Chapter 9

Proteomics of Cancer of Hormone-Dependent Tissues

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Abstract

Serum and tissue biomarkers have begun to play an increasingly important role in the detection and management of many cancers of hormone-sensitive tissues. Specifically, the introduction of serum PSA measurements into clinical practice has dramatically altered detection and treatment of prostate cancer and serum tumor markers play a critical role in the management of testicular cancer. Serum biomarkers are used for ovarian and pancreatic cancers, but their usefulness is limited by poor specificity. Tissue biomarkers are used to help guide breast cancer treatment but are not widely used in other cancers. Even the "best" biomarkers such as PSA have substantial limitations. The discovery of new biomarkers for both early detection and prognosis of cancer is critical to the hope of better clinical outcomes. Recently there has been an expanding understanding of the underlying molecular etiology of cancer and molecular targeted therapies for some particularly aggressive cancers such as renal cell carcinoma have been developed. Better understanding of the molecular etiology of cancer and identification of additional therapeutic targets remain important research goals. Currently, there are very few patient-tailored therapies and there is a great need to better understand the molecular alterations associated with cancer and to use this information to design need cancer therapies and prevention strategies.

Advances in proteomic technologies have created tremendous opportunities for biomarker discovery and biological studies of cancer. The potential that proteomics will impact clinical practice is currently greater than ever, but there main several obstacles in making this a reality. A major hurdle to overcome continues to be the proper acquisition of patient tissues and body fluids for investigation and clinical diagnostics. Each cancer has specific issues in this regard and it is incumbent upon investigators and collaborating clinicians to understand the various nuances of tissue and biofluid procurement. This chapter not only reviews the clinical need and potential impact of proteomic studies of hormone-sensitive cancers, but details specific technologies and discusses the issues surrounding tissue/biofluid procurement.

Introduction and Overview

Clinical Perspective

In the United States, cancer of hormone-sensitive tissues represent a majority of solid tumors, with prostate and breast cancers being the most common types of noncutaneous malignancies in men and women, respectively. Ovarian and pancreatic cancers are less common but usually lethal when they do occur. Pancreatic cancer is responsible for 6% of cancer deaths in both men

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and women and ovarian cancer for 6% of cancer deaths in women.[1] In contemporary practice, serum biomarkers have the greatest role in prostate cancer management. Prostate specific antigen (PSA) is widely accepted as a serum biomarker for prostate cancer (CaP) and is used extensively for screening, staging and monitoring patients after treatment.[2] CA-125 is considered the best biomarker for ovarian cancer, although it is not recommended for widespread screening due to a lack of specificity.[3] Similarly, CA19-9 is a widely used marker of pancreatic cancer, but it lacks sufficient specificity and sensitivity to be used for screening purposes.[4] Biomarkers play an important role in staging and monitoring patient with testicular cancer. Alpha-fetoprotein (AFP) is highly specific for nonseminomatous germ cell tumors and human chorionic gonadotropin (bHCG) is elevated in more than 50% of nonseminomatous germ cell tumors and approximately 10% of pure seminomas.[5] There are currently no useful serum biomarkers for the detection of breast or adrenal cancers. In most cases, early detection improves the outcome for cancer treatment. Although biomarkers have begun to play a role in detection and management of some cancers, for most there is either no useful serum biomarkers or the available biomarker lacks sufficient specificity and sensitivity for use as a screening tool.

Although serum (or potentially another body fluid) is the most useful source to measure biomarkers for early detection, tissue biomarkers are now being used more often to help determine the most appropriate treatment for a specific patient or follow response to therapy. For example, tissue biomarkers such as HER-2 and estrogen and progesterone receptor levels are used for prognostication and to direct treatment in breast cancer. Unfortunately, targeting these molecules has not proven to be a sufficient means to completely eradicate these cancers as they tend to develop resistance to uni-targeted therapies.[6] CA-125 is often used to monitor treatment of ovarian cancer, but is not always helpful since many of these tumors do not express this protein.[3] For testicular cancer, AFP and bHCG are used to monitor response to chemotherapy and are used to direct further therapy.[7] The molecular determinants of prostate, ovarian, endometrial and pancreatic carcinogenesis remain ill-defined; therefore, no molecular based prognostic tools are commonly used for these cancers.

