Plasma Membrane Proteomics and Its Application in Clinical Cancer Biomarker Discovery*

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Plasma membrane proteins that are exposed on the cell surface have important biological functions, such as signaling into and out of the cells, ion transport, and cell-cell and cell-matrix interactions. The expression level of many of the plasma membrane proteins involved in these key functions is altered on cancer cells, and these proteins may also be subject to post-translational modification, such as altered phosphorylation and glycosylation. Additional protein alterations on cancer cells confer metastatic capacities, and some of these cell surface proteins have already been successfully targeted by protein drugs, such as human antibodies, that have enhanced survival of several groups of cancer patients. The combination of novel analytical approaches and subcellular fractionation procedures has made it possible to study the plasma membrane proteome in more detail, which will elucidate cancer biology, particularly metastasis, and guide future development of novel drug targets. The technical advances in plasma membrane proteomics and the consequent biological revelations will be discussed herein. Many of the advances have been made using cancer cell lines, but because the main goal of this research is to improve individualized treatment and increase cancer patient survival, further development is crucial to direct analysis of clinically relevant patient samples. These efforts include optimized specimen handling and preparation as well as improved proteomics platforms. Identification of potentially useful proteomics-based biomarkers must be validated in larger, well defined retrospective and prospective clinical studies, and these combined efforts should result in identification of biomarkers that will greatly improve early detection, prognosis, and prediction of treatment response.   *Molecular & Cellular Proteomics 9:1369–1382, 2010.*

During the past two decades, there has been a growing interest in systems biology approaches toward discovering new biomarkers that may allow early diagnosis, prognosis, classification of disease subtypes, prediction of treatment response, and identification of potential targets for drug therapy. For most of these applications, a single marker is likely insufficient for stratification, and a panel of markers, the so-called molecular profiles or biosignatures, can be more informative. Such biomarker profiles can be identified at different molecular levels, such as DNA, RNA, microRNA, and protein, and include a plethora of different modifications from DNA deletions/amplifications and gene and microRNA expression alterations to post-translational modifications (PTMs)[1] of proteins. A significant portion of the biomarker discovery efforts using -omics approaches has been in the area of cancer, and several markers are already in routine clinical practice, such as K-Ras mutation and HER-2 amplification. In particular, global gene expression analysis has been extensively utilized, and the cancer management results are currently being translated into clinical tests, such as MammaPrint and Oncotype DX. Proteome analysis of cancer tissue samples as well as identification of potential protein biomarkers within serum or plasma is also a growing field. The direct analysis of protein, the functional unit of the cell, using proteomics analysis has several advantages over indirect measures, such as transcriptomics analysis, despite requiring more tissue and being more time-consuming. Proteomics technologies for analysis of clinical specimens are rapidly being refined. A particular area of interest is the cell surface proteome because it is easily accessible and may serve as an ideal target for novel protein drugs. We focus herein on the most recent proteomics developments in the cancer biomarker discovery area with a particular focus on plasma membrane (PM) proteins.

PLASMA MEMBRANE PROTEINS

Membranes play a critical role in cell structure by providing a physical barrier between the cell, the environment, and the various subcellular compartments. The cell surface membrane, or PM, encloses the cell and maintains the essential

[1] The abbreviations used are: PTM, post-translational modification; PM, plasma membrane; ECM, extracellular matrix; FFPE, formalin-fixed, paraffin-embedded; ER, estrogen receptor; EGFR, epidermal growth factor receptor; SILAC, stable isotope labeling by amino acids in cell culture; iTRAQ, isobaric tag for relative and absolute quantitation; TMA, tissue microarray; AQUA, absolute quantification.

boundaries between the cytoplasm and the extracellular environment. PM proteins constitute ~50% of the mass of the PM and exhibit essential specific or multidisciplinary functions (1–4). Membrane-associated proteins include proteins physically embedded in the lipid bilayers, such as ion channels and transmembrane proteins, and proteins anchored to the membrane that sense external signals, transport specific molecules, and connect the membrane to the cytoskeleton, the extracellular matrix (ECM), and adjacent cells (5). Thus, a number of PM proteins are permanently bound to the lipid bilayer as integral or peripheral proteins, whereas others associate to the membrane only briefly under specific conditions (6).

PM receptors are transmembrane proteins that bind hydrophilic signaling molecules that cannot cross the lipid bilayer and ultimately act as signal transducers to regulate cell processes. They can be subdivided into four main classes: G-protein-linked, enzyme-linked, ligand-gated ion channel-linked, and receptors of cellular adhesion. Thus, receptors are key molecules for cell survival and consequently primary therapeutic targets. Many receptors are found tightly associated with other proteins, and information gained from proteomics of receptor complexes in PMs can elucidate the mechanisms of drug action and possibly the mechanisms of diseases (7). Receptor-protein interactions are independent of protein synthesis, making proteomics the principal approach, as opposed to genomics and transcriptomics, for analyzing signaling and regulation of receptors (8, 9).

A current estimate indicates that 30% of all the predicted open reading frames in a typical genome encode membrane proteins and PM proteins in numbers that represent a significant portion of the human proteome. However, because PM proteins are low abundant compared with many soluble proteins, the overall fraction of PM proteins in a cell/tissue lysate is very low, making them difficult to study even with the recent advances in proteomics technologies (10–12).

PLASMA MEMBRANE PROTEIN ALTERATIONS ASSOCIATED WITH CANCER

The phenotypic changes associated with malignant transformation, including cell proliferation, adhesion, and migration, are often mediated or initiated by proteins associated with the PM, making these central in the biological process and potentially effective drug targets (13–16). The difference between the cancer cells and the normal cells from which the cancer cells mutated are often alterations in the expression level of particular PM proteins. This can be either higher expression of a certain receptor, such as HER-2, that can contribute to tumor cell growth when activated by circulating or locally produced ligands or down-regulation of certain adhesion molecules that allow the cells to detach from the primary tumor and spread (17). In many comparative proteomics MS studies of cancer cell lines, such as cancer *versus* normal cells or metastatic *versus* non-metastatic can-

cer cells, a cutoff of 2–3-fold difference is set to determine whether proteins, including PM proteins, are differently expressed in the two groups, whereas in other studies as little as 1.2–1.5-fold differences are considered a significant difference (18–20). Whether these alterations have biological meaning is difficult to evaluate, but smaller expression alterations in several interacting proteins may have additive effects. Confirmatory studies using different biochemical assays may elucidate this. In addition, the expression of the differently expressed PM proteins in biologically relevant clinical tissues is often not evaluated, although it is hypothesized that these proteins may be potential therapeutic targets or biomarkers. In some studies, however, the identified proteins were examined in detail, including evaluation of their expression in clinically relevant tissue samples (21–23).

More rarely, specific PM proteins that are expressed in normal cells are not expressed in the corresponding malignant cells. An example of this is down-regulation or lack of HLA class I antigen expression in some tumors. In other rare cases, proteins not normally expressed by most normal cells are expressed in the cancer cells, possibly representing oncofetal antigens, such as survivin (24), and cancer/testis antigens (25). These proteins are important in specific stages of fetal development but are not, with the exception of germ cells, expressed in normal adult tissues. Most of these cancer-specific antigens are intracellular proteins and not PM proteins (25).

Altered Post-translational Modification of Cancer Cells— Although the amino acid sequence of the PM proteins predominantly determines their three-dimensional structure, the PTMs, such as glycosylation and phosphorylation, of the proteins modulate their physical and chemical properties and thus their stability and molecular function. The diversity of PTMs expands the functional properties of key proteins in the cell and plays an important role in the processes of cell division, differentiation, growth, cell death, and interaction with stromal tissue for both normal and cancer cells (26). Several proteomics methods (described later in this review) for studying the various PTMs of proteins in complex biological systems have been developed, but the pattern of PTMs in normal and cancer cells at a given time and under given conditions remains to be systematically investigated (27).

*Glycosylation of Cancer Cells—*Among the various PTMs, glycosylation is the most common, being present in ~50% of the total number of proteins (28). Cancer cells frequently display glycoproteins with increased branching of the glycan structures and/or altered expression levels compared with normal cells, and these alterations most often involve Asn (*N*)- and Thr/Ser (*O*)-linked glycans and proteoglycans (29). An increase in the branching creates additional sites for terminal sialic acid residues, negatively charged acidic sugars that can be recognized by lectins (30). The alterations can affect interactions between cancer cell-associated PM glycoproteins and endogenous lectins that subsequently may in-

fluence the metastatic potential of the cancer cell (31, 32). Enhanced expression of terminal α2–6-linked sialic acid on N-linked glycans and of sialyl-Lewis X on O-linked glycans (typically found on mucins) often correlates with poor prognosis (33–35). Although the glycan structure or expression level of many PM glycoproteins may be altered, alterations may also occur on secreted glycoproteins and serve as biomarkers for early detection of cancers (29, 36–38).

Glycan analysis can be challenging for several reasons. Unlike proteins, glycan biosynthesis is governed by a series of enzymes present in the cell and does not rely on an underlying template. As a result, the cell can reproduce a single amino acid sequence each time it synthesizes a specified protein, whereas the glycosylation pattern in cancer can be highly aberrant due to changes in the expression or activity of glycosyltransferases and glycosidases (e.g. sialyltransferases and fucosyltransferases) (39–41). Proteomics approaches to elucidate glycoprotein patterns have mainly focused on cell lines or serum/plasma from which the glycoproteins have been enriched followed by identification of glycan structures or deglycosylated peptide sequencing through MS analysis (42–45). Profiling glycans on invasive versus noninvasive breast cancer cell lines identified statistically significant differences in certain neutral, sialylated, and fucosylated structures, suggesting that these profiles may contain distinct glycan biomarkers that may correspond to glycan signatures of cancer (46). Well characterized N-glycosylation alterations in sera of breast cancer patients compared with disease-free controls include increased sialylation and fucosylation of glycan structures (47, 48). Profiling glycoproteins, without specific identification of the glycans, of premalignant versus malignant/metastatic cell lines identified a range of unique glycoproteins associated with breast cancer metastasis (49).

A complete characterization of altered glycoproteins between cancer versus normal cells requires identification of the protein, the glycan composition, and the glycosylation site. Characterization of the peptide or the glycan structure alone will not determine whether the alteration is due to a more abundant glycoprotein, more frequent occupation of a specific glycosylation site, or an alteration in the glycan structure alone where altered expression of glycosyltransferases is likely involved. Performing such a complete analysis is complicated by the complexity of biological samples, low levels and heterogenous structures of glycans, and lack of a single technique that can provide a full qualitative or quantitative structural analysis of glycoproteins or glycopeptides (50).

Mucins are a family of high molecular weight, heavily glycosylated proteins that are overexpressed in most carcinomas (51). Some mucins are PM-bound due to the presence of a hydrophobic membrane-spanning domain that favors retention in the PM, whereas most mucins are secreted and can serve as ligands for adhesion receptors, such as selectins, that promote the ability of tumor cells to interact with host platelets, leukocytes, and endothelial cells (29). MUC1, which is one of the PM mucins, is anchored to the apical surface of many epithelial cells. In breast, colon, and a number of other epithelial tumors, MUC1 is overexpressed and exhibits truncated O-glycan structures (52). MUC1 is the main component of Stimuvax®, a vaccine that stimulates the immune response of the patient against MUC1-expressing cancer cells, that is currently being evaluated in two phase III trials in non-small cell lung cancer and estrogen receptor (ER)-positive breast cancer (53).

Tumor markers in current clinical use, such as human chorionic gonadotropin-β, α-fetoprotein, carcinoembryonic antigen, prostate-specific antigen, HER-2, and mucins (e.g. CA 19.9, CA 125, and CA 15.3), are all glycoproteins that are either membrane-associated or secreted to the serum (37, 54, 55). Typically, the disease marker is the protein part and not the glycan moiety of the glycoprotein or mucin.

