features of the proteomic tools which have proven to be useful in cancer research, showing their advantages and disadvantages. The application of these proteomic tools could provide early biomarkers detection in various cancer types and could improve the understanding the mechanisms of tumor growth and dissemination. J. Cell. Biochem. 117: 828–835, 2016.

KEY WORDS: CANCER; BIOMARKERS; PLASMA PROTEOMIC

It is now well establish that to increase the cancer patient survival, an early detection of the disease is fundamental. To reach this objective, researchers are engaged for a long time in the search for biomarkers that can detect the disease in the early stages of its development. An hypothetical biomarker should be a protein or a metabolite with enough specificity and sensitivity to discriminate between normal and pathological conditions. The National Institutes of Health (NIH) Biomarker working group defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic response to a therapeutic intervention.

Biomarkers are discriminate in these following classes:

- Predictive biomarkers: predict the response to treatment.
- Diagnostic biomarkers, as a guide to identify the presence and the progression of pathology.
- Prognostic biomarkers: useful during the pharmacological treatment and to improve the quality of life in patients affected.

The choice of biomarker source plays an essential role, the most widely used source is undoubtedly plasma or serum other sources could be urine, saliva, spinal fluid, or biopsies. Serum or plasma are sources of choice because are easily available and contain large

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species of proteins and metabolites, however, proteins of clinically relevance in plasma span over 10 orders of magnitude in abundance, thus making it difficult to determine proteins poorly represented. The overall protein content in an organism is now named "Proteome," this term was coined in 1994 by Mark Wilkins as model of term genome, since then much has been made thanks to the development of technologies such as two-dimensional electrophoresis and mass spectrometry. Proteome analysis is much more complex than that of the genome because although the genome of a cell is almost unchangeable, the proteome is continuously remodeling due to post-translational modifications occurring on proteins and that can vary as a function of the cell metabolic conditions. Post-translational modifications like phosphorylation, lipidation, glycosylation, methylation, acetylation, and nitrosylation, which in healthy cells serve as key regulator for physiological functions can been found altered in tumor cells, thus making proteins subject to these changes useful biomarker. Two-dimensional electrophoresis dates back to the 1970s when O'Farrel described for the first time the separation of proteins by isoelectric point as a first dimension, using anfolines, followed by separation in the second dimension by molecular weight with the use of SDS. Successively the separation by isoelectric point was improved by the use of immobilines. Although this technique is largely manual, it suffers for a limited reproducibility. Now an improvement was obtained with the improvement of the difference gel electrophoresis (2D-DIGE) that consists of labeling proteins by fluorescent probe prior to electrophoretic separation. The reference and sample are labeled with two different probe which have different excitation and emission wavelength, they are then mixed together and separated in one single gel run. The gel scanning under two different wavelengths results in two gel images that can be superimposed by a software thus showing difference for each protein migration essentially due to post-translational modification. As complement to two-dimensional electrophoresis, mass spectrometry analysis can be used. In fact, spots can be excised from the gel, proteins digested with trypsin and peptides identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF).