The Cancer Phenotype

It is a widely held belief that in most cases the malignant phenotype originates from inherited germline genetic alterations, acquired somatic mutations, or by epigenetic phenomena. Examination of the changes occurring in cancer at the nucleic acid level has resulted in invaluable information about disease development and progression. More recently, advances in gene expression profiling technologies have allowed for global analysis of expression levels of thousands of mRNA transcripts simultaneously. This information has begun to be used for disease classification to help clinicians with prognosis and treatment. Malignant transforming genetic alterations are typically manifested as either a loss or a gain of function of a specific regulatory protein. These tumor suppressors or oncogenes are commonly responsible for how a cell responds to its environment and may cause inappropriate proliferation, migration, survival or other cancer-defining responses.

Although many biomarker discovery studies have focused on RNA expression analysis, there are, however, distinct advantages of proteomic studies; above all, proteins are ultimately responsible for the disease phenotype. In addition, proteomics can identify alterations in posttranslational modifications, subcellular localization and proteolytic cleavage events, and protein levels are not necessarily reflective of RNA-based expression studies. Furthermore, since most FDA-approved diagnostic tests are protein based, directly studying proteins and their variants should expedite the development of clinically useful tests. Traditionally, proteomic studies have focused on biomarker discovery and clinical tests are typically antibody-based and directed at individual biomarkers. Technological advances, however, have increased the throughput and accuracy of protein analysis and it is possible that some of these analytical instruments will be usable for proteomic-based clinical assays rather than relying on the development of antibodies, a laborious and time-consuming process that is not guaranteed to succeed.[8]

Many different scientific strategies have utilized a variety of biospecimens to identify novel cancer biomarkers. Some studies have focused on the molecular alterations occurring in cancerous tissue as well as the surrounding stroma; others have concentrated on circulating blood, other body fluids, or distant tissues that may be affected by the developing tumor. The study of these tissues and fluids at the protein level is broadly referred to as proteomics.

Proteomics Defined

Proteomics can be defined in many ways depending on the desired scope and complexity of the analysis. In the main, proteomic analyses aim to characterize all the proteins present within a particular cell, tissue or organ. However, since a single gene can encode multiple proteins via different exon usage or splicing events and proteins are invariably modified posttranslationally (e.g., phosphorylation and acetylation), a single gene can produce tens to hundreds and possibly thousands of unique proteins within a single cell. In addition, proteins are constantly being modified and any single analysis only represents a snapshot of the ongoing milieu. Not surprisingly, variability is a considerable problem in the field of proteomics. In spite of these difficulties, proteomic analyses have provided substantial new insight into our understanding of cancer as well as powerful new techniques for finding biological markers to detect and analyze cancer development, progression and response to treatment. The goal of this chapter is to summarize some of the recent studies using proteomic analyses on endocrine-regulated cancers, to describe advantages and limitations of these approaches, to discuss potential clinical applications of these findings and to provide insight into the future directions that proteomics will take cancer biology and clinical management of these common cancers. Proteomic techniques that have been used to identify biomarkers in different sample sources (e.g., blood or tumor tissue) will also be discussed.

Biomarkers versus Biology

Numerous tools have been developed to provide both quantitative and qualitative information about protein composition in tumor tissue and biofluids. Application of these tools to the study of cancer has generally focused on two distinct yet complementary goals: (1) understanding how cancer develops and progresses and (2) cataloguing new biomarkers associated with a particular tumor type. In each case, the application of proteomics is primarily discovery-driven and does not have a specific hypothesis as a prerequisite. Regardless, validation of results generated using discovery-based approaches are critical.[9] To date, most of the efforts in this arena have been put toward the discovery phase with very little follow through on the validation. Few, if any, biomarkers identified with proteomic technologies have been validated by clinical trials and approved by regulatory agencies.[8]

In the past, studies designed to understand the biological basis of cancer have involved a reductionist approach aimed to reduce the complexity of analysis.[10] However, with recent major advances in the fields of bioinformatics and computational technology the inherent complexity can now be examined en masse in what is referred to as systems biology.[10-12] Thus, the use of highly sensitive and quantitative proteomic techniques coupled with the new computational capabilities permit an unbiased cataloging of molecular changes associated with cancer initiation and progression. This provides an unprecedented opportunity for discovering new clinically useful biomarkers and gaining new insight into tumor biology.