Phosphorylation of Cancer Cells—Protein phosphorylation of tyrosine, serine, and threonine amino acid residues is a reversible PTM and a key mechanism in the regulation of cellular signaling pathways that control biological processes. Protein phosphorylation is strictly controlled by the interplay between protein kinases and protein phosphatases (56, 57). Phosphoproteins are present at very low levels, and the phosphorylation process is a transient modification, enabling the cell to respond to cellular or environmental changes by phosphorylation/dephosphorylation (58). Abnormal protein phosphorylation is known to cause, or be a consequence of, many diseases, including cancer (59, 60).

Tyrosine kinases are among the most important oncogenes yet known. Receptor tyrosine kinases are found on the PM and bind to peptides, such as epidermal growth factor, platelet-derived growth factor, fibroblast growth factor, and insulin and insulin-like growth factor. Following ligand binding, the receptor tyrosine kinases undergo dimerization and autophosphorylation, resulting in phosphorylation of tyrosine sites in other proteins (61). Phosphorylation often occurs at multiple residues within a protein and, in most cases, by different protein kinases activated by diverse mechanisms, making proteomics analysis of these changes very challenging. Most phosphoproteomics studies involve the enrichment of phosphorylated proteins from tissue or cell lines prior to MS identification and quantification of protein phosphorylation sites (62, 63). Various new cancer drugs and drug candidates are aimed at protein kinase targets, such as epidermal growth factor receptor (EGFR)/HER-1 and HER-2 (64–66).

Plasma Membrane Proteins and Cancer Metastasis—Metastases are established through a complex set of events that is yet not fully elucidated but that requires detachment of single cells from the primary tumor, penetration of the tissue matrix, and migration to distant locations where they induce angiogenesis and undergo expansive growth (67). The metastatic process is highly dependent on interactions between tumor cell PM proteins and the microenvironment,

and metastatic cells develop PM protein alterations that enable them to bind more strongly to corresponding ECM proteins, including laminins, collagens, and proteoglycans (67, 68). The interaction between PM proteins and the ECM activates signaling cascades that regulate gene expression, cytoskeletal organization, cell adhesion, and cell survival mechanisms, resulting in degradation of ECM by specific enzymes, such as the matrix metalloproteinases and the urokinase-type plasminogen activator. As a result, cancer cells become more invasive, migratory, and able to survive in different microenvironments (69).

Integrins, a diverse family of glycoproteins that form heterodimeric receptors for ECM molecules, comprise one of the primary links between tumor cells and the ECM and play essential roles in several steps of the metastatic process (70). The heterodimeric receptors are composed of an α and a β subunit; there are 18 α-subunits and eight β-subunits, giving rise to at least 24 different integrin heterodimers. In a recent quantitative proteomics study of PM protein alterations associated with the ability of disseminated breast tumor cells to establish lung metastasis, we demonstrated that integrin β_1, α_v, and α_6 were more highly expressed on cells capable of generating metastasis. Immunohistochemical analysis of clinical breast cancer biopsies confirmed a significant correlation between high integrin β_1 expression and poor outcome, measured as tumor spread or distant recurrence within a 10-year follow-up (22, 71). Chen *et al.* (72) also identified integrin β_1 as being up-regulated in cancer when they performed quantitative proteomics profiling to compare cancerous and normal pancreatic tissues. The elevated level of integrin β_1 in pancreatic cancers was confirmed by Western blots and immunohistochemistry. Using cell surface biotinylation, Kischel *et al.* (73) compared the profiles of PM proteins in the human breast cancer cell line MDA-MB-231 and a bone metastatic subclone. This strategy allowed the identification of several proteins, including $\alpha_v\beta_3$ integrins, that were up-regulated in the osteotropic cell line.

PROTEOMICS METHODS FOR STUDY OF PLASMA MEMBRANE
PROTEINS

The complexity and concentration of individual proteins in the sample are crucial when performing proteomics analyses because abundant proteins may hinder the detection of more infrequent proteins, *e.g.* PM proteins. Separation of crude extracts according to the different biochemical features of the subcellular compartments and organelles increases the likelihood of detecting the more infrequent proteins. Proteins expressed at low levels may be enriched from larger volumes by selective fractionation, immunoprecipitation, chromatographic, or electrophoretic methods (74–77).

Proteomics Analysis of Cell Lines—Most strategies used for enrichment of PM proteins from cultured cells use either homogenization followed by membrane density separation or whole cell protein tagging followed by affinity purification. The

former is usually initiated by a step in which the cells are incubated in a hypotonic buffer followed by mechanical homogenization and removal of nuclei and cell debris by centrifugation at low speeds (8, 23, 71, 78–80). The membranes, including the associated membrane proteins that are maintained in the supernatant, can be separated based on the different lipid-to-protein ratios in the different cellular membranes either in a discontinuous sucrose gradient or on a 35% sucrose cushion (8, 79). The latter approach was used to identify differentially expressed, functionally related proteins in three breast cancer cell lines *versus* a human mammary epithelial cell line (8). Alternatively, a crude membrane fraction containing all membrane types can be obtained by sedimenting the membranes by ultracentrifugation and separation by SDS-PAGE (23, 78). Membrane purification may also be achieved by combining different methods, such as sedimentation and a discontinuous sucrose gradient (80) or Percoll/sucrose density separation (71, 78); both strategies separate the membranes of different organelles according to their varying densities (81). We have obtained good results with PM protein separation using a Percoll/sucrose density gradient to compare a metastatic *versus* a non-metastatic cell line and identified 526 membrane proteins of which 16 exhibited altered expression between the two cell lines. Two of the proteins were also expressed at significantly higher levels in primary tumors that had spread within a 10-year follow-up period compared with tumors that had not (22, 71).

Enrichment of PM proteins using whole cell protein tagging is often based on a membrane-impermeable biotin labeling reagent followed by cell lysis and affinity purification using streptavidin-coated beads (18, 19, 21, 73). Other systems, including coating with cationic silica beads and purification through a density gradient, have also been used (82). Conn *et al.* (21) used biotinylation and streptavidin precipitation to isolate PM proteins from a fibrosarcoma cell line pair that differed 50–100-fold in their ability to intravasate and disseminate. They found that NCAM, JAM-C, and tissue factor were expressed at higher and TIMP-2 was expressed at lower levels in the highly invasive *versus* less invasive cell line. Other alternative purification approaches include lysing cells directly in the cell culture flask and sequentially recovering the basolateral cell membranes (83) or purifying subpopulations of PM proteins, such as glycoproteins, through lectin affinity purification (49). Wang *et al.* (49) identified a number of proteins that exhibited altered expression in a precancerous *versus* a malignant metastatic breast cancer cell line by extracting glycoproteins using lectin affinity columns.

Proteomics Analysis of Patient Material—Cancer cell lines are easy to handle and comprise a homogeneous and almost inexhaustible source of biological material, including proteins (84, 85). However, each cell line represents only one tumor unaffected by signals from the microenvironment, and the cells may have been subjected to clonal drift and *in vitro*

selection, which may render them less representative of the tumor from which they originated (84–86). It is more clinically relevant, but less straightforward, to perform a proteomics analysis directly on patient tumor tissue from which proteins may be extracted immediately upon removal from the patient (87) or later from frozen or formalin-fixed, paraffin-embedded (FFPE) tissue (88–91).

Protein purification from tissue specimens can be achieved similarly to that of cell line material, although there are some important differences. Tissue, especially from solid tumors, is harder/more compact and requires harsher mechanical homogenization methods, *e.g.* various types of bead mill, rotor-stator, blade homogenizers, grinders, or ultrasonic disintegrators (92). The preparation protocols for the tissue samples should ensure reproducibility and minimize loss of polypeptides and bias of the composition while removing interfering compounds, such as aggregates, lipids, and carbohydrates, especially if the aim is to compare different samples. As described for cultured cells, enrichment strategies need to be used for PM proteins to identify and compare their levels in different tissue samples. Nielsen *et al.* (93) purified PM proteins from murine brain tissue using centrifugation and density separation, procedures similar to those described for cell line material, and by adding a reverse phase chromatography separation step, up to 60% of membrane proteins were identified. *Ex vivo* biotinylation offers another PM protein purification strategy. By injecting biotinylation solution into the blood vessels of surgically excised colon specimens from colon cancer patients, Conrotto *et al.* (94) were able to affinity purify cell surface proteins from the tumors and identified proteins primarily expressed in colon cancer and not in healthy tissues.

The experimental parameters must be carefully controlled to distinguish between true clinical differences and variations in sample collection and experimental setting or normal biological variability (95). Stringent sample collection, storage, preparation, and analysis are required, and several standardized protocols have been established (96). To avoid protein degradation, biological samples should be aliquoted and stored at −80 °C or lower, and protease inhibitors should be added as soon as possible (97, 98).

In addition to technical considerations, when performing proteomics analysis of clinical tumor samples, issues, such as tumor heterogeneity, percentage of necrotic tumor tissue, and percentage of tumor *versus* surrounding cells (connective tissue, blood vessels, and infiltrating immune cells), must be considered (99). When using tissue samples from patients with well defined medical histories and long term clinical follow-up, the conditions under which these historical samples have been stored must also be evaluated. Moreover, it is crucial to analyze an adequate number of tissues to ensure statistical validity; proteomics are time-consuming, and many studies use too few samples. Finally, the study design is crucial; an initial discovery phase to identify candidate pro-

teomic markers associated with a particular disease condition should be followed by a validation study using an independent sample set. For this purpose, collections of FFPE or frozen tissues stored in biobanks around the world are especially useful because they permit studies with long term end points, such as recurrence of breast cancer that can take 10 or more years to manifest. The use of such archival tissue for proteomics analysis requires introduction of additional purification steps, *e.g.* to remove paraffin, which renders the proteins very hydrophobic and thus difficult to solubilize. Furthermore, both paraffin and formalin may give rise to additional peaks in the mass spectra obtained from such tissue. A few groups have successfully obtained useful proteomics data from analysis of FFPE tissue (88, 91, 100). Hood *et al.* (91) compared protein expression in prostate cancer and benign prostate hyperplasia tissue from the same patient using cells from different areas (*i.e.* cancer and hyperplasia) of a 10-μm-thick section of FFPE tissue block. The proteins were isolated using laser microdissection, digested directly in the deparaffinized tissue, and analyzed by linear ion trap MS. For quantification, the peptides were labeled with ^{18}O and ^{16}O, and 68 proteins were found to exhibit altered expression (91). Assessment of the cellular localization of the identified proteins revealed another important issue regarding MS analysis of archival tissue. The purification procedures often only contain few steps to compensate for the low amounts of protein available or protein degradation. Consequently, there is no PM protein enrichment step, and the percentages of identified membrane and PM proteins are often lower in studies using tissue than in studies using cell lines. Hood and Cheresh (71) identified ~25% of membrane proteins from FFPE tissue compared with 66% in our recent cell line study.

The most prevalent method for analysis of frozen and fresh tumor tissue is two-dimensional gel electrophoresis combined with MALDI-TOF MS, but PM proteins are rarely identified using this technique (90, 101). Celis *et al.* (87) used this strategy to examine fresh mammary adipose tissue and corresponding fat interstitial fluid from 21 high risk breast cancer patients. A total of 359 unique proteins, including numerous signaling molecules, hormones, cytokines, and growth factors involved in a variety of biological processes, were identified. Sprung *et al.* (102) compared shotgun proteomics analyses of frozen *versus* FFPE specimens prepared from the same colon adenoma tissues. Following deparaffinization, rehydration, and tryptic digestion under mild conditions, analysis of the combined frozen and FFPE data showed a 92% overlap in the protein groups identified, suggesting that proteomics analysis could be performed on retrospective FFPE tissue. Furthermore, equally low (~10%) amounts of PM proteins were identified.