To overcome some of the problems of gel proteomics, researchers have developed several methods. These methods often use the so called bottom-up approach (or shotgun) for analyzing the proteome; they require that the proteins are digested to peptide fragments prior to their separation. Several different electrophoretic and chromatographic options are available; the most common approach consists in a separation of peptides by 2-dimensional HPLC chromatography, followed to electrospray mass spectrometry. The first dimension is usually performed on a strong cation exchange (SCX) column that fractionates the peptides, eluting them with increasing ionic strength into a reversed-phase (RP) column, directly coupled by a nano-electrospray device to a MS/MS spectrometer. Differently from the 2-D Gel, this technique can be made automated, furthermore it is very good at enhancing fractionation of complex peptide mixtures, is highly reproducible and easily integrated with MS and computational search techniques. Further improvement to HPLC-MS analysis was made by the observation that although proteolysis generate a large number of peptides, only a small part of them are really helpful to identify a protein. These peptides are called proteotypics and can be used as a suitable identifier of a protein. A proteotypic peptide should have the following characteristics: (i) should be observed in more than 50% of all identifications of the corresponding protein; (ii) should be unique for each protein in the database; (iii) should not possess missing cleavage sites; (vi) its sequence should not include amino acid susceptible to post-translational modifications [Dittrich et al., 2014]. Thus, to identify a protein at least one or two proteotypic peptides are adequate, from this evidence the MS technique called single reaction monitor (SRM) or multiple reaction monitor (MRM) originally developed for organic molecules, was applied to peptides tanks also to the technological improvement of mass spectrometry equipments with increasing mass scanning speed and better sensitivity. In a typical MRM analysis, peptides obtained from the digestion of complex matrices like plasma or tissues are separated by HPLC and only the proteotypic peptides relative to proteins of interest are searched by mass in the spectrometer, the fragmentation pattern of those peptides (transition) confirm unambiguously their sequence and the most intense fragment can be used for quantization. Using appropriate instrumentation and software, about 50 transitions can be monitored per each HPLC run . However, the search of peptide MS/MS data against a database, although is the method of choice, is not able to detect new or altered proteins since the database is constituted only by known proteins. In the cancer proteome, very often are present aberrant proteins synthesized by mutated genes or by different frame of translation. Therefore, these cancer-related proteins will be missed searching in a protein databases. It has been calculated that about 20% of transcripts do not correspond to any protein and the translation of aberrant genes is difficult using only the genomic approach. To overcome this problem, a combination of proteomic and genomic technologies is needed. In fact, the advent of high-throughput nucleic acid sequencing technologies together with the development of the mass-spectrometry-based proteomics now allows to validate at protein level the RNA or DNA sequence found in cancer tissues. However, considering that samples from two patients with the same cancer may differ from each other, a large amount of data is necessary for a proteogenomics database construction and consequently efficient searching software are required [Woo et al., 2014].

THE BIOMARKER CONCEPT

Cancer etiology and development depend on multigenic factors driven by environmental factors and cellular events. Due to this complexity, data from protein expression can be considered a important resource for comprehension of the molecular pathology of cancer. The identification of proteins associated with cancer has started to enable early diagnosis and possible treatments at an early stage; this is crucial and strongly correlated with the chance of survival. The analysis of protein patterns is a crucial step in developing the suitable combination of tailored therapies for each individual. The detection of markers is not easy because cancer proteome is exceptionally complex and is affected by biological processes taking place in cancer cells, cancer tissue microenvironment, and cancer cell–host interactions. A major problem is to distinct proteins associate to the disease from proteins that can be produced by the cellular inflammatory state produced by the disease. Protein profiling can also differ substantially between different areas of the tumor mass. Moreover, several factors like race, age, diet,

genetic variability, circadian rhythms, feeding, smoking, stress, and use of drugs can affect the physiological parameters, hence statistical significance is achieved with numerous analyses. Psychological stress, which promoting relevant changes in neuronal activity and gene regulations, also can play huge role in cancer development. Psychological distress, which can begin with cancer diagnosis and persist during the treatment, is associated with circadian and endocrine disorder. For this reason, circadian/endocrine factors are potent modulators of cancer progression. Some authors [Cash et al., 2015] hypothesized that circadian activity disruption, distress, and diurnal cortisol rhythms would be associated with biomarkers of tumor progression in breast cancer. In this study, patients with poor circadian coordination and distress present high levels of VEGF, MMP-9, and TGF-beta factors suggesting tumor invasion/immunosuppression. For this reason, we can affirm that depression and cancer commonly co-occur. Cancer patients generally are affected by depression which increases with the disease severity. On the other hand, severe depression may be associated to high risk of cancer.

Possible markers must be confirmed with additional genomic or metabolomic analysis and quite often, even for the same samples, the results do not correspond. Advances in proteomics have so far provided a large list of potential protein cancer markers in the scientific literature. To date, a small number of them are clinically approved, for staging, prognosis or selection of therapy; most of them are single-protein, serum-derived [Polanski and Anderson, 2007] . Those few are still affected by poor sensitivity and specificity [Williams et al., 2007], for example, in liver cancer diagnosis, alpha-fetoprotein (AFP) has been reported a 50% sensitivity, HER2/NEU is showing 40% sensitivity in predicting breast cancers, and non-malignant prostatic diseases can increase levels in serum of prostate-specific antigen (PSA) that is routinely used to detect prostate cancer.