Cancer Proteomics: Sample Sources and Methodological Approaches

Cancerous Human Tissue

Typically, cancerous tissue is the most fertile source to procure relevant molecular information. However, for many human cancers an invasive procedure is required to obtain tissue samples for analysis. For example, in order to procure prostate cancer specimens a transrectal needle biopsy of the prostate or a radical prostatectomy is required. In addition, due to widespread screening and better detection modalities for cancers of the breast and prostate, most of these cancers are detected as low-volume disease; there is often only a limited amount of cancerous tissue present

even within a radical prostatectomy specimen. Furthermore, the infiltrative nature of prostatic adenocarcinomas makes isolation of pure cell populations of cancer cells difficult. This is also true for pancreatic ductal adenocarcinoma in which the tumor is comprised of 30-90% tumor cells with a large amount of fibroblastic infiltration.[13] While it is becoming much more appreciated that the surrounding stroma is a major contributor to tumor biology,[14] the primary focus of most studies of the tumor involve the cancerous epithelial cells themselves (the vast majority of cancers are adenocarcinomas).

In order to minimize the contribution of contaminated stroma and inflammatory cells in the proteomic analyses, different methods have been developed for procuring pure populations of cells from human tissues. Laser capture microdissection is a relatively new technique that allows researchers to visualize a tissue section via light microscopy and procure the desired cells by activating a 7.5 to 30 μm diameter infrared laser beam to "weld" the tissue to a plastic cap. Intact DNA, RNA and protein can then be extracted from the "welded" tissue and analyzed by conventional methods.[15,16] Proteomic studies utilizing two-dimensional gel electrophoresis (2DGE) analysis of LCM procured benign and cancerous prostate cells have been successfully performed. Through this approach annexin I was found to be under-expressed in early stage CaP,[17,18] and subsequent studies have confirmed that annexin I and annexin II are commonly reduced in CaP and that these molecules may be useful tissue biomarkers.[19] LCM has been used extensively to isolate tumor cells from breast cancer for subsequent proteomic analyses,[20-29] and at least two groups have used LCM to aid in the isolation of pure populations of tumor cells from ovarian tumors that are frequently highly infiltrative at initial detection.[30,31] Similarly, studies on pancreatic,[32] and renal cancers[33] have also relied on LCM to enrich the tumor cell population for proteomic-based studies. The major limitations of LCM are: 1) it is extremely labor intensive (although new systems that provide automated cell selection and cutting have at least partially alleviated this) and 2) for optimal extraction of macromolecules the input tissue should be cryopreserved rather than formalin-fixed.

Although LCM is the most frequently employed tool for separating tumor cells from benign cells and stroma, other techniques have also been employed. These include short-term culture of enzymatically disaggregated cells[34-36] or immunomagnetic bead separation of individual cells.[37,38] Short-term culture is useful to provide a cellular amplification step to increase sample size when available tissue is limiting. However, even short-term culture of cells may induce changes in response to nonnative growth conditions that may mask relevant markers of malignancy. The use of immunocapture beads to isolate cells from disaggregated tumors allows tumor cell enrichment without requiring the cells to proliferate and would therefore alleviate the risk of cellular changes induced by culture but would eliminate the amplification step.