Identification of Post-translational Modifications—Elucidating a full range of PTMs, such as phosphorylations and glycosylations, from highly complex biological systems is complicated by their low abundance and heterogeneity in the

starting material. Most phospho- and glycoproteomics studies use MS as the most sensitive method for detection typically combined with an enrichment step or derivatization/tagging as phosphorylated and glycosylated peptides tend to display a decreased signal intensity in MS in the presence of non-modified peptides (41, 103, 104).

Glycosylation is the most complex, but also one of the most common, types of modification. Glycoproteomics usually includes enzymatic digestion of the glycoprotein-containing samples to generate peptides and glycopeptides. The glycopeptides are then enriched using selective chromatographic methods, typically using immobilized lectins, hydrophilic interaction LC, titanium dioxide (TiO_2), or graphite (41, 49, 50, 105–108). Lectins, *e.g.* concanavalin A, differ in their specificity and selectivity toward glycan compositions, and serial lectin affinity chromatography with immobilized lectins will recover various subsets of glycopeptides from complex biological samples (104, 109, 110). Hydrophilic interaction chromatography makes use of the polar interactions between the hydroxy groups of glycans and the stationary phase, and in this method, the retention mechanism is governed by the size of the glycan, resulting in separating glycoforms with the same peptide moiety (111). Titanium dioxide is a very selective method for quantitative and qualitative assessment of sialic acid-containing peptides from complex peptide mixtures (45). Porous graphitized carbon is well suited for enriching glycopeptides with smaller peptide portions, but sufficient selectivity is not achieved with larger tryptic glycopeptides (112). The glycopeptides recovered by one or a combination of enrichment methods are then analyzed using MS (MALDI or ESI), which can be used to obtain spectra of intact glycoproteins, glycopeptides, or released glycans (26). However, individual glycoforms can only be resolved from peptides containing a single glycosylation site. The challenge with quantitative and qualitative analysis of glycopeptides is that it is not always possible to obtain glycopeptides with just one glycosylation site, and not all glycosylation sites are necessarily occupied by glycan moieties, whereas others may be partially occupied. Details of the glycan structure can be obtained by exoglycosidase digestion or mass spectrometric fragmentation (113, 114). A database of human *N*-linked glycosylation sites (UniPep) can be used for targeted biomarker identification, but *O*-linked protein glycosylations, which are often related to cancer, are not covered by the database (115).

The analysis of phosphorylation related to membrane proteins on cancer cells has been very challenging because of frequent alterations in phosphorylation patterns and inconsistent reproducibility. The methods of phosphoproteomics have recently been reviewed (56, 62, 116), but only a few studies have described the methodology on biologically relevant samples using a combination of isotopic or chemical labeling, enrichment of phosphopeptides by immobilized metal affinity chromatography, and MS to compare and sequence phosphopeptides presented by multiple cancer cell lines (62, 117–119).

Phosphoproteomics methods use MS analysis of fractions enriched in phosphopeptides by different ion exchange chromatographic techniques, including strong cation exchange, IMAC, and TiO_2 (103, 120). Phosphospecific antibodies may also be used if tyrosine, serine, and threonine phosphorylations are specifically examined. A comprehensive study of protein phosphorylation should include the identification of phosphoproteins and sites of phosphorylation, identification of the kinases and phosphatases involved in the phosphorylation process, and a description of the biological events following the phosphorylation (103). Current methods for phosphopeptide enrichment, such as IMAC and TiO_2 chromatography, provide varying degrees of selectivity and specificity (58). One of the major drawbacks of those strategies is the nonspecific binding of peptides containing acidic amino acids and the strong binding of multiphosphorylated peptides with IMAC and monophosphorylated peptides with TiO_2. Multiphosphorylated peptides are generally suppressed in the ionization process to a higher extent than monophosphorylated peptides, and thus fractionation of mono- and multiphosphorylated peptides through sequential elution from immobilized metal affinity chromatography overcomes this obstacle (121). Thingholm *et al.* (122) described an efficient method that combines PM protein fractionation with TiO_2-based phosphopeptide enrichment in a cell model of human mesenchymal stem cells; they assigned 703 unique phosphorylation sites in 376 phosphoproteins.

Protein Quantification Methods—Because most disease-associated markers are not exclusively expressed in either the disease or the "healthy" state, quantification of protein expression differences must be included in marker identification strategies. Such quantitative proteomics approaches include comparison of proteins expressed in specific subcellular components, such as the PM as well as their PTMs (123). Several quantitative profiling studies of PM proteins on different cell types and at specific differentiation or disease stages have identified novel molecular markers that may be recognized by monoclonal antibodies and other protein molecules (124–126). The optimal quantification strategy for proteins and PTMs is independent of subcellular origin; however, some strategies may be advantageously used when examining tissue specimen, whereas others may be more effective when examining cell lines.

The quantification strategies used in combination with MS-based proteomics are often based on the introduction of stable isotopes into the samples, which can be done either by metabolic, chemical, or proteolytic labeling. The most widely used metabolic labeling strategy is stable isotope labeling by amino acids in cell culture (SILAC). SILAC is simple and powerful because the label is introduced prior to protein purification but can mainly be applied to cells in culture. Several of the previously cited studies have used SILAC to identify PM proteins differentially expressed between two cell lines (18, 19, 22, 23, 71), and the method was carefully reviewed by Mann (127).

Quantitative proteomics using chemical and proteolytic labels is, in contrast to SILAC, sensitive to variations in protein purifications between the compared samples because the labels are introduced after protein purification. On the other hand, labeling following protein purification enables labeling of material without metabolic activity, such as PM proteins obtained from surgically excised frozen tissue specimens. Examples of chemical and proteolytic labels include ICAT (cleavable isotope-coded affinity tags), iTRAQ (stable isotope-tagged amine-reactive reagents), and ^{18}O (128–132). ICAT consists of a reactive group, which reacts with cysteine residues; a linker containing the stable isotopes; and a biotin tag for purification of labeled peptides. The specificity for cysteines and the subsequent affinity purification reduce the complexity of the peptide mixtures but may also eliminate peptides of special interest (129, 133). Ramus *et al.* (134) developed a strategy using ICAT in the presence of high concentrations of SDS and urea followed by electrophoresis and enzymatic digestion to obtain quantitative data from the highly hydrophobic membrane proteins of murine embryonic stem cells. ICAT was also used by Pawlik *et al.* (135) to compare nipple aspirate fluid from patients with early breast cancer *versus* healthy controls. They found a higher content of vitamin D-binding protein in nipple aspirate fluid in breast cancer *versus* healthy controls, but the menopausal status of the two groups differed significantly, which may have influenced the results.

In the iTRAQ system, the tags react with the N termini of the peptide and lysine residues, thus tagging all peptides. The iTRAQ tags are designed to make the derivatized peptides isobaric and chromatographically indistinguishable and yield a signature of reporter ions in MS/MS mode (131). Han *et al.* (136) reported a strategy for PM proteins that combines gel-assisted digestion, iTRAQ labeling, and LC-MS/MS for characterization of differentially expressed proteins of kidney cell plasma membranes from wild-type *versus* PKD1 knock-out mice. More than 100 proteins showed at least 2-fold up- or down-regulation (136). Rajcevic *et al.* (137) focused on membrane-associated proteins in glioma xenografts transplanted in rats and profiled protein expression during the progression from an invasive to an angiogenic phenotype by iTRAQ combined with two-dimensional LC and MALDI-TOF/TOF MS. They extracted 1460 quantifiable proteins (137). Ho *et al.* (138) used the iTRAQ labeling system to identify seven novel breast cancer metastasis-associated proteins in four cell lines with different metastatic potential and verified the differential expression of four of the proteins on 50 matched invasive and metastatic breast carcinomas.

Heavy oxygen (^{18}O) can be introduced into peptides through proteolytic labeling by digesting the proteins in the presence of $H_2^{18}O$ using trypsin, Lys-C, or Glu-C, which introduces one or two ^{18}O molecules into the peptides (132). This technique is simple and works well with small amounts of sample, but the major drawback is that the label-
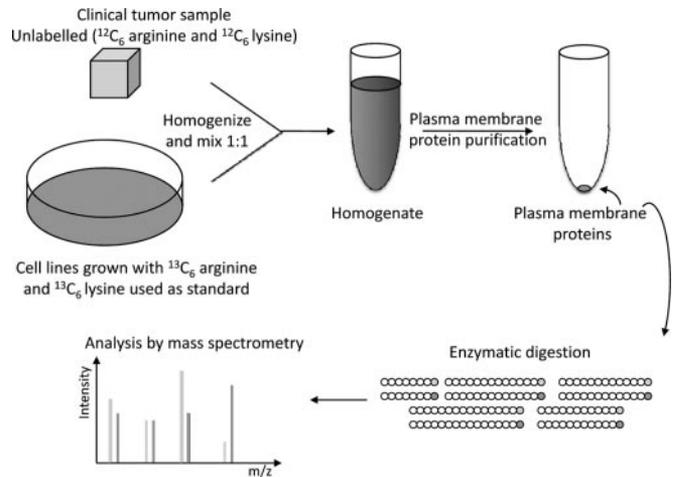

FIG. 1. **Proteins derived from SILAC-labeled cell line can be used to provide internal standard for quantification of plasma membrane proteins obtained from tissue specimens.**

ing is not homogeneous, *i.e.* sometimes one ^{18}O is incorporated, whereas at other times, two ^{18}O molecules are incorporated (139). Kristiansen *et al.* (140) used ^{18}O labeling to compare proteins exhibiting altered expression between frozen xenografts derived from two human cholangiocarcinomas and frozen normal human biliary tract tissue. Chi *et al.* (141) used ^{18}O labeling in combination with laser capture microdissection to identify proteins expressed more highly in oral cancer *versus* adjacent non-tumor tissue. Stockwin *et al.* (142) performed a quantitative proteomics analysis of PM from hypoxia-adapted murine B16F10 melanoma using differential ^{18}O stable isotopic labeling and multidimensional liquid chromatography-tandem MS. The analysis resulted in quantitative information for 2433 proteins (142).

An alternative to the stable isotope-based strategies is the label-free approach, which is based on ion intensity quantification either by counting the fragment spectra of the peptides identifying the different proteins or by measuring the chromatographic peaks of the peptides. This technique is cheaper to perform but requires the experiment to be repeated more times and required more robust computing power and algorithms. Röwer *et al.* (143) applied label-free protein quantification on tissue samples from healthy breast and tumor to generate an invasive ductal breast carcinoma proteome signature consisting of 60 protein entries. Proteins from tumor tissue are most often quantified by either a label-free technique (144) or a postharvest labeling technique, *e.g.* chemical or enzymatic (135, 140). However, metabolic labeling strategies can also be adapted for quantification of proteins in tissue samples (145, 146). For example, Sihlbom *et al.* (146) used proteins derived from SILAC-labeled cells as internal standards to study the regeneration process in murine brain tissue. This method could potentially also be combined with PM protein purification to quantify and compare PM proteins within tissue specimens as depicted in Fig. 1.
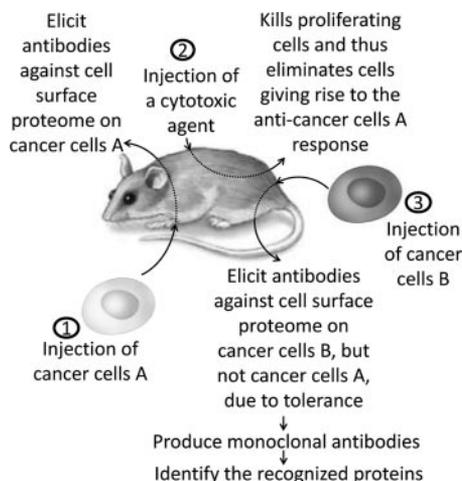
FIG. 2. **Proteomic scanning of PM using subtractive immunization strategy in mice.** An antibody response toward PM proteins predominantly expressed on cell line B compared with cell line A is elicited and can subsequently be cloned, allowing identification of PM proteins associated with particular phenotypes, *e.g.* PM proteins exhibiting altered expression in a metastatic cancer cell line compared with the corresponding isogenic non-metastatic cancer cell line.