Due to the multifactorial nature of cancer, it is clear that a combination of numerous markers is necessary for an accurate diagnosis and a tailored of therapy. Proteins need further characterization both for their structure (potential post-translational modifications) and for their amount to develop diagnostic patterns specific for each cancer state. Polanski and Anderson built a database of potential cancer biomarkers reported by many different cancer studies. They identified 1,261 proteins that could, if validated, contribute to panels of markers that may be useful in early diagnosis of cancers [Polanski and Anderson, 2007]. The authors have also indicated a subset of 260 high-priority candidates [Polanski and Anderson, 2007]. Extracellular proteins represent possible candidates for cancer biomarkers as well as proteins involved in apoptosis, cell cycle, and proliferation. Changes in protein levels do not provide full information about protein function. The promising fields of phosphoproteomics [Rocchetti et al., 2014] and glycoproteomics [Zhang et al., 2014] are unraveling protein modifications. Many proteins experience post-translational modifications; changes of phosphorylation degree is a possible marker of a pathological condition. Phosphorylation of tyrosine residues is the most studied phosphorylation event; although phosphorylation of serine and threonine are more frequent [Cito et al., 2015]. Tyrosine phosphorylation is critical for mitogenic and angiogenic signaling regulation and tyrosine kinases are often overexpressed or mutated in various human cancers [Karimi et al., 2014], hence they have also become attractive targets for anticancer drugs. Studies using reverse-phase arrays have shown that the phosphorylation and activation of the serine/threonine kinase AKT is a central early step in prostate cancer and follicular lymphoma (FL) [Yahiaoui et al., 2014]. Reactive oxygen or nitrogen can also modify the function or action of proteins, thus, this protein can represent a great target for proteomic methods. Gel-based methods are very useful in protein modification studies because they highlight PTMs; the isoelectric point of a phosphorylated protein is, for example, shifted toward a lower pH and it could be easily separated from a not phosphorylated form in 2-D maps.

Current diagnostic screening requiring the use of protein markers. Some protein markers that are currently in clinical use have substantial limitations regarding their utility for screening. They include the marker CA125 for ovarian cancer, the PSA for prostate cancer, the carcinoembryonic antigen CEA for colon cancer, and pancreatic cancer (CA19-9). For other common cancers, in particular, breast and lung cancer there are, at this time, no specific markers demonstrating clinical utility that can be used for primary screening. Thus, there is the need for biomarkers with diagnostic sensitivity and specificity for detecting common types of cancer. The considerable number of protein in the blood and the multiplicity of their modified forms can be used to provide information on the health of an individual and of most organs of the body and provides an opportunity to develop a non-invasive diagnostic for cancer. Thus, the diagnostics field forward will drive the future generation of personalized medicine where the individual patient will receive the best possible medicine for the correct disease [Di Domenico et al., 2013].

LIMITATIONS OF CURRENT CANCER SCREENING

The detection of different cancer types depends heavily on imaging modalities such as computed tomography (CT) scans for lung cancer, mammograms for breast cancer, and pelvic ultrasounds for ovarian cancer. The progress of imaging technology, capable of detecting even small injuries, is accompanied by an increase in false positives, needing invasive procedures to make a definitive diagnosis. Many advances have been made in proteomic technologies to validate biological indicators or biomarkers for cancer. The improvement of biomarker panels will lead to prediction of cancer at early stage of growth and its response to therapy. Through a protein-based biomarker analysis for each of the most common cancer types, their screening will be possible on a single platform.