Historically, the stalwart platform for proteomics has been two dimensional gel electrophoresis (2DGE).[39] Although the technique has been in use for over three decades, recent modifications to the technique have enhanced the dynamic range and resolution of protein discrimination enabling this technique to remain as a common platform for proteomic analyses. Despite these advances, 2DGE is still limited by a relatively small dynamic range (two to three orders of magnitude), difficulty separating highly basic or acidic proteins or those of low molecular mass and the relatively low throughput. Advances in separation technologies and bioinformatics have greatly enhanced the use of mass spectrometry (MS)-based approaches and have begun to replace 2DGE as the proteomic analytic technique of choice.

Determining the cellular source of protein production is of critical importance for the proper identification of molecular alterations occur during the transition from benign to malignant tissue. For 2DGE, relative expression levels can now be determined much more accurately using differentially labeled samples that are run simultaneously on the same gel.[39] Gel-to-gel variability, a well-known problem of 2DGE, is also mitigated by the use of dually labeled samples run simultaneously. The identification of individual spots on 2D gels is generally accomplished using liquid chromatographic separation of trypsin-digested fragments subjected to tandem mass spectrometry (LC-MS/MS) or matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

As outlined in Table 1, many other techniques have been employed to examine the proteome of clinical cancer specimens from hormone-regulated organs, but mass spectrometry (MS) has become the preferred technology. The description and use of MS for proteomic studies has been extensively reviewed.[40-45] However, several innovative MS technologies are worth describing further. Relevant to the analysis of tumor tissue, the elegant work conducted by Caprioli and colleagues has provided a new dimension to MS spectra, specifically tissue localization.[46-48] This is accomplished by directly adding micron-sized matrix droplets onto whole tissue sections and subjecting the tissue to direct MALDI-TOF analysis.[49] This technique has been used to analyze normal mammary epithelium, ductal carcinoma in situ, invasive breast adenocarcinoma and surrounding stroma from sectioned human breast cancer samples.[50] Although no specific peaks were identified, this approach demonstrated definite alterations of spectral patterns from tissue sections containing the various histological phenotypes easily allowing their discrimination on the spectral data alone.[50] The use of spectral data alone has previously been suggested as a diagnostic tool for analyzing serum constituents as described below.

Body Fluids

In clinical practice, most useful biomarkers are measured in serum or plasma. There is an emerging body of data suggesting that for most cancers the assessment of a pattern of multiple biomarkers provides more robust diagnostic and prognostic information than the measurement of a single biomarker. Advances in proteomic technologies have made it possible to rapidly assess complex protein expression patterns in a large number of clinical samples. Surface-enhanced laser desorption ionization time-of-flight (SELDI-TOF) mass spectrometry is a relatively new technology that can profile low molecular weight peptides. SELDI-TOF is a proprietary modification of MALDI-TOF that incorporates an affinity resin on the MALDI plate to facilitate protein capture and purification in a single step prior to subsequent MALDI-TOF analysis.[51-53] This technology produces crude but rapid protein purification and signal amplification with very high throughput and provides a strong platform for cancer biomarker screening by generating a reproducible low molecular protein fingerprint from a miniscule amount of sample (i.e., 1 μl of blood). In addition, no a priori knowledge of specific protein components is required. SELDI-TOF has been used extensively to profile cancer of hormone-sensitive tissues. For example, it has been used to identify protein signatures from nipple aspirates for the discrimination of women with breast cancer from healthy women,[54-56] to discriminate between microdissected benign and malignant cells from prostate tissue[57,58] and to screen for presence of kidney cancer in serum[59] and urine.[60] SELDI-TOF has also been used to detect alterations in serum profiles of men undergoing androgen ablation therapy[61] or radiation[62] for prostate cancer and to screen for diagnostic markers in thyroid cancer[63] and renal cancer.[64]