Other Methods for Identification and Quantification of Membrane Proteins—Methods based on principles other than MS are also used for biomarker discovery and include antibody arrays and subtractive immunization. Subtractive immunization allows scanning of the entire cell surface proteome and identification of molecules that exhibit altered expression between two cell types (147). Concurrently, this method gives rise to monoclonal antibodies for further characterization of the identified proteins. The strategy is based on subtractive immunization of mice. Following immunization with one cell line, the mice are treated with a cytotoxic agent, which eliminates the stimulated B cells. Subsequently, the mice are immunized with the second cell line, giving rise to an immune response to those proteins that exhibit altered expression between the two cell lines (Fig. 2).

APPROCHES FOR VALIDATION OF CLINICAL PROTEOMIC BIOMARKERS

High throughput proteomics, such as MS, is an excellent tool for biomarker discovery, but for further validation of a limited number of the identified proteins, including PM proteins, other high throughput methods, such as tissue arrays, antibody arrays, or ELISAs, may be advantageous. An exception is the use of MS to monitor selected peptides, *e.g.* using QconCAT, for identification and quantification of a set of predefined representative peptides.

Tissue Microarray—Tissue microarrays (TMAs), which allow simultaneous immunohistochemical analysis of tens to hundreds of samples on a single glass slide, have become an attractive validation strategy and also are sometimes described as a proteomics technique (148, 149). This type of validation of potential novel biomarkers, including PM proteins, relies on access to large numbers of biological samples, *e.g.* biopsies of primary tumors and metastases collected and stored for research at hospitals (150). Different cylindrical core sizes of tissue can be used ranging from 0.6 to 2 mm. More reliable assessment of the expression of a given protein includes two cores from a given tumor in the same block, either randomly selected or from predefined areas, such as the invasive front and a central area of the tumor. The readout of these TMAs, which should be based on predefined criteria, depends on the protein in question. In some instances, the readout is a simple positive/negative result, whereas for some other markers, particular features are evaluated, such as the staining intensity, percentage of positive tumor cells, and subcellular localization. The classical breast cancer markers ER, progesterone receptor, and HER-2/neu are typical examples of how different criteria define whether a clinical sample is positive or negative (151). The readout of these assays often requires a highly skilled pathologist, and it may be beneficial to have two independent pathologists score the arrays to limit subjectivity. To make the readout more objective, different instruments have been developed for automated quantitative analysis of TMA (152–154).

Antibody Arrays—Antibody arrays are used to detect the presence of a specific set of proteins in a given sample (serum, tissue homogenates, etc.) and are generated by spotting antibodies on a solid surface. They are the protein analog of cDNA arrays but are technically more difficult to make because proteins, including antibodies, are more complex in their composition, protein folding, denaturation, aggregation, and multimerization (155, 156). The availability of specific antibodies in sufficient amounts is also a challenge. A particular problem related to PM proteins is that they may have altered conformation when they are no longer in the context of the PM, which might render them unrecognizable by the antibodies. Antibody arrays are useful for high throughput analysis of candidate biomarkers in patient samples (155, 157). However, the method has a limited role in discovery-based identification of novel biomarkers where other platforms, such as MS, are better suited (155, 156).

QconCAT—The QconCAT strategy enables absolute quantification of 10–25 preselected peptides in a complex protein sample by using stable isotope-labeled peptides as reference. The strategy relies on the absolute quantification method AQUA in which a stable isotope-labeled synthetic internal standard peptide, which mimics a peptide produced during proteolysis of the target protein, is introduced at a known concentration to the sample prior to digestion. AQUA has been shown to be useful for quantification of both proteins and post-translational modifications (158). The QconCAT peptides are designed as a concatemer, *i.e.* a chain of DNA sequences. The concatemer is expressed in *e.g. Escherichia coli*, and the resulting polypeptide, consisting of concatenated peptides, is metabolically labeled with stable iso-

topes similarly to SILAC. By spiking a protein sample with a known amount of the polypeptide prior to digestion, the absolute content of the peptides of interest can be determined by MS (159, 160). The drawbacks of this method include a number of "trial and error" steps prior to selecting the correct peptides for each protein followed by purification and determination of the protein concentration.

CURRENT STATUS OF CLINICAL BIOMARKER DISCOVERY

Early Detection of Cancer—Clearly, the earlier a primary tumor is detected, the better the prognosis and response to treatment, including the possibility of a complete cure. However, current methods for early detection are insufficient, and most tumors are first detected at a late stage in which the tumor size is extensive and has invaded other tissues or spread to other organs. Identification of new methods for early detection is therefore of the utmost importance, and proteomics analysis of body fluids, such as serum, spinal fluid, saliva, or urine, is a promising area. In addition to the detection of early stage primary tumors, such tests may also be useful to follow cancer patients that are deemed clinically cured but for whom recurrence may occur. Some cancer-associated PM proteins or fragments thereof are released into the plasma/serum in increased amounts in patients with cancers. This can occur by exterior protein cleavage, membrane sloughing, or cell lysis, and these proteins may represent unique markers that might be useful for *e.g.* predicting disease progression (161–165).

The extracellular domains of the two cancer-associated PM receptors HER-2/neu and EGFR are proteolytically released upon receptor activation and can be detected in serum. The value of serum HER-2/neu and EGFR levels has been evaluated in different cancers with somewhat conflicting results. Importantly, soluble EGFR is also present in healthy individuals, and one study found serum levels to be significantly higher in normal individuals than in patients with primary breast cancer (166). In another study, a small subgroup of patients with decreased serum EGFR was identified and shown to exhibit significantly reduced survival compared with patients with normal serum EGFR levels. However, no significant differences in objective response rate, time to progression, or time to treatment failure were observed (167). Another breast cancer study found that the serum EGFR and serum HER-2/neu levels at the onset of metastatic disease were not associated with overall survival, and no correlation was observed between expression of EGFR in primary tumors and EGFR serum levels (168). In contrast, HER-2/neu serum levels at the time of metastatic disease correlated with HER-2/neu expression in the primary tumors as determined by immunohistochemistry (168). Serum HER-2/neu therefore seems somewhat more promising and has been shown to be a useful marker for early prediction of probability of response, progression-free survival, and overall survival in patients with advanced breast cancer treated with metronomic chemothera-

py (167). An elevated serum HER-2/neu level as measured after 2 months of chemotherapy treatment was significantly associated with reduced long term clinical benefit. Serum HER-2/neu was also found to predict response to trastuzumab-based therapies as individuals who did not achieve a significant decline in serum HER-2/neu levels had decreased benefit from trastuzumab-based therapy (169). Many of these findings need confirmation in other independent cohorts; however, overall, they seem promising.

Other fragments of serum PM proteins often detected in sera of cancer patients include soluble forms of PM glycoprotein CD44 that functions as a receptor for hyaluronan and is involved in cell-cell and cell-matrix adhesion, cell migration, and signaling. CD44 is found in several isoforms, including a standard CD44 (CD44s) and different variable forms (CD44v). CD44 may be shed or released into circulation by proteolytic enzymatic mechanisms. Alternative splicing of CD44 and aberrant levels of soluble CD44v in the serum of cancer patients have been correlated to tumor progression and metastasis in different tumors, including breast, colon, and head, and neck cancer (170). Soluble CD44v can be modified by different glycotopes, some of which have been found to be highly expressed in patients with cancers, whereas other glycotopes are frequently found in patients with benign disorders (171).

Prognostic Markers—Different sources of patient tissue, such as the tumor itself, serum, or other bodily fluids, may be used for prediction of prognosis, although currently most studies have focused on serum. In a recent proteomics study aimed at identifying prognostic serum biomarkers of breast cancer, preoperative serum samples obtained from 48 breast cancer patients and 28 controls were used to generate MALDI-TOF MS protein profiles (172). Among 533 common peaks, the investigators identified 72 peaks exhibiting statistically significant intensity differences between cases and controls. They subsequently constructed a diagnostic rule based on these 72 mass values with cross-validated sensitivity and specificity of ~85%. Although the study yielded relatively high sensitivity and specificity, which are in the same range as those of a few other protein profiling studies in breast cancer where sensitivities and specificities were reported (173, 174), these values are still too low for direct clinical implementation of these methods. In addition, cross-center validation to verify the clinical significance of the results is needed. The identities and general applicability of these markers were not defined in this study. Although some of these profiles can distinguish between cancer and healthy controls, the discriminating peaks/proteins may not be cancer-related but rather a sign of inflammation, which may be seen in multiple disease conditions. This limits their utility as a stand-alone tumor marker, but they may still be useful in a multimarker panel for early detection of cancer.

Prediction of Benefit to Given Treatment—Very few studies have used discovery-based proteomic approaches, such as MS, to develop predictive marker profiles. In breast cancer,

the expression of ER, as measured by immunohistochemistry, is predictive of response to adjuvant endocrine treatments with tamoxifen and aromatase inhibitors (175, 176). Although tamoxifen is of great benefit for many patients, recurrence occurs in ~30% of patients within 15 years despite adequate treatment (177). Thus, there is a need to identify a marker that indicates whether a given patient would benefit from endocrine treatment or whether chemotherapy should be added. Umar *et al.* (178) performed proteomics analysis of clinical primary ER-positive breast cancer samples from patients that had either objective response or disease progression following tamoxifen treatment of metastatic disease. For the marker discovery phase, laser-captured microdissected cancer cells from each of the two groups of patients were pooled and compared using nano-LC-FTICR-MS. Sample pooling limits the number of samples that need to be analyzed but also makes it impossible to determine whether the increase of a given protein is intense in just one sample or a more moderate increase in all the samples. The verification phase of such studies is thus very important. Here, the authors used whole tissue sections from the patients who were already included in the discovery phase and processed them individually using targeted MS/MS (178). The authors verified 47 of the initial 100 proteins that exhibited altered expression between the two groups. Only a few of these were membrane proteins. The expression of the top candidate, extracellular matrix metalloproteinase inducer, was validated in an independent patient cohort using TMAs and shown to be expressed more highly in therapy-resistant *versus* -sensitive tumors.

CONCLUSION

PM proteins have attracted significant attention as biomarkers for disease diagnosis, prognosis, monitoring, and treatment benefit prediction. In addition, they may serve as potential targets for drug therapy, a fact already proven as two-thirds of all drugs targeting proteins are directed against PM proteins. However, PM proteins are hydrophobic and possess a variety of PTMs, which require more sophisticated proteomics methods for their separation and identification. Although most proteomics studies of PM proteins are currently using cell lines, newer proteomics strategies for PM biomarker identification in clinical tissue samples, such as tumors, are being developed. Future effort should focus on optimizing these strategies and performing MS-based proteomics on clinically well characterized tissue samples. Study design is essential to effectively address the clinical problem in question, including selection of proper patient materials and sufficient numbers of samples stored under optimal conditions. These efforts require close collaborations between translational researchers, biostatisticians, pathologists, and clinicians. Moving markers from the discovery phase to daily clinical practice is also a crucial area. It is a cumbersome process that requires marker verification, validation and assay development, and testing of large numbers of clinical samp-les, but these efforts should determine the clinical utility of proteomics in cancer patient care.