PROTEOMIC SURVEYS IN DIFFERENT SAMPLES

Biopsies and blood are the most widely used samples for cancer proteomic surveys [Yang et al., 2014], but also animal tissues, cell cultures [Serkova and Glunde, 2009], urine [Linden et al., 2012], saliva [Yang et al., 2014], amniotic [Kim et al., 2014], bile [Grunnet and Mau-Sorensen, 2014], cerebrospinal [Patel et al., 2014], follicular, and pancreatic fluids have been used. Tissue-based proteomic studies are very attractive as they correlate protein biomarkers directly to the disease. But intra- and inter-cell

heterogeneity of cancer tissues, due to the contamination of the healthy tissues and other tissues present, increase the uncertainty degree of proteins quantization. Moreover, representative samples are not always in accessible sites, and to obtain them in large numbers from a biopsy, or during surgical removal of a tumor may be problematic. It would be appropriate to isolate cancer cells from the stroma, because the necrotic tissue contaminates serum proteins and blood cells. We need to remind different methods of success useful for the capture of cancer cells, as the needle aspiration or scrapped surface.

Other non-enzymatic methods such as calcium starvation and immunomagnetic separation are also valid methods for isolation of cancer cells, as well as the laser-capture microdissection (LCM) which is a highly sophisticated technique requiring a labor-intensive procedure.

The urine is an ideal biological sample for the detection of cancer biomarkers, in fact it is easily available in almost all patients, it is simple to collect and does not require any invasive procedures. However, there are several difficulties in the use of urinary proteomics such as low protein concentration, high levels of interfering compounds (particularly salts), and high degree of variations (both intra-individual and inter-individual variabilities).

Use of amino acids labeled with stable isotope in cell culture (SILAC) to compare the secreted proteins (secretome) from pancreatic cancer-derived cells with that from non-neoplastic pancreatic ductal cells showed the differential expression of 145 proteins, some of which not previously detected, that have been also validated by Western blot analysis [Gronborg et al., 2006].

ICAT (isotope-coded affinity tag) approach is a useful tool in the identification of diagnostic biomarkers in several body fluids, including cerebrospinal, sinovial, and nipple aspirate fluids [Pawlik et al., 2006]. The cerebrospinal fluid (CSF) shows a similar protein content as blood plasma, although with a lesser concentration. Chen et al. [2006] reported, for the first time, results of quantitative proteomic analysis of pancreatic cancer juice obtained with ICAT. Qualitative and quantitative analyses with ICAT of proteins differentially expressed in nipple aspirate fluid (NAF) samples were able to differentiate between patients with early-stage breast cancer and healthy women [Pawlik et al., 2006]. However, ICAT technique can be only applied to protein with sulfhydryl group because it involves cysteine-specific tagging of intact proteins followed by proteolytic digestion. The ITRAQ (isobaric tag for relative and absolute quantization) technique is more useful because it consists of peptide chemical derivatizated in the amino groups using a set of multiplexed amine-reactive reagents with isobaric tags. Another difference between the ICAT and iTRAQ techniques is that peptides coming from a sample and control labeled with ICAT possesses different masses in MS1 wile iTRAQ peptides possesses the same mass in MS1 and different masses after MS2 fragmentation [Wiese et al., 2007].

PLASMA PROTEOMICS IN TUMORS

The blood is an excellent source of candidate biomarkers as it collects proteins released from tumor and can be easily obtained by a non invasive procedure. In most proteomic studies, it is not actually blood that is directly analyzed but rather plasma or serum portion of blood. The type of blood component that is best for protein profiling and peptidome analysis has been debated. Some investigators favor the use of plasma because they presume that, in serum, the enzymatic activity occurring during clotting can produce a cleavage of proteins involved in biologically relevant pathways.

Among the potential biomarkers of human plasma for cancer (Table I), only some are recognized; in fact, currently proteomics technology has a limited sensitivity to detect low-abundance cancer biomarkers against the background of high-abundance plasma proteins. Low abundant proteins are often involved in the regulatory processes thus cancer candidates show generally a lower concentration range than general other plasma proteins [Polanski and Anderson, 2007]. To overcome this problem, a depletion of high abundant proteins is performed as an essential treatment for serum and plasma samples; these treatments delete albumin and globulins that represent more than 60% of plasma proteins [Dittrich et al., 2014]. Removal of high abundance protein can deplete up to 90 % of total protein content and reveal hundreds of additional low abundance proteins. However, high abundance proteins can serve as carrier proteins and can bind potentially useful biomarkers and low abundance proteins can be involved in a nonspecific binding to the affinity column used. Proteomic analysis with 2-DE can simultaneously detect changes of multiple proteins in plasma often detected differentially expressed proteins are common abundant plasma proteins and their diagnostic value may be limited. Two-dimensional electrophoresis (2-DE) has been used for the discovery of circulating auto-antibodies in cancer patients and annexins I and II were reported as specific antigens in sera from patients with lung