Because of its ability to rapidly analyze a large number of samples, SELDI-TOF is particularly well suited to generate informative proteomic patterns from serum. Because visual analysis only detects gross changes in protein expression, bioinformatics tools are required to detect subtle differences in patterns of protein expression. Importantly, because of the huge dimensionality of the data, advanced pattern recognition algorithms are required to find the hidden, non-apparent signatures in a background of noise and chaos. Bioinformatics tools, some of which have utilized artificial intelligence based pattern recognition algorithms that evolve and learn, can facilitate the analysis of complex data sets and have been applied to the detection of ovarian and prostate cancer. Using this approach, a diagnostic algorithm was generated that yielded an overall positive predictive value (PPV) of 94% for the diagnosis of ovarian cancer and all 18 women with stage I ovarian cancer were correctly classified by the algorithm.[65] Although these preliminary studies generated highly promising data and demonstrated feasibility of a new diagnostic paradigm, a lack of reproducibility and the inability to identify the proteins and peptides comprising the spectra drew significant criticism of the approach. The use of high-end mass spectrometers like the API QSTAR Pulsar LC/MS/MS System (Applied Biosystems Inc.) has increased mass accuracy that reduces machine-to-machine difference in mass drift. Moreover, the QSTAR can perform direct

Table 1. Examples of proteomic analyses performed on cancers of hormone-regulated organs

Cancer Type	Sample Source	Prefractionation	Analysis Technology	Quantitation	Study Goal	Ref.
Prostate	urine	reverse phase	LC-MS/MS	protein coverage	diagnostic	72
	urine	none	2DGE	spot intensity	diagnostic	73
	serum	none	SELDI-TOF	peak height	diagnostic	68
	serum	cation X	LC-MS/MS	protein coverage	diagnostic	67
	serum	none	antigen array	spot intensity	diagnostic	85
	serum	none	SELDI-TOF	peak height	prognostic	61
	serum	none	Autoantibody array	spot intensity	diagnostic	86
	tumors	LCM	SELDI-TOF	peak height	diagnostic	58
	tumors	manual dissection	high-throughput IB	band intensity	diagnostic	87
	tumor	LCM	SELDI-TOF	peak height	diagnostic	57
	tumor	manual dissection	2DGE	radioiodine	diagnostic	88
	tumor	manual dissection	2DGE	spot intensity	diagnostic	89
	tumor	LCM	2DGE	spot intensity	diagnostic	90
	tumor biopsies	none	LC-MS/MS	none	diagnostic	91
	FFPE tumors	LCM	LC-MS/MS	O^{16}/O^{18}	diagnostic	92
	tumor, LNCaP	LCM	2DGE	spot intensity	diagnostic	93
	LNCaP	microsomal prep	LC-MS/MS	ICAT	diagnostic	94
	LNCaP	secretome	LC-MS/MS	ICAT	diagnostic	95
	LNCaP	none	2DGE	spot intensity	prognostic	96
	LNCaP	none	2DGE	ICAT	diagnostic	97
Breast	nipple aspirate	none	2DGE	spot intensity	diagnostic	75
	nipple aspirate	none	SELDI-TOF	peak height	diagnostic	54
	nipple aspirate	metal affinity cation X	SELDI-TOF	peak height	diagnostic	55
	nipple aspirate	1D-PAGE	LC-MS/MS	ICAT	diagnostic	76
	nipple aspirate	hydrophobic anion X	SELDI-TOF	peak height	diagnostic	56
	serum	none	immunobead array	fluorescence	diagnostic	83
	adipose tissue/fluid	none	2DGE and antibody array	spot intensity	diagnostic	80
	tumor	LCM	reverse-phase array	spot intensity	diagnostic	29
	tumor (HER-$2^{-/+}$)	metal affinity	SELDI	peak height	prognostic	25
	tumor ($PR^{-/+}$)	LCM	2DGE	radioiodine	prognostic	26
	tumor	LCM	2DGE	spot intensity	diagnostic	21
	tumor	LCM	MALDI-TOF	peak height	diagnostic	22
	tumor	LCM	2DGE	O^{16}/O^{18}	diagnostic	23
	tumor(HER-$2^{-/+}$)	LCM	2DGE	spot intensity	prognostic	98