REFERENCES

1. Mouritsen, O. G., and Bloom, M. (1993) Models of lipid-protein interactions in membranes. *Annu. Rev. Biophys. Biomol. Struct.* **22,** 145–171
2. Mukherjee, S., and Maxfield, F. R. (2004) Membrane domains. *Annu. Rev. Cell Dev. Biol.* **20,** 839–866
3. Guidotti, G. (1972) Membrane proteins. *Annu. Rev. Biochem.* **41,** 731–752
4. Bretscher, M. S., and Raff, M. C. (1975) Mammalian plasma membranes. *Nature* **258,** 43–49
5. Zheng, Y. Z., and Foster, L. J. (2009) Biochemical and proteomic approaches for the study of membrane microdomains. *J. Proteomics* **72,** 12–22
6. Escribá, P. V., González-Ros, J. M., Goñi, F. M., Kinnunen, P. K., Vigh, L., Sánchez-Magraner, L., Fernández, A. M., Busquets, X., Horváth, I., and Barceló-Coblijn, G. (2008) Membranes: a meeting point for lipids, proteins and therapies. *J. Cell. Mol. Med.* **12,** 829–875
7. Kabbani, N. (2008) Proteomics of membrane receptors and signaling. *Proteomics* **8,** 4146–4155
8. Patwardhan, A. J., Strittmatter, E. F., Camp, D. G., 2nd, Smith, R. D., and Pallavicini, M. G. (2005) Comparison of normal and breast cancer cell lines using proteome, genome, and interactome data. *J. Proteome Res.* **4,** 1952–1960
9. Cox, J., and Mann, M. (2007) Is proteomics the new genomics? *Cell* **130,** 395–398
10. Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E. L. L. (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J. Mol. Biol.* **305,** 567–580
11. Wallin, E., and von Heijne, G. (1998) Genome-wide analysis of integral membrane proteins from eubacterial, archaean, and eukaryotic organisms. *Protein Sci.* **7,** 1029–1038
12. Pike, L. J. (2009) The challenge of lipid rafts. *J. Lipid Res.* **50,** S323–328
13. Harvey, S., Zhang, Y., Landry, F., Miller, C., and Smith, J. W. (2001) Insights into a plasma membrane signature. *Physiol. Genomics* **5,** 129–136
14. Landry, Y., and Gies, J. P. (2008) Drugs and their molecular targets: an updated overview. *Fundam. Clin. Pharmacol.* **22,** 1–18
15. Dorsam, R. T., and Gutkind, J. S. (2007) G-protein-coupled receptors and cancer. *Nat. Rev. Cancer* **7,** 79–94
16. Gschwind, A., Fischer, O. M., and Ullrich, A. (2004) The discovery of receptor tyrosine kinases: targets for cancer therapy. *Nat. Rev. Cancer* **4,** 361–370
17. Swanton, C., Futreal, A., and Eisen, T. (2006) Her2-targeted therapies in non-small cell lung cancer. *Clin. Cancer Res.* **12,** 4377s–4383s
18. Qiu, H., and Wang, Y. (2008) Quantitative analysis of surface plasma membrane proteins of primary and metastatic melanoma cells. *J. proteome Res.* **7,** 1904–1915
19. Aggelis, V., Craven, R. A., Peng, J., Harnden, P., Cairns, D. A., Maher, E. R., Tonge, R., Selby, P. J., and Banks, R. E. (2009) Proteomic identification of differentially expressed plasma membrane proteins in renal cell carcinoma by stable isotope labelling of a von Hippel-Lindau transfectant cell line model. *Proteomics* **9,** 2118–2130
20. Gou, L. T., Tong, A. P., Chen, L. J., Tang, M. H., Chen, B., Liang, S. F., Huang, C., and Wei, Y. Q. (2008) Comparative plasma membrane-associated proteomics of immortalized human hepatocytes. *Biochem-*

istry **73,** 1200–1206

21. Conn, E. M., Madsen, M. A., Cravatt, B. F., Ruf, W., Deryugina, E. I., and Quigley, J. P. (2008) Cell surface proteomics identifies molecules functionally linked to tumor cell intravasation. *J. Biol. Chem.* **283,** 26518–26527

22. Leth-Larsen, R., Lund, R., Hansen, H. V., Laenkholm, A. V., Tarin, D., Jensen, O. N., and Ditzel, H. J. (2009) Metastasis-related plasma membrane proteins of human breast cancer cells identified by comparative quantitative mass spectrometry. *Mol. Cell. Proteomics* **8,** 1436–1449

23. Liang, X., Zhao, J., Hajivandi, M., Wu, R., Tao, J., Amshey, J. W., and Pope, R. M. (2006) Quantification of membrane and membrane-bound proteins in normal and malignant breast cancer cells isolated from the same patient with primary breast carcinoma. *J. proteome Res.* **5,** 2632–2641

24. Falleni, M., Pellegrini, C., Marchetti, A., Oprandi, B., Buttitta, F., Barassi, F., Santambrogio, L., Coggi, G., and Bosari, S. (2003) Survivin gene expression in early-stage non-small cell lung cancer. *J. Pathol.* **200,** 620–626

25. Simpson, A. J., Caballero, O. L., Jungbluth, A., Chen, Y. T., and Old, L. J. (2005) Cancer/testis antigens, gametogenesis and cancer. *Nat. Rev. Cancer* **5,** 615–625

26. Larsen, M. R., Trelle, M. B., Thingholm, T. E., and Jensen, O. N. (2006) Analysis of posttranslational modifications of proteins by tandem mass spectrometry. *BioTechniques* **40,** 790–798

27. Jensen, O. N. (2006) Interpreting the protein language using proteomics. *Nat. Rev. Mol. Cell Biol.* **7,** 391–403

28. Apweiler, R., Hermjakob, H., and Sharon, N. (1999) On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochim. Biophys. Acta* **1473,** 4–8

29. Fuster, M. M., and Esko, J. D. (2005) The sweet and sour of cancer: glycans as novel therapeutic targets. *Nat. Rev. Cancer* **5,** 526–542

30. Lehmann, F., Tiralongo, E., and Tiralongo, J. (2006) Sialic acid-specific lectins: occurrence, specificity and function. *Cell. Mol. Life Sci.* **63,** 1331–1354

31. Blomme, B., Van Steenkiste, C., Callewaert, N., and Van Vlierberghe, H. (2009) Alteration of protein glycosylation in liver diseases. *J. Hepatol.* **50,** 592–603

32. Kim, Y. J., and Varki, A. (1997) Perspectives on the significance of altered glycosylation of glycoproteins in cancer. *Glycoconj. J.* **14,** 569–576

33. Seales, E. C., Jurado, G. A., Brunson, B. A., Wakefield, J. K., Frost, A. R., and Bellis, S. L. (2005) Hypersialylation of beta1 integrins, observed in colon adenocarcinoma, may contribute to cancer progression by upregulating cell motility. *Cancer Res.* **65,** 4645–4652

34. Hedlund, M., Ng, E., Varki, A., and Varki, N. M. (2008) alpha 2–6-Linked sialic acids on N-glycans modulate carcinoma differentiation in vivo. *Cancer Res.* **68,** 388–394

35. Varki, N. M., and Varki, A. (2007) Diversity in cell surface sialic acid presentations: implications for biology and disease. *Lab. Invest.* **87,** 851–857

36. Dube, D. H., and Bertozzi, C. R. (2005) Glycans in cancer and inflammation-potential for therapeutics and diagnostics. *Nat. Rev. Drug Discov.* **4,** 477–488

37. Peracaula, R., Barrabés, S., Sarrats, A., Rudd, P. M., and de Llorens, R. (2008) Altered glycosylation in tumours focused to cancer diagnosis. *Dis. Markers* **25,** 207–218

38. Block, T. M., Comunale, M. A., Lowman, M., Steel, L. F., Romano, P. R., Fimmel, C., Tennant, B. C., London, W. T., Evans, A. A., Blumberg, B. S., Dwek, R. A., Mattu, T. S., and Mehta, A. S. (2005) Use of targeted glycoproteomics to identify serum glycoproteins that correlate with liver cancer in woodchucks and humans. *Proc. Natl. Acad. Sci. U.S.A.* **102,** 779–784

39. Ohyama, C. (2008) Glycosylation in bladder cancer. *Int. J. Clin. Oncol.* **13,** 308–313

40. Hägglund, P., Bunkenborg, J., Elortza, F., Jensen, O. N., and Roepstorff, P. (2004) A new strategy for identification of N-glycosylated proteins and unambiguous assignment of their glycosylation sites using HILIC enrichment and partial deglycosylation. *J. Proteome Res.* **3,** 556–566

41. Taylor, A. D., Hancock, W. S., Hincapie, M., Taniguchi, N., and Hanash, S. M. (2009) Towards an integrated proteomic and glycomic approach to finding cancer biomarkers. *Genome Med.* **1,** 57

42. Mechref, Y., Hussein, A., Bekesova, S., Pungpapong, V., Zhang, M.,

Dobrolecki, L. E., Hickey, R. J., Hammoud, Z. T., and Novotny, M. V. (2009) Quantitative serum glycomics of esophageal adenocarcinoma and other esophageal disease. *J. Proteome Res.* **8,** 2656–2666

43. Ito, H., Kuno, A., Sawaki, H., Sogabe, M., Ozaki, H., Tanaka, Y., Mizokami, M., Shoda, J., Angata, T., Sato, T., Hirabayashi, J., Ikehara, Y., and Narimatsu, H. (2009) Strategy for glycoproteomics: identification of glyco-alteration using multiple glycan profiling tools. *J. Proteome Res.* **8,** 1358–1367

44. Vercoutter-Edouart, A. S., Slomianny, M. C., Dekeyzer-Beseme, O., Haeuw, J. F., and Michalski, J. C. (2008) Glycoproteomics and glycomics investigation of membrane N-glycosylproteins from human colon carcinoma cells. *Proteomics* **8,** 3236–3256

45. Larsen, M. R., Jensen, S. S., Jakobsen, L. A., and Heegaard, N. H. (2007) Exploring the sialiome using titanium dioxide chromatography and mass spectrometry. *Mol. Cell. Proteomics* **6,** 1778–1787

46. Goetz, J. A., Mechref, Y., Kang, P., Jeng, M. H., and Novotny, M. V. (2009) Glycomic profiling of invasive and non-invasive breast cancer cells. *Glycoconj. J.* **26,** 117–131

47. Kyselova, Z., Mechref, Y., Kang, P., Goetz, J. A., Dobrolecki, L. E., Sledge, G. W., Schnaper, L., Hickey, R. J., Malkas, L. H., and Novotny, M. V. (2008) Breast cancer diagnosis and prognosis through quantitative measurements of serum glycan profiles. *Clin. Chem.* **54,** 1166–1175

48. Arnold, J. N., Saldova, R., Hamid, U. M., and Rudd, P. M. (2008) Evaluation of the serum N-linked glycome for the diagnosis of cancer and chronic inflammation. *Proteomics* **8,** 3284–3293

49. Wang, Y., Ao, X., Vuong, H., Konanur, M., Miller, F. R., Goodison, S., and Lubman, D. M. (2008) Membrane glycoproteins associated with breast tumor cell progression identified by a lectin affinity approach. *J. Proteome Res.* **7,** 4313–4325

50. Selby, D. S., Larsen, M. R., Calvano, C. D., and Jensen, O. N. (2008) Identification and characterization of N-glycosylated proteins using proteomics. *Methods Mol. Biol.* **484,** 263–276

51. Hollingsworth, M. A., and Swanson, B. J. (2004) Mucins in cancer: protection and control of the cell surface. *Nat. Rev. Cancer* **4,** 45–60

