Review

Metastatic Biomarker Discovery Through Proteomics

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Abstract. *Tumor heterogeneity has been a stumbling block in the development of effective cancer treatments. Personalized medicine has evolved with the theory that matching therapies with the unique misregulated pathways often present in tumors will increase patient prognosis. Of particular interest is prediction or determination of the metastatic potential of a tumor. Thus, biomarkers that can predict metastases represent an enormous advance to our understanding over the clinical treatment of cancer. Considerable effort has been expended to characterize the cancer proteome for early detection, however, fewer efforts have been made to develop biomarkers to distinguish the potential for and the nature of metastasis. In this review, we discuss proteomic technologies as well as existing potential metastatic biomarkers for various cancers. In the conclusion, we discuss forward thinking as to what the field needs to enable translation to the clinic.*

The connection between metastasis and poor prognosis is well-established, and as such, identifying the metastatic potential of a tumor becomes of paramount importance when deciding treatment regimens. Over-treatment causes superfluous exposure to cytotoxic chemotherapeutic drugs while under-treatment leads to recurrence. Additionally, attempts to treat a resistant tumor can lead to complications and diminished quality of life. In this vein, personalized therapy has become an important concept in tumor treatment strategies with the goal of tailoring treatments through knowledge of the molecular defects of an individual tumor.

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For patients with late-stage disease, metastatic biomarkers can help detect initial stages of tumor spread, probability of recurrence and even predict preferred sites of metastasis. Such biomarkers can be found in the blood, other biological fluids and can be used as targets for imaging agents. Thus, biomarkers have the advantage of being a minimally invasive way to personalize cancer treatment at all stages of disease.

Most biomarkers are proteins and proteomics is an ideal and highly translatable research tool to find novel biomarkers. Proteomic techniques assess proteins within a physiological setting, and unlike reductionist approaches, the techniques can provide an unbiased evaluation for discovery as well as targeted capability for quantifying assays. Thanks to advances in methodology and technology, large-scale proteomic studies can simultaneously identify a substantial number of potential biomarkers and even assess combinations of biomarkers in order to improve diagnostic accuracy. The results of proteomic studies are highly translatable to the clinic, where they could greatly improve the standard-of-care for cancer patients. In this review, we will discuss recently identified biomarkers of metastasis (Table I) as well as the proteomic methods important to their discovery and clinical development. Many biomarkers are found in blood because serum samples are convenient, both in research and in the clinic. The first part of this review focuses on serum biomarkers of different cancers. Other biological fluids, such as saliva and urine, can be obtained with minimal invasiveness and constitute alternative good sources for biomarker discovery and detection. The second part of this review discusses recent biomarkers derived from tissue that can be applied to molecular imaging, an emerging field of cancer diagnostics and therapeutics.

Serum Biomarkers

Primary tumors spread by releasing tumor cells into the blood stream and lymphatic system. However, presence of circulating tumor cells is not sufficient to indicate metastasis has occurred. Less than 0.1% of cancer cells that enter the blood circulation actually form a metastasis (1). Much research has occurred to

Table I. *Summary of metastatic biomarkers*.