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Table 1. Continued

Cancer Type	Sample Source	Prefractionation	Analysis Technology	Quantitation	Study Goal	Ref.
Ovaries	serum	albumin-bound	1D-PAGE/ LC-MS/MS	none	diagnostic	69
	serum	albumin-bound	MALDI-TOF	none	diagnostic	99
	serum	hydrophobic	SELDI-TOF	peak height	diagnostic	65
	tumor	LCM	2DGE	spot intensity	diagnostic	31
	tumor	LCM	reverse-phase array	spot intensity	diagnostic	100
Pancreas	serum	anion X	SELD-TOF	peak height	diagnostic	101
	serum	none	2DGE	fluorescence	diagnostic	102
	plasma	anion X	SELDI-TOF	peak height	diagnostic	103
	plasma	none	2DGE	fluorescence	diagnostic	104
	plasma	none	2DGE	spot intensity	diagnostic	105
	pancreatic juice	1D-PAGE	LC-MS/MS	protein coverage	diagnostic	77
	tumor	LCM	2DGE	spot intensity	diagnostic	32
	tumor	none	high-throughput IB	band intensity	diagnostic	106
	tumor	cation X	LC-MS/MS	ICAT	diagnostic	37
	tumor	LCM	2DGE	fluorescence	diagnostic	107
	cell line	secretome	LC-MS/MS	ICAT	diagnostic	108
	cell line (–/+ Tx)	none	2DGE	spot intensity	prognostic	109
	cell line (–/+ Tx)	none	2DGE	spot intensity	prognostic	110
Kidney	serum	anion X	SELDI-TOF	peak height	diagnostic	59
	urine	cation X	SELDI-TOF	peak height	diagnostic	60
	urine	none	2DGE	spot intensity	diagnostic	74
	tumor	none	2DGE	spot intensity	diagnostic	111
	tumor	LCM	2DGE	radioiodine	diagnostic	112
	tumor	manual dissection	2DGE	spot intensity	diagnostic	33
	tumor	LCM	2DGE	spot intensity	diagnostic	113
	primary cells	none	2DGE	spot intensity	diagnostic	36
	primary cells	none	2DGE	spot intensity	diagnostic	114
	tumor	cation X/metal affinity	SELDI-TOF	peak height	diagnostic	63
Thyroid	serum	C8 reverse phase	MALDI-TOF	peak height	diagnostic	115
	tumor	none	2DGE	fluorescence	diagnostic	116
	tumor	manual dissection	2DGE	spot intensity	diagnostic	117
Endometrium	serum	none	immunobead array	fluorescence	diagnostic	84

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Table 1 Abbreviations: LC-MS/MS, liquid chromatography—tandem mass spectrometry; 2DGE, two dimensional gel electrophoresis; SELDI-TOF, surface-enhanced laser desorption/ionization—time of flight mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization—time of flight mass spectrometry; LCM, laser capture microdissection; X, exchange; ICAT, isotope-coded affinity tag; FFPE, formalin-fixed, paraffin-embedded; Tx, treatment

MS/MS protein identification, alleviating a major drawback to the use of SELDI, the lack of peak identification.[66]

This concept is not limited to just one type of cancer. An algorithm capable of predicting the presence of prostate cancer with 41% sensitivity has been generated. The artificial intelligence-type pattern recognition algorithm identified correctly 36 of 38 men with prostate cancer (i.e., 95% sensitivity) and 177 of 228 men with benign biopsies (i.e., specificity of 76%). For men with total PSA levels between 4.0 and 10.0 ng/ml, 97 of 137 (71%) were correctly classified as having benign prostates. Thus, if serum proteomic analysis had been used to determine the need for prostate biopsy, 70% of "unnecessary" biopsies could have been prevented while only 5% of cancers would have been missed. Importantly, the algorithm correctly classified all of these men with prostate cancer.[67] Another analytical strategy utilizes a decision tree algorithm that relies on binomial decisions based on heights of a predefined set of specific protein peaks. Using this approach in a blinded test set of 60 men (30 with prostate cancer and 30 with benign prostates) yielded a sensitivity of 83% and a specificity of 97%.[68]