52. Burchell, J. M., Mungul, A., and Taylor-Papadimitriou, J. (2001) O-Linked glycosylation in the mammary gland: changes that occur during malignancy. *J. Mammary Gland Biol. Neoplasia* **6,** 355–364

53. Sangha, R., and Butts, C. (2007) L-BLP25: a peptide vaccine strategy in non small cell lung cancer. *Clin. Cancer Res.* **13,** s4652–4654

54. Perkins, G. L., Slater, E. D., Sanders, G. K., and Prichard, J. G. (2003) Serum tumor markers. *Am. Fam. Physician* **68,** 1075–1082

55. Shariat, S. F., Karam, J. A., Margulis, V., and Karakiewicz, P. I. (2008) New blood-based biomarkers for the diagnosis, staging and prognosis of prostate cancer. *BJU Int.* **101,** 675–683

56. Chong, P. K., Lee, H., Kong, J. W., Loh, M. C., Wong, C. H., and Lim, Y. P. (2008) Phosphoproteomics, oncogenic signaling and cancer research. *Proteomics* **8,** 4370–4382

57. Sefton, B. M., and Shenolikar, S. (2001) Overview of protein phosphorylation. *Curr. Protoc. Protein Sci.* Chapter 13, Unit 13.1

58. Thingholm, T. E., Jensen, O. N., and Larsen, M. R. (2009) Analytical strategies for phosphoproteomics. *Proteomics* **9,** 1451–1468

59. Castellvi, J., Garcia, A., Ruiz-Marcellan, C., Hernández-Losa, J., Peg, V., Salcedo, M., Gil-Moreno, A., and Ramon y Cajal, S. (2009) Cell signaling in endometrial carcinoma: phosphorylated 4E-binding protein-1 expression in endometrial cancer correlates with aggressive tumors and prognosis. *Hum. Pathol.* **40,** 1418–1426

60. McArdle, L., Rafferty, M., Maelandsmo, G. M., Bergin, O., Farr, C. J., Dervan, P. A., O'Loughlin, S., Herlyn, M., and Easty, D. J. (2001) Protein tyrosine phosphatase genes downregulated in melanoma. *J. Invest. Dermatol.* **117,** 1255–1260

61. Schlessinger, J. (2000) Cell signaling by receptor tyrosine kinases. *Cell* **103,** 211–225

62. Ashman, K., and Villar, E. L. (2009) Phosphoproteomics and cancer research. *Clin. Transl. Oncol.* **11,** 356–362

63. Larsen, M. R., Thingholm, T. E., Jensen, O. N., Roepstorff, P., and Jørgensen, T. J. (2005) Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. *Mol. Cell. Proteomics* **4,** 873–886

64. Scaltriti, M., Verma, C., Guzman, M., Jimenez, J., Parra, J. L., Pedersen, K., Smith, D. J., Landolfi, S., Ramon y Cajal, S., Arribas, J., and Baselga, J. (2009) Lapatinib, a HER2 tyrosine kinase inhibitor, induces stabiliza-

tion and accumulation of HER2 and potentiates trastuzumab-dependent cell cytotoxicity. *Oncogene* **28,** 803–814

65. Capdevila, J., Elez, E., Macarulla, T., Ramos, F. J., Ruiz-Echarri, M., and Tabernero, J. (2009) Anti-epidermal growth factor receptor monoclonal antibodies in cancer treatment. *Cancer Treat. Rev.* **35,** 354–363

66. Moran, M. F., Tong, J., Taylor, P., and Ewing, R. M. (2006) Emerging applications for phospho-proteomics in cancer molecular therapeutics. *Biochim. Biophys. Acta* **1766,** 230–241

67. Chambers, A. F., Groom, A. C., and MacDonald, I. C. (2002) Metastasis: Dissemination and growth of cancer cells in metastatic sites. *Nat. Rev. Cancer* **2,** 563–572

68. Dowling, P., Walsh, N., and Clynes, M. (2008) Membrane and membrane-associated proteins involved in the aggressive phenotype displayed by highly invasive cancer cells. *Proteomics* **8,** 4054–4065

69. Bidard, F. C., Pierga, J. Y., Vincent-Salomon, A., and Poupon, M. F. (2008) A "class action" against the microenvironment: do cancer cells cooperate in metastasis? *Cancer Metastasis Rev.* **27,** 5–10

70. Hood, J. D., and Cheresh, D. A. (2002) Role of integrins in cell invasion and migration. *Nat. Rev. Cancer* **2,** 91–100

71. Lund, R., Leth-Larsen, R., Jensen, O. N., and Ditzel, H. J. (2009) Efficient isolation and quantitative proteomic analysis of cancer cell plasma membrane proteins for identification of metastasis-associated cell surface markers. *J. Proteome Res.* **8,** 3078–3090

72. Chen, R., Yi, E. C., Donohoe, S., Pan, S., Eng, J., Cooke, K., Crispin, D. A., Lane, Z., Goodlett, D. R., Bronner, M. P., Aebersold, R., and Brentnall, T. A. (2005) Pancreatic cancer proteome: the proteins that underlie invasion, metastasis, and immunologic escape. *Gastroenterology* **129,** 1187–1197

73. Kischel, P., Guillonneau, F., Dumont, B., Bellahcène, A., Stresing, V., Clézardin, P., De Pauw, E. A., and Castronovo, V. (2008) Cell membrane proteomic analysis identifies proteins differentially expressed in osteotropic human breast cancer cells. *Neoplasia* **10,** 1014–1020

74. Garbis, S., Lubec, G., and Fountoulakis, M. (2005) Limitations of current proteomics technologies. *J. Chromatogr. A* **1077,** 1–18

75. Zhao, Y., Zhang, W., Kho, Y., and Zhao, Y. (2004) Proteomic analysis of integral plasma membrane proteins. *Anal. Chem.* **76,** 1817–1823

76. Blonder, J., Goshe, M. B., Moore, R. J., Pasa-Tolic, L., Masselon, C. D., Lipton, M. S., and Smith, R. D. (2002) Enrichment of integral membrane proteins for proteomic analysis using liquid chromatography and tandem mass spectrometry. *J. Proteome Res.* **1,** 351–360

77. Josic, D., and Clifton, J. G. (2007) Mammalian plasma membrane proteomics. *Proteomics* **7,** 3010–3029

78. Kristensen, D. B., Brønd, J. C., Nielsen, P. A., Andersen, J. R., Sørensen, O. T., Jørgensen, V., Budin, K., Matthiesen, J., Venø, P., Jespersen, H. M., Ahrens, C. H., Schandorff, S., Ruhoff, P. T., Wisniewski, J. R., Bennett, K. L., and Podtelejnikov, A. V. (2004) Experimental Peptide Identification Repository (EPIR): an integrated peptide-centric platform for validation and mining of tandem mass spectrometry data. *Mol. Cell. Proteomics* **3,** 1023–1038

79. Mannová, P., Fang, R., Wang, H., Deng, B., McIntosh, M. W., Hanash, S. M., and Beretta, L. (2006) Modification of host lipid raft proteome upon hepatitis C virus replication. *Mol. Cell. Proteomics* **5,** 2319–2325

80. Bartee, E., McCormack, A., and Früh, K. (2006) Quantitative membrane proteomics reveals new cellular targets of viral immune modulators. *PLoS Pathog.* **2,** e107

81. Righetti, P. G., Castagna, A., Antonioli, P., and Boschetti, E. (2005) Prefractionation techniques in proteome analysis: the mining tools of the third millennium. *Electrophoresis* **26,** 297–319

82. Hör, S., Ziv, T., Admon, A., and Lehner, P. J. (2009) Stable isotope labeling by amino acids in cell culture and differential plasma membrane proteome quantitation identify new substrates for the MARCH9 transmembrane E3 ligase. *Mol. Cell. Proteomics* **8,** 1959–1971

83. Rahbar, A. M., and Fenselau, C. (2005) Unbiased examination of changes in plasma membrane proteins in drug resistant cancer cells. *J. Proteome Res.* **4,** 2148–2153

84. Vargo-Gogola, T., and Rosen, J. M. (2007) Modelling breast cancer: one size does not fit all. *Nat. Rev. Cancer* **7,** 659–672

85. Chen, E. I., and Yates, J. R., 3rd (2007) Cancer proteomics by quantitative shotgun proteomics. *Mol. Oncol.* **1,** 144–159

86. Lacroix, M., and Leclercq, G. (2004) Relevance of breast cancer cell lines as models for breast tumours: an update. *Breast Cancer Res. Treat.* **83,** 249–289

87. Celis, J. E., Moreira, J. M., Cabezón, T., Gromov, P., Friis, E., Rank, F., and Gromova, I. (2005) Identification of extracellular and intracellular signaling components of the mammary adipose tissue and its interstitial fluid in high risk breast cancer patients: toward dissecting the molecular circuitry of epithelial-adipocyte stromal cell interactions. *Mol. Cell. Proteomics* **4,** 492–522

88. Hwang, S. I., Thumar, J., Lundgren, D. H., Rezaul, K., Mayya, V., Wu, L., Eng, J., Wright, M. E., and Han, D. K. (2007) Direct cancer tissue proteomics: a method to identify candidate cancer biomarkers from formalin-fixed paraffin-embedded archival tissues. *Oncogene* **26,** 65–76

89. Kim, J., Kim, S. H., Lee, S. U., Ha, G. H., Kang, D. G., Ha, N. Y., Ahn, J. S., Cho, H. Y., Kang, S. J., Lee, Y. J., Hong, S. C., Ha, W. S., Bae, J. M., Lee, C. W., and Kim, J. W. (2002) Proteome analysis of human liver tumor tissue by two-dimensional gel electrophoresis and matrix assisted laser desorption/ionization-mass spectrometry for identification of disease-related proteins. *Electrophoresis* **23,** 4142–4156

90. Niméus, E., Malmström, J., Johnsson, A., Marko-Varga, G., and Fernö, M. (2007) Proteomic analysis identifies candidate proteins associated with distant recurrences in breast cancer after adjuvant chemotherapy. *J. Pharm. Biomed. Anal.* **43,** 1086–1093

91. Hood, B. L., Darfler, M. M., Guiel, T. G., Furusato, B., Lucas, D. A., Ringeisen, B. R., Sesterhenn, I. A., Conrads, T. P., Veenstra, T. D., and Krizman, D. B. (2005) Proteomic analysis of formalin-fixed prostate cancer tissue. *Mol. Cell. Proteomics* **4,** 1741–1753

92. Schmitt, M., Mengele, K., Schueren, E., Sweep, F. C., Foekens, J. A., Brünner, N., Laabs, J., Malik, A., and Harbeck, N. (2007) European Organisation for Research and Treatment of Cancer (EORTC) Pathobiology Group standard operating procedure for the preparation of human tumour tissue extracts suited for the quantitative analysis of tissue-associated biomarkers. *Eur. J. Cancer* **43,** 835–844

93. Nielsen, P. A., Olsen, J. V., Podtelejnikov, A. V., Andersen, J. R., Mann, M., and Wisniewski, J. R. (2005) Proteomic mapping of brain plasma membrane proteins. *Mol. Cell. Proteomics* **4,** 402–408

94. Conrotto, P., Roesli, C., Rybak, J., Kischel, P., Waltregny, D., Neri, D., and Castronovo, V. (2008) Identification of new accessible tumor antigens in human colon cancer by ex vivo protein biotinylation and comparative mass spectrometry analysis. *Int. J. Cancer* **123,** 2856–2864

95. Clarke, W., Zhang, Z., and Chan, D. W. (2003) The application of clinical proteomics to cancer and other diseases. *Clin. Chem. Lab. Med.* **41,** 1562–1570

96. Diaz, J. I., Cazares, L. H., and Semmes, O. J. (2008) Tissue sample collection for proteomics analysis. *Methods Mol. Biol.* **428,** 43–53