TABLE I. Some of Cancer Biomarkers Discovered by Proteomics

Cancer types	Biomarkers
Ovarian cancer	Cancer antigen 125 (CA125) [Nolen and Lokshin, 2013]; β-2 microglobulin [Yang et al., 2009] apolipoprotein [Podzielinski et al., 2013]; gene-expression in HE4 [Ferraro et al., 2013]
Breast cancer	Fibrinogen A fragment [van den Broek et al., 2010]; BAG6, DDX39, ANXA8, COX4 [Calderon-Gonzalez et al., 2015]; GCDFP-15 [Darb-Esfahani et al., 2014]; Versican [Du et al., 2013]; AGR2 [Li et al., 2015c]; ubiquitin ligase [Goka and Lippman, 2015]; ferritin light chain [Jezequel et al., 2012]
Gastric cancer	α1-antitrypsin precursor [Hsu et al., 2007]; pepsinogen C [Terasawa et al., 2014]; Cathepsin B [Ebert et al., 2005]; galectin-1/-3 [Thijssen et al., 2015]; miR-214 [Zhang et al., 2015b]; angiopoietin-like protein 2 [Yoshinaga et al., 2015]; MOR1 [Yao et al., 2015]; circular RNA [Li et al., 2015a]
Liver cancer	Galectin-1/-3 [Thijssen et al., 2015]; CHI3L1/MASP2 [Ding et al., 2014]; Sall4, Glypican-3, dickkopf-1 and talin-1 [Chatterjee and Mitra, 2015]
Pancreatic cancer	Vinculin [Wang et al., 2012]; PAM4 [Liu et al., 2015]; cystatin [Jiang et al., 2015]; salivary micro RNAs [Xie et al., 2015]; glicoprotein panel [Nie et al., 2014]
Colorectal cancer	Vimentin [Bukhari et al., 2015];N-cadherin [Yan et al., 2015b]; GRObeta [Zheng et al., 2015]; GATA5 and SFRP2 [Zhang et al., 2015c]; kinesin 26B [Wang et al., 2015b]; microRNA-155 [Lv et al., 2015]; KRAS [Bruera et al., 2015]
Lung cancer	Tyrosine kinase-3 [Zhang et al., 2015a]; Annexin A2 [Yang et al., 2015]; osteopontin [Yan et al., 2015a]; SOX4 [Wang et al., 2015a]; GM2-activator protein [Potprommanee et al., 2015]; IL1A [Li et al., 2015b]; alpha-actinin 4 [Wang et al., 2015c]

cancer [Brichory et al., 2001]. Humoral immune response against tumor antigens in cancer patients have been used in a serum-based assays to monitor the disease progression or in the development of anticancer vaccines [GuhaThakurta et al., 2015].

The innate variability of serum among individuals makes it difficult to compare and validate disease indicators using proteomic techniques based on gel separation and MS identification. High-throughput automated methods like SELDI-TOF have been extensively used to generate characteristic proteomic patterns of disease, and search for markers in serum and plasma; many types of tumor have been investigated [Simsek et al., 2013].

PLASMA PROTEOMIC IN PANCREATIC CANCER

Pancreatic juice contains proteins directly secreted from the pancreatic ducts, hence it could be the best source for biomarker from pancreas cancer cells. However, to gain samples from pancreatic juice is not easy; for this reason, the attention of investigators has shifted on the search of potential biomarkers in plasma. However, due to the low abundance in plasma of possible cancer biomarkers, proteomic technologies are constantly improved to enhance and refine analysis for biomarker quantification. Other problem arising from biomarker detection in pancreas is due to the false-positive results coming from pancreatitis because this inflammatory condition shares several molecular features with pancreatic cancer. Examples of candidate biomarkers that have been identified such as asporin, CD9, CXC chemokine ligand 7, fibronectin 1, galectin-1, gelsolin, intercellular adhesion molecule 1, insulin-like growth factor binding protein 2, metalloproteinase inhibitor 1, stromal cell derived factor 4, and transforming growth factor beta-induced protein.