Although body fluids are fertile sources for biomarker discovery they pose several challenges that complicate biomarker discovery. A major difficulty in the direct identification of serum or plasma biomarkers is the high abundance of albumin and other larger carrier molecules, which has historically made it impossible to identify small molecule biomarkers directly from serum or plasma. Traditionally, serum-based biomarker studies have utilized strategies to deplete albumin and immunoglobulins to increase the sensitivity for the lesser abundant proteins. Recent data challenges this experimental paradigm as it has become increasingly apparent that an immense archive of potentially relevant clinical biomarkers exists bound to albumin. In fact, it has been demonstrated that depletion strategies for high-abundant carrier proteins can be exploited as a means to amplify low abundant serum proteins and peptide fragments.[69] This approach and other examples of innovative solutions to technical challenges in clinical proteomics are listed in Table 2.

Blood likely contains only minute quantities of tumor-specific biomarkers due to its presence throughout the body. Hence, organ-proximal fluids (e.g., pancreatic juice and nipple aspirate fluid) may be more useful as source materials, albeit with a loss of ease of acquisition. For urogenital malignancies, urine provides an easily obtainable source material that is likely enriched in tumor-specific molecules. However, urine is also known to vary significantly in protein content even from the same individual, making its analysis more challenging.[70,71] Urine has been screened for markers of prostate[72,73] and kidney[60,74] cancer and has provided several potential markers for each. Nipple aspirate fluid has been studied extensively for the presence of tumor markers and may be a particularly useful sample source for diagnosis of breast cancer.[54-56,75,76] Several potential markers of breast cancer found in nipple aspirate fluid include vitamin D binding protein, lipophilin B, hemopexin, alpha1-acid glycoprotein and GCDFP-15.[75,76] Likewise, human pancreatic juice has also been analyzed for the presence of cancer-specific biomarkers.[77] This extensive study produced a very large list of proteins present in pancreatic juice from patients undergoing pancreatectomy for pancreatic cancer and many of the proteins identified have previously been shown to be markers of pancreatic cancer.[77]

There is an emerging body of evidence supporting the role of adipose tissue as an endocrine organ and fat has recently garnered attention as a source of biomarkers for breast and other cancers. Adipose tissue is a major component of mammary glands and has been shown to contribute to the development of the glands[78,79] and several studies have suggested a direct role of mammary adipose tissue in the progression of breast tumors. Initial studies of mammary adipose tissue and

Table 2. Examples of limitations and challenges of clinical proteomics and recent innovative solutions

Limitations/Challenges	Needs	Recent Innovative Solutions
Lack of sensitivity during discovery phase	Signal amplification, removal of abundant proteins, more sensitive discovery methods	Carrier-protein amplification (e.g., characterization of LMW peptides bound to albumin[99]) Use of antibodies during discovery stage (e.g., multiplex formats of antibody-bound beads[83,84] or arrays;[86] reverse-phase lysate arrays[82]) Computer model of protein abundance distributions to assist experimental design[118]
Enormous datasets with different levels of quantitation and unknown associations	Bioinformatic and computational tools to handle multidimensional data	Development of software for examining multi-dimensional datasets using interval estimation[119] Development of software for analyzing potential interacting molecules[120-123] Development of a computer algorithm that uses neural network processing to discern discriminatory patterns from mass spectrometry data[68]
Limited supply of clinical specimens with long-term clinical annotation to better determine risk of recurrence or death	Tissue procurement programs incorporating annotated databases, alternative source materials in more abundance	Use of archived formalin-fixed paraffin-embedded tissue as source material[92]
Cells in low abundance within tumors (e.g., cancer stem cells) are not well represented	Direct analysis or prior isolation of low protein abundance cells in complex tissues	Microdissection of cells based on expression[124,125]

its interstitial fluid from human patients undergoing mastectomy for breast cancer were analyzed by 2DGE and an antibody array to detect signaling proteins in tissue lysates.[80] This extensive characterization provides a substantial list of proteins (359 identifiable proteins) found within and around the adipose tissue, including numerous growth factors and cytokines well documented as mediators of cancer progression.[80] These studies suggest that mammary adipose tissue should be considered as part of the tumor stroma since it contributes significantly to the secreted factors surrounding the tumor cells. This may also be the case for other malignancies. Adipose tissue surrounding organs likely provide organ-specific functions and can be expected to actively participate in organ homeostasis. Therefore, organ-proximal adipose tissue may interact bi-directionally with developing tumors.