97. Rai, A. J., Gelfand, C. A., Haywood, B. C., Warunek, D. J., Yi, J., Schuchard, M. D., Mehigh, R. J., Cockrill, S. L., Scott, G. B., Tammen, H., Schulz-Knappe, P., Speicher, D. W., Vitzthum, F., Haab, B. B., Siest, G., and Chan, D. W. (2005) HUPO Plasma Proteome Project specimen collection and handling: towards the standardization of parameters for plasma proteome samples. *Proteomics* **5,** 3262–3277

98. Pieragostino, D., Petrucci, F., Del Boccio, P., Mantini, D., Lugaresi, A., Tiberio, S., Onofrj, M., Gambi, D., Sacchetta, P., Di Ilio, C., Federici, G., and Urbani, A. (2010) Pre-analytical factors in clinical proteomics investigations: impact of ex vivo protein modifications for multiple sclerosis biomarker discovery. *J. Proteomics* **73,** 579–592

99. Hondermarck, H., Tastet, C., El Yazidi-Belkoura, I., Toillon, R. A., and Le Bourhis, X. (2008) Proteomics of breast cancer: the quest for markers and therapeutic targets. *J. Proteome Res.* **7,** 1403–1411

100. Patel, V., Hood, B. L., Molinolo, A. A., Lee, N. H., Conrads, T. P., Braisted, J. C., Krizman, D. B., Veenstra, T. D., and Gutkind, J. S. (2008) Proteomic analysis of laser-captured paraffin-embedded tissues: a molecular portrait of head and neck cancer progression. *Clin. Cancer Res.* **14,** 1002–1014

101. Neubauer, H., Clare, S. E., Wozny, W., Schwall, G. P., Poznanovic, S., Stegmann, W., Vogel, U., Sotlar, K., Wallwiener, D., Kurek, R., Fehm, T., and Cahill, M. A. (2008) Breast cancer proteomics reveals correlation between estrogen receptor status and differential phosphorylation of PGRMC1. *Breast Cancer Res.* **10,** R85

102. Sprung, R. W., Jr., Brock, J. W., Tanksley, J. P., Li, M., Washington, M. K., Slebos, R. J., and Liebler, D. C. (2009) Equivalence of protein inventories obtained from formalin-fixed paraffin-embedded and frozen tissue in multidimensional liquid chromatography-tandem mass spectrometry

shotgun proteomic analysis. *Mol. Cell. Proteomics* **8,** 1988–1998

103. Paradela, A., and Albar, J. P. (2008) Advances in the analysis of protein phosphorylation. *J. Proteome Res.* **7,** 1809–1818

104. Tissot, B., North, S. J., Ceroni, A., Pang, P. C., Panico, M., Rosati, F., Capone, A., Haslam, S. M., Dell, A., and Morris, H. R. (2009) Glycoproteomics: past, present and future. *FEBS Lett.* **583,** 1728–1735

105. Dalpathado, D. S., and Desaire, H. (2008) Glycopeptide analysis by mass spectrometry. *Analyst* **133,** 731–738

106. Cho, W., Jung, K., and Regnier, F. E. (2008) Use of glycan targeting antibodies to identify cancer-associated glycoproteins in plasma of breast cancer patients. *Anal. Chem.* **80,** 5286–5292

107. Jung, K., Cho, W., and Regnier, F. E. (2009) Glycoproteomics of plasma based on narrow selectivity lectin affinity chromatography. *J. Proteome Res.* **8,** 643–650

108. Powlesland, A. S., Hitchen, P. G., Parry, S., Graham, S. A., Barrio, M. M., Elola, M. T., Mordoh, J., Dell, A., Drickamer, K., and Taylor, M. E. (2009) Targeted glycoproteomic identification of cancer cell glycosylation. *Glycobiology* **19,** 899–909

109. Yang, Z., and Hancock, W. S. (2005) Monitoring glycosylation pattern changes of glycoproteins using multi-lectin affinity chromatography. *J. Chromatogr. A* **1070,** 57–64

110. Drake, R. R., Schwegler, E. E., Malik, G., Diaz, J., Block, T., Mehta, A., and Semmes, O. J. (2006) Lectin capture strategies combined with mass spectrometry for the discovery of serum glycoprotein biomarkers. *Mol. Cell. Proteomics* **5,** 1957–1967

111. Wuhrer, M., Koeleman, C. A., Hokke, C. H., and Deelder, A. M. (2005) Protein glycosylation analyzed by normal-phase nano-liquid chromatography-mass spectrometry of glycopeptides. *Anal. Chem.* **77,** 886–894

112. Koizumi, K. (1996) High-performance liquid chromatographic separation of carbohydrates on graphitized carbon columns. *J. Chromatogr. A* **720,** 119–126

113. Harvey, D. J. (2005) Proteomic analysis of glycosylation: structural determination of N- and O-linked glycans by mass spectrometry. *Expert Rev. Proteomics* **2,** 87–101

114. Kirmiz, C., Li, B., An, H. J., Clowers, B. H., Chew, H. K., Lam, K. S., Ferrige, A., Alecio, R., Borowsky, A. D., Sulaimon, S., Lebrilla, C. B., and Miyamoto, S. (2007) A serum glycomics approach to breast cancer biomarkers. *Mol. Cell. Proteomics* **6,** 43–55

115. Zhang, H., Loriaux, P., Eng, J., Campbell, D., Keller, A., Moss, P., Bonneau, R., Zhang, N., Zhou, Y., Wollscheid, B., Cooke, K., Yi, E. C., Lee, H., Peskind, E. R., Zhang, J., Smith, R. D., and Aebersold, R. (2006) UniPep—a database for human N-linked glycosites: a resource for biomarker discovery. *Genome Biol.* **7,** R73

116. Cordwell, S. J., and Thingholm, T. E. (2010) Technologies for plasma membrane proteomics. *Proteomics* **10,** 611–627

117. Zarling, A. L., Polefrone, J. M., Evans, A. M., Mikesh, L. M., Shabanowitz, J., Lewis, S. T., Engelhard, V. H., and Hunt, D. F. (2006) Identification of class I MHC-associated phosphopeptides as targets for cancer immunotherapy. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 14889–14894

118. Kim, J. E., and White, F. M. (2006) Quantitative analysis of phosphotyrosine signaling networks triggered by CD3 and CD28 costimulation in Jurkat cells. *J. Immunol.* **176,** 2833–2843

119. Wolf-Yadlin, A., Hautaniemi, S., Lauffenburger, D. A., and White, F. M. (2007) Multiple reaction monitoring for robust quantitative proteomic analysis of cellular signaling networks. *Proc. Natl. Acad. Sci. U.S.A.* **104,** 5860–5865

120. Alcolea, M. P., Kleiner, O., and Cutillas, P. R. (2009) Increased confidence in large-scale phosphoproteomics data by complementary mass spectrometric techniques and matching of phosphopeptide data sets. *J. Proteome Res.* **8,** 3808–3815

121. Thingholm, T. E., Jensen, O. N., Robinson, P. J., and Larsen, M. R. (2008) SIMAC (sequential elution from IMAC), a phosphoproteomics strategy for the rapid separation of monophosphorylated from multiply phosphorylated peptides. *Mol. Cell. Proteomics* **7,** 661–671

122. Thingholm, T. E., Larsen, M. R., Ingrell, C. R., Kassem, M., and Jensen, O. N. (2008) TiO(2)-based phosphoproteomic analysis of the plasma membrane and the effects of phosphatase inhibitor treatment. *J. Proteome Res.* **7,** 3304–3313

123. Zhao, Y., Lee, W. N., and Xiao, G. G. (2009) Quantitative proteomics and biomarker discovery in human cancer. *Expert Rev. Proteomics* **6,** 115–118

124. Hanash, S. M., Pitteri, S. J., and Faca, V. M. (2008) Mining the plasma proteome for cancer biomarkers. *Nature* **452,** 571–579

125. Foster, L. J., Zeemann, P. A., Li, C., Mann, M., Jensen, O. N., and Kassem, M. (2005) Differential expression profiling of membrane proteins by quantitative proteomics in a human mesenchymal stem cell line undergoing osteoblast differentiation. *Stem Cells* **23,** 1367–1377

126. Van Hoof, D., Dormeyer, W., Braam, S. R., Passier, R., Monshouwer-Kloots, J., Ward-van Oostwaard, D., Heck, A. J., Krijgsveld, J., and Mummery, C. L. (2010) Identification of cell surface proteins for antibody-based selection of human embryonic stem cell-derived cardiomyocytes. *J. Proteome Res.* **9,** 1610–1618

127. Mann, M. (2006) Functional and quantitative proteomics using SILAC. *Nat. Rev. Mol. Cell Biol.* **7,** 952–958

128. Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F., Gelb, M. H., and Aebersold, R. (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* **17,** 994–999

129. Li, J., Steen, H., and Gygi, S. P. (2003) Protein profiling with cleavable isotope-coded affinity tag (cICAT) reagents: the yeast salinity stress response. *Mol. Cell. Proteomics* **2,** 1198–1204

130. Butler, G. S., Dean, R. A., Tam, E. M., and Overall, C. M. (2008) Pharmacoproteomics of a metalloproteinase hydroxamate inhibitor in breast cancer cells: dynamics of membrane type 1 matrix metalloproteinase-mediated membrane protein shedding. *Mol. Cell. Biol.* **28,** 4896–4914

131. Ross, P. L., Huang, Y. N., Marchese, J. N., Williamson, B., Parker, K., Hattan, S., Khainovski, N., Pillai, S., Dey, S., Daniels, S., Purkayastha, S., Juhasz, P., Martin, S., Bartlet-Jones, M., He, F., Jacobson, A., and Pappin, D. J. (2004) Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. *Mol. Cell. Proteomics* **3,** 1154–1169

132. Yao, X., Freas, A., Ramirez, J., Demirev, P. A., and Fenselau, C. (2001) Proteolytic 18O labeling for comparative proteomics: model studies with two serotypes of adenovirus. *Anal. Chem.* **73,** 2836–2842

133. Zieske, L. R. (2006) A perspective on the use of iTRAQ reagent technology for protein complex and profiling studies. *J. Exp. Bot.* **57,** 1501–1508

134. Ramus, C., Gonzalez de Peredo, A., Dahout, C., Gallagher, M., and Garin, J. (2006) An optimized strategy for ICAT quantification of membrane proteins. *Mol. Cell. Proteomics* **5,** 68–78

135. Pawlik, T. M., Hawke, D. H., Liu, Y., Krishnamurthy, S., Fritsche, H., Hunt, K. K., and Kuerer, H. M. (2006) Proteomic analysis of nipple aspirate fluid from women with early-stage breast cancer using isotope-coded affinity tags and tandem mass spectrometry reveals differential expression of vitamin D binding protein. *BMC Cancer* **6,** 68

136. Han, C. L., Chien, C. W., Chen, W. C., Chen, Y. R., Wu, C. P., Li, H., and Chen, Y. J. (2008) A multiplexed quantitative strategy for membrane proteomics: opportunities for mining therapeutic targets for autosomal dominant polycystic kidney disease. *Mol. Cell. Proteomics* **7,** 1983–1997

137. Rajcevic, U., Petersen, K., Knol, J. C., Loos, M., Bougnaud, S., Klychnikov, O., Li, K. W., Pham, T. V., Wang, J., Miletic, H., Peng, Z., Bjerkvig, R., Jimenez, C. R., and Niclou, S. P. (2009) iTRAQ-based proteomics profiling reveals increased metabolic activity and cellular cross-talk in angiogenic compared with invasive glioblastoma phenotype. *Mol. Cell. Proteomics* **8,** 2595–2612