Many of these proteins are involved in various steps in pancreatic tumor progression including cell proliferation, adhesion, migration, invasion, metastasis, immune response, and angiogenesis.

These new protein candidates may provide essential information for the development of protein diagnostics and targeted therapies [Mirus et al., 2014]. In the near future, the increasing of sensitivity and reproducibility of proteomic techniques together with transcriptomic data will enable a more precise identification and validation of specific biomarkers of pancreatic cancer that will produce a sure benefit for patients who will benefit from a targeted treatment.

PLASMA PROTEOMIC IN OVARIAN CANCER

Although there are new drug treatments for ovarian cancer, the 70% of women affected still die. Therefore, it is necessary a diagnosis at an early stage of the disease, unfortunately, biomarkers discovered to date, had not good results in clinical trials.

The first biomarker studied in the screening of ovarian cancer was cancer antigen 125 (CA125) [Bast et al., 1981]. It is a serum marker whose values may, however, be high in many situations both gynecological and non-gynecological cancer, both in non-neoplastic diseases such as chronic liver disease, pancreatitis. So it does not give sufficient guarantees to be extended as a screening of the female population. In women with suspected gynecological disease, HE4 showed a higher specificity (93% vs. 78%) and similar sensitivity (79%) to CA125 for distinguishing benign ovarian cancer. Studies have shown a potential benefit in combining HE4 and CA125 when quantify the potential risk of malignancy in the evaluation of a pelvic mass [Nolen et al., 2010].

In the 1990, Jacobs et al. defined the risk of malignancy index (RMI) considering the CA125 level, the menopausal status and the ultrasound score to determine likelihood of malignancy in the setting of an adnexal mass. Later, it has been developed the risk of malignancy algorithm (ROMA) and the OVA1 test [Nolen and Lokshin, 2013] for the malignancy risk in women with adnexal masses: the former is based on HE4 and CA125 serum levels and menopausal status; the latter on biomarkers discovered through mass spectrometry, such as β-2 microglobulin, transferrin, transthyretin, and apolipoprotein [Nolen and Lokshin, 2013].

PLASMA PROTEOMIC IN LUNG CANCER

Lung cancer is one of the main cause of death in the world and is often diagnosed in the advanced or late stages. The discovery of candidate biomarkers in human lung cancer materials holds clinical potential as well as a significant challenge. Many proteomic approaches have been used to investigate the biomarkers in human lung cancer materials. Human plasma, serum and tissues are mostly used for analysis of biomarkers, whereas urine, cell lines, and pleural effusion samples are used less often [Fiorelli et al., 2015]. The most common proteomic approaches used for analysis of the lung cancer proteome are 2-D electrophoresis (2-DE), two-dimensional difference in-gel electrophoresis (2-D DIGE), and mass spectrometry (MS) using matrix-assisted laser desorption/ ionization (MALDI) or electrospray ionization (ESI) as ion sources. Two-dimensional electrophoresis is a powerful method and is used to identify the large scale of the proteome, it still has some limitations: the poor resolution of separation for less abundant proteins; its detection of proteins with extreme properties (small, large, hydrophobic, and strong acidic or basic) is limited; and it is time consuming and expensive.

Recently, many efforts have been made to improve the resolution of protein separation in proteomic analysis, for example, a combination of multi-dimensional liquid chromatography (LC) and very small amounts of a sample, and can analyze the complex biological mixtures directly. It has been used to analyze the proteome of lung cancer materials. The immobilizing specific monoclonal antibodies or phage-expressed proteins on the chips, known as protein arrays, allow for quantitation of distinct proteins from different lung cancer samples.

The test of seropositivity for tumor antigens to determine the presence of cancer is a humoral response that occurs during the outset of the tumor and generates an amplified signal appreciable in the blood in the form of autoantibodies. Proteomic technologies fit well for the identification of tumor antigens that induce autoantibodies. A protein microarray approach was applied to discover and validate lung cancer tumor antigens associated with autoantibodies.