Cultured Cells

The use of clinical samples provides the most relevant tissue for discovery-based approaches to cancer. However, the limiting supply of tissue and the extreme heterogeneity of samples provide substantial hurdles to these studies. Model systems by design are reductive approaches to understanding a particular system and are limited in the global applicability, but cultured cells can be extremely useful in alleviating the problems of sample supply and heterogeneity. For these reasons, cultured cells have been used extensively to study many human diseases, especially cancer and comprise the main source material for molecular analyses. Primary cell lines are isolated directly from tissue and grown in culture. Sufficient cell separation techniques are required to assure a high enrichment of cancer cells, otherwise the benign cells within the culture can mask any cancer-specific alterations. Short-term cultures generally have a short life span (often under five passages) but have been shown to maintain many of their phenotypic properties over this time.[34] A potential problem with short-term cultures is that the growth medium may be selective for a particular cell population, thereby misrepresenting the true cellular population of the initial tumor.[81]

Recent Innovations and Technological Advances

Aside from 2DGE and MS-based techniques there are a wide variety of protein arrays that can provide alternative modalities for detecting cancer-specific factors, such as reverse phase lysate arrays, antibody arrays, kinase substrate arrays and others.[82] Numerous techniques have been developed that aim to reduce the complexity of the samples by focusing on specific subsets of proteins (e.g., kinases by measuring activity with peptide substrate arrays) and the use of array-based proteomics for clinical management of cancer has been excellently reviewed by Gulmann et al.[82] In addition to solid phase arrays, a new quantitative platform based on flow cytometric separation of fluorescently labeled beads (xMAP™) is becoming more widely used. The technology allows for linkage of many types of molecules, including antibodies, peptides, carbohydrates, etc. to beads with different fluorescent properties that can then be used as affinity capture reagents. As many as 100 different beads can be discriminated in a single tube, which allows for a highly multiplexed analysis of samples. This technology has already been used to examine several components in blood of patients with prostate cancer[83] or endometrial cancer.[84] A limitation of this technology is that knowledge is required a priori to determine which types of molecules to detect. Major advantages of this approach are that the beads are small enough to provide binding kinetics similar to those in solution, the results are quantitative over approximately five orders of magnitude and only very small sample sizes are required.

The Future of Clinical Proteomics

The utilization of proteomics for discovery-based studies has generated extensive lists of proteins and peptides that may be clinically useful biomarkers. Although the generation of these lists has been the focus of the majority of clinical proteomic studies, discerning the true relevance of these biomarkers to a particular disease state is much more important and presents a much greater challenge. The evaluation of clinical biomarkers is an arduous process and will likely lead to the removal of many of the candidates from the list. However, it is imperative that these studies are performed so that the truly relevant and useful biomarkers can be applied toward minimizing pain and suffering from endocrine-related cancers. There are several critical factors that are of utmost importance for achieving this goal: (1) the development of data and sample repositories with accurate and thorough clinical annotation, (2) the continual development of new technologies to address the deficiencies of current approaches, (3) standardized protocols and data management procedures to ensure that results from multiple groups can be directly compared, (4) the development of new computational and informatic systems that can integrate the multidimensional data from multiple investigators into unifying theories relevant to disease development and progression, (5) incorporation of other data sets (e.g., genomic, transcriptomic and metabolomic information) into these models and (6) continual basic research at the cellular and molecular level to aid in our understanding of carcinogenesis and tumor progression.

The application of proteomics to patient-tailored diagnosis and treatment has not yet come to fruition, but with vigilant efforts this goal may still be achieved. Until such time that cancer is no longer a major cause of morbidity and mortality, such efforts remain imperative.

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