138. Ho, J., Kong, J. W., Choong, L. Y., Loh, M. C., Toy, W., Chong, P. K., Wong, C. H., Wong, C. Y., Shah, N., and Lim, Y. P. (2009) Novel breast cancer metastasis-associated proteins. *J. Proteome Res.* **8,** 583–594

139. Ye, X., Luke, B., Andersson, T., and Blonder, J. (2009) 18O stable isotope labeling in MS-based proteomics. *Brief. Funct. Genomic. Proteomic.* **8,** 136–144

140. Kristiansen, T. Z., Harsha, H. C., Grønborg, M., Maitra, A., and Pandey, A. (2008) Differential membrane proteomics using 18O-labeling to identify biomarkers for cholangiocarcinoma. *J. Proteome Res.* **7,** 4670–4677

141. Chi, L. M., Lee, C. W., Chang, K. P., Hao, S. P., Lee, H. M., Liang, Y., Hsueh, C., Yu, C. J., Lee, I. N., Chang, Y. J., Lee, S. Y., Yeh, Y. M., Chang, Y. S., Chien, K. Y., and Yu, J. S. (2009) Enhanced interferon signaling pathway in oral cancer revealed by quantitative proteome analysis of microdissected specimens using 16O/18O labeling and integrated two-dimensional LC-ESI-MALDI tandem MS. *Mol. Cell. Proteomics* **8,** 1453–1474

142. Stockwin, L. H., Blonder, J., Bumke, M. A., Lucas, D. A., Chan, K. C., Conrads, T. P., Issaq, H. J., Veenstra, T. D., Newton, D. L., and Rybak,

S. M. (2006) Proteomic analysis of plasma membrane from hypoxia-adapted malignant melanoma. *J. Proteome Res.* **5,** 2996–3007

143. Röwer, C., Vissers, J. P., Koy, C., Kipping, M., Hecker, M., Reimer, T., Gerber, B., Thiesen, H. J., and Glocker, M. O. (2009) Towards a proteome signature for invasive ductal breast carcinoma derived from label-free nanoscale LC-MS protein expression profiling of tumorous and glandular tissue. *Anal. Bioanal. Chem.* **395,** 2443–2456

144. Zhu, Z., Boobis, A. R., and Edwards, R. J. (2008) Identification of estrogen-responsive proteins in MCF-7 human breast cancer cells using label-free quantitative proteomics. *Proteomics* **8,** 1987–2005

145. Ishihama, Y., Sato, T., Tabata, T., Miyamoto, N., Sagane, K., Nagasu, T., and Oda, Y. (2005) Quantitative mouse brain proteomics using culture-derived isotope tags as internal standards. *Nat. Biotechnol.* **23,** 617–621

146. Sihlbom, C., Wilhelmsson, U., Li, L., Nilsson, C. L., and Pekny, M. (2007) 14-3-3 expression in denervated hippocampus after entorhinal cortex lesion assessed by culture-derived isotope tags in quantitative proteomics. *J. Proteome Res.* **6,** 3491–3500

147. Rasmussen, N., and Ditzel, H. J. (2009) Scanning the cell surface proteome of cancer cells and identification of metastasis-associated proteins using a subtractive immunization strategy. *J. Proteome Res.* **8,** 5048–5059

148. Giltnane, J. M., and Rimm, D. L. (2004) Technology insight: Identification of biomarkers with tissue microarray technology. *Nat. Clin. Pract. Oncol.* **1,** 104–111

149. Thomson, T. A., Zhou, C., Chu, C., and Knight, B. (2009) Tissue microarray for routine analysis of breast biomarkers in the clinical laboratory. *Am. J. Clin. Pathol.* **132,** 899–905

150. Mager, S. R., Oomen, M. H., Morente, M. M., Ratcliffe, C., Knox, K., Kerr, D. J., Pezzella, F., and Riegman, P. H. (2007) Standard operating procedure for the collection of fresh frozen tissue samples. *Eur. J. Cancer* **43,** 828–834

151. Wasielewski, R., Hasselmann, S., Rüschoff, J., Fisseler-Eckhoff, A., and Kreipe, H. (2008) Proficiency testing of immunohistochemical biomarker assays in breast cancer. *Virchows Arch.* **453,** 537–543

152. Camp, R. L., Chung, G. G., and Rimm, D. L. (2002) Automated subcellular localization and quantification of protein expression in tissue microarrays. *Nat. Med.* **8,** 1323–1327

153. Rubin, M. A., Zerkowski, M. P., Camp, R. L., Kuefer, R., Hofer, M. D., Chinnaiyan, A. M., and Rimm, D. L. (2004) Quantitative determination of expression of the prostate cancer protein alpha-methylacyl-CoA racemase using automated quantitative analysis (AQUA): a novel paradigm for automated and continuous biomarker measurements. *Am. J. Pathol.* **164,** 831–840

154. Harigopal, M., Heymann, J., Ghosh, S., Anagnostou, V., Camp, R. L., and Rimm, D. L. (2009) Estrogen receptor co-activator (AIB1) protein expression by automated quantitative analysis (AQUA) in a breast cancer tissue microarray and association with patient outcome. *Breast Cancer Res. Treat.* **115,** 77–85

155. Kozarova, A., Petrinac, S., Ali, A., and Hudson, J. W. (2006) Array of informatics: applications in modern research. *J. Proteome Res.* **5,** 1051–1059

156. Sanchez-Carbayo, M. (2006) Antibody arrays: technical considerations and clinical applications in cancer. *Clin. Chem.* **52,** 1651–1659

157. MacBeath, G. (2002) Protein microarrays and proteomics. *Nat. Genet.* **32,** (suppl.) 526–532

158. Gerber, S. A., Rush, J., Stemman, O., Kirschner, M. W., and Gygi, S. P. (2003) Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. *Proc. Natl. Acad. Sci. U.S.A.* **100,** 6940–6945

159. Pratt, J. M., Simpson, D. M., Doherty, M. K., Rivers, J., Gaskell, S. J., and Beynon, R. J. (2006) Multiplexed absolute quantification for proteomics using concatenated signature peptides encoded by QconCAT genes. *Nat. Protoc.* **1,** 1029–1043

160. Mirzaei, H., McBee, J. K., Watts, J., and Aebersold, R. (2008) Comparative evaluation of current peptide production platforms used in absolute quantification in proteomics. *Mol. Cell. Proteomics* **7,** 813–823

161. Zucker, S., Hymowitz, M., Conner, C., Zarrabi, H. M., Hurewitz, A. N., Matrisian, L., Boyd, D., Nicolson, G., and Montana, S. (1999) Measurement of matrix metalloproteinases and tissue inhibitors of metalloproteinases in blood and tissues: clinical and experimental applications.

162. Anderson, N. L., and Anderson, N. G. (2002) The human plasma proteome: history, character, and diagnostic prospects. *Mol. Cell. Proteomics* **1,** 845–867

163. Pardo, M., García, A., Antrobus, R., Blanco, M. J., Dwek, R. A., and Zitzmann, N. (2007) Biomarker discovery from uveal melanoma secretomes: identification of gp100 and cathepsin D in patient serum. *J. Proteome Res.* **6,** 2802–2811

164. Whelan, S. A., Lu, M., He, J., Yan, W., Saxton, R. E., Faull, K. F., Whitelegge, J. P., and Chang, H. R. (2009) Mass spectrometry (LC-MS/MS) site-mapping of N-glycosylated membrane proteins for breast cancer biomarkers. *J. Proteome Res.* **8,** 4151–4160

165. Gross, M., Top, I., Laux, I., Katz, J., Curran, J., Tindell, C., and Agus, D. (2007) Beta-2-microglobulin is an androgen-regulated secreted protein elevated in serum of patients with advanced prostate cancer. *Clin. Cancer Res.* **13,** 1979–1986

166. Souder, C., Leitzel, K., Ali, S. M., Demers, L., Evans, D. B., Chaudri-Ross, H. A., Hackl, W., Hamer, P., Carney, W., and Lipton, A. (2006) Serum epidermal growth factor receptor/HER-2 predicts poor survival in patients with metastatic breast cancer. *Cancer* **107,** 2337–2345

167. Sandri, M. T., Johansson, H. A., Zorzino, L., Salvatici, M., Passerini, R., Maisonneuve, P., Rocca, A., Peruzzotti, G., and Colleoni, M. (2007) Serum EGFR and serum HER-2/neu are useful predictive and prognostic markers in metastatic breast cancer patients treated with metronomic chemotherapy. *Cancer* **110,** 509–517

168. Witzel, I., Thomssen, C., Krenkel, S., Wilczak, W., Bubenheim, M., Pantel, K., Neumann, R., Jänicke, F., and Müller, V. (2006) Clinical utility of determination of HER-2/neu and EGFR fragments in serum of patients with metastatic breast cancer. *Int. J. Biol. Markers* **21,** 131–140

169. Ali, S. M., Carney, W. P., Esteva, F. J., Fornier, M., Harris, L., Köstler, W. J., Lotz, J. P., Luftner, D., Pichon, M. F., and Lipton, A. (2008) Serum HER-2/neu and relative resistance to trastuzumab-based therapy in patients with metastatic breast cancer. *Cancer* **113,** 1294–1301

170. Mayer, S., zur Hausen, A., Watermann, D. O., Stamm, S., Jäger, M., Gitsch, G., and Stickeler, E. (2008) Increased soluble CD44 concentrations are associated with larger tumor size and lymph node metastasis in breast cancer patients. *J. Cancer Res. Clin. Oncol.* **134,** 1229–1235

171. Lim, K. T., Miyazaki, K., Kimura, N., Izawa, M., and Kannagi, R. (2008) Clinical application of functional glycoproteomics—dissection of glycotopes carried by soluble CD44 variants in sera of patients with cancers. *Proteomics* **8,** 3263–3273

172. Callesen, A. K., Vach, W., Jørgensen, P. E., Cold, S., Tan, Q., Depont Christensen, R., Mogensen, O., Kruse, T. A., Jensen, O. N., and Madsen, J. S. (2008) Combined experimental and statistical strategy for mass spectrometry based serum protein profiling for diagnosis of breast cancer: a case-control study. *J. Proteome Res.* **7,** 1419–1426

173. Becker, S., Cazares, L. H., Watson, P., Lynch, H., Semmes, O. J., Drake, R. R., and Laronga, C. (2004) Surfaced-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) differentiation of serum protein profiles of BRCA-1 and sporadic breast cancer. *Ann. Surg. Oncol.* **11,** 907–914

174. Vlahou, A., Laronga, C., Wilson, L., Gregory, B., Fournier, K., McGaughey, D., Perry, R. R., Wright, G. L., Jr., and Semmes, O. J. (2003) A novel approach toward development of a rapid blood test for breast cancer. *Clin. Breast Cancer* **4,** 203–209

175. Knoop, A. S., Bentzen, S. M., Nielsen, M. M., Rasmussen, B. B., and Rose, C. (2001) Value of epidermal growth factor receptor, HER2, p53, and steroid receptors in predicting the efficacy of tamoxifen in high-risk postmenopausal breast cancer patients. *J. Clin. Oncol.* **19,** 3376–3384

176. Early Breast Cancer Trialists' Collaborative Group (1998) Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* **351,** 1451–1467

177. Early Breast Cancer Trialists' Collaborative Group (2005) Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* **365,** 1687–1717

178. Umar, A., Kang, H., Timmermans, A. M., Look, M. P., Meijer-van Gelder, M. E., den Bakker, M. A., Jaitly, N., Martens, J. W., Luider, T. M., Foekens, J. A., and Pasa-Toliæ, L. (2009) Identification of a putative protein profile associated with tamoxifen therapy resistance in breast cancer. *Mol. Cell. Proteomics* **8,** 1278–1294