Array analysis, which can define up- and down-regulated proteins in lung cancer samples with high accuracy, is a promising approach for additional diagnosis of lung cancer. Two-dimensional

electrophoresis or mass spectrometry (LC-MS) has been applied to plasma proteomics in lung cancer [Liotta and Petricoin, 2011].

Validation of cancer biomarkers requires different experimental approaches due to protein heterogeneities. In fact, chemical modifications, cleavages, complex with antibody, and other proteins makes them hard to be identified. Plasma biomarkers have been investigated in an integrative study on lung cancer [Serkova and Glunde, 2009] in which plasmas from three mouse models of lung adenocarcinoma have been analyzed in a quantitative proteomic analysis. The adenocarcinoma models were obtained by a mutation of epidermal growth factor receptor (EGFR) or Kras, induced by urethane exposure, and a mouse model of small-cell lung cancer lacking of Trp53 and Rb.

Data obtained from proteome investigation of lung cancer were compared with those obtained from other cancer mouse models like ovarian, pancreatic, colon, prostate and breast in addition to 2 models of inflammation [Di Domenico et al., 2011].

In plasmas of mouse models of lung adenocarcinoma were identified several proteins regulated by Nkx2–1, a transcription factor in cells from the peripheral airways, and a known survival oncogene in lung cancer. Furthermore, in plasma of mice with lung tumors determined by a mutant of human EGFR was found a upregulated group of proteins whose concentration decreased following a treatment with a tyrosine kinase inhibitor. In order to assess the correspondence between mouse models and human lung cancer an array of protein identified as marker in mice lung cancer were assayed in samples of human plasma. Since in lung cancer, through the proteomic analysis, antibodies against tumor antigens have been identified, results obtained with this method have been implemented with those obtained by autoantibodies with the microarray technology. If the results obtained with proteomic investigations of post-translational modifications like glycosylation could be further integrated, a panel of markers with high specificity and diagnostic sensitivity for lung cancer could be obtained. The proteomic technologies for the detection of tumor markers are very promising, but they are also difficult to transfer to diagnostic analysis that requires a high number of investigations, to date no reference standards have been developed. However, in the plasma, in addition to proteins, there are other molecules and cell populations that can be used as cancer biomarkers. Very promising are the microRNAs and circulating tumor cells, but also metabolites, mutated DNA, or DNA methylated fragments. The metabolome offers the opportunity for the discovery of new biomarkers due to its constant changes as a result of the organism state. However, the difficulty lies in identifying what is the concentration range in which a metabolite may be considered physiological or pathological; to solve this problem, a large amount of data are required to obtain statistically significant results. Although the proteomic technologies require high labor intensity with high costs, the results that can produce fully justify the investment, but little consideration is given to the use of proteomics in clinical diagnostic. Genomic analysis often requires the availability of tissue, which is usually obtained through a biopsy or during surgery, while proteomics, in addition to the analysis of tissue, is suitable for body fluid samples more easily available. Therefore, an important advantage of proteomics is the ability to analyze the plasma of an individual in pre-diagnosis to detect the disease at an early stage and after treatment to assess response to therapy. Furthermore, proteomics can be used to monitor the patient status during all the treatment steps by means of a simple blood test. There is still the need for a comparison of the results produced by proteomic analyses against other diagnostic approaches based on blood test for assessing the risk of other diseases [Fiorelli et al., 2014].

CONCLUSIONS

Proteomic technologies still face important technical challenges, as it is still lacking of standardized methodologies, sensitivity, and reproducibility. Common technical shortcomings include limitations in the sensitivity of detecting low abundant biomarkers and possible systematic biases in the observed data. Current research is focused on the development of high-resolution proteomic instrumentation for high-throughput monitoring of protein changes occurring in cancer. However, it is now clear that proteome analysis alone could not satisfy all requisites necessary to obtain reproducible and significative results, hence in the post-genomic era a new field of investigation is growing: proteogenomic. Proteogenomic, in fact, can overtake the problem of identifying mutated proteins from peptides in a in bottom-up proteomics. Of course to reach this result, a consolidated database containing sequences of mutated protein obtained from translation of mutated nucleic acids is required.

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