Protein	Biomarker of	Fluid	Cancer	Method	Reference
AGR2	Metastasis	Serum	Colorectal	LC-MS, WB, ELISA	8
AGR3	Aggression; Subtype; Prognosis; Therapeutic response	Tissue	Ovarian	IHC, IF, WB, Radioimmunoassay	71, 74
Alpha-enolase	Metastasis	Serum	Colorectal	LC-MS, 2D-DIGE	14
CA125	Recurrence; Prognosis; Lung invasion; Survival	Serum	Ovarian, NSCLC, Cervical, Breast	AUC	34, 36-37
CA125, CRP, SAA, IL6, IL8	Recurrence	Serum	Ovarian	AUC	33, 35
CacyBP	Metastasis	Serum	Colorectal	iTRAQ, LC-MS, WB, Quantitative IF	12
CCR7	Bone metastasis; Lymph node metastasis	Tissue	Breast	IHC	48-49
E-cadherin	Metastasis; Poor Prognosis; Brain metastasis	Serum, Tissue	Lung, NSCLC, other cancers	2D-DIGE, nano-LC-MS, IHC, Fucosylation index	38-42, 53
CXCR4	Bone metastasis; Lymph node metastasis; Poor prognosis	Tissue	Breast	IHC	48-51
CYFRA21-1	Distant metastasis; Progression	Serum	Lung	ELISA	43-45
EGFR	Lymph node metastasis; Histological grade	Tissue	Breast	IHC	50
EMP2	Prognosis; Recurrence	Tissue	Endometrial	IHC	84-86
EphA2	Metastasis; Invasion	Tissue	Colorectal	IHC	42
Galectin-1	Prognosis; Survival	Stroma	Pancreatic	iTRAQ, MS, IHC	68
GDF15	Metastasis	Serum	Colorectal	LC-MS, WB,ELISA	8
H2K18ac	Survival	Tissue	Pancreatic	IHC	67
H3K4me2	Survival	Tissue	Pancreatic	IHC	67
H3K9me2	Survival	Tissue	Pancreatic	IHC	67
HE4	Diagnosis	Serum	Ovarian	Lowess Curves	34
HER2-neu	Lymph node metastasis	Tissue	Breast	IHC	49
HSP27	Metastasis; Progression	Serum	Colorectal	MALDI-TOF MS	15
HSP60	Lymph node metastasis	Tissue	Breast	IT-MS, IHC	52
IGFBP2	Progression; Stage	Serum	Prostate	ELISA, Radioimmunoassay	18-19
IGFBP3	Skeletal metastasis	Serum	Prostate	ELISA, Radioimmunoassay	18-19
IGFBP7	Metastasis	Serum	Colorectal	LC-MS, WB, ELISA	8
IL6, IL6sR	Metastatic potential	Serum	Prostate	Quantitative immunoassay	20
ILK	Lymph node metastasis; TNM stage; EMT	Tissue	NSCLC	IHC	54
Integrin $\alpha_v\beta_6$	Metastasis; Survival	Tissue	Pancreatic, Gastric, Lung, Colon, Cervical	IHC	87, 89-93
LCN2	Metastasis	Serum	Colorectal	LC-MS, WB, ELISA	8
MSLN	Diagnosis; Prognosis	Serum, Tissue	Ovarian, Pancreatic	Lowess Curves, s-TMA, IHC	34, 65
Muc-1	Prognosis	Tissue	PDAC	s-TMA, IHC	65
PDX6	Lymph node metastasis	Tissue	Breast	IT-MS, IHC	52
Plectin	Lymph node metastasis; Liver metastasis	Tissue	Pancreatic	Phage display, IHC	80, 88
SAA	Advanced stage; Prognosis; Distant Metastasis; Progression; Recurrence; lung, liver, or Bone metastasis	Serum	Nasopharyngeal, Renal, Breast, Colorectal, Lung, Prostate, Gastric, Ovarian	SELDI-TOF MS, ZipTip desalting, acetronitrile precipitation, HPLC, MALDI-TOF MS	21, 23-33
SPARC	Stage; Metastasis	Tissue	Prostate	Phage display, IHC, FMT	96-97
TFF3	Metastasis	Serum	Colorectal	LC-MS, WB, ELISA	8
TGF-β_1	Metastasis; Lymph node metastasis	Serum	Prostate	Quantitative immunoassay	20
TGM2	Metastasis	Serum	Colorectal	LC-MS, WB, ELISA	8
TPM4	Lymph node metastasis; Clinical stage	Tissue	Breast	IT-MS, IHC	52
Triosephosphate isomerase	Metastasis	Serum	Colorectal	LC-MS, 2D-DIGE	14
USP9X	Survival; Metastasis	Tissue	Pancreatic	SB mutagenesis, RT-PCR, WB, IHC	69
VCAM-1	Peritoneal metastasis	Tissue, Peritoneal Washing	Ovarian	IHC	70
VEGF-C	Recurrence; Lymph node metastasis	Serum, Tissue	Cervical, Gastric, other cancers	ELISA, IHC, RT-PCR	58-61, 63-64
VEGF-D	Lymph node metastasis; Survival	Tissue	Gastric, other cancers	IHC	58-59
VEGFR-3	Survival	Tissue	Gastric	IHC	58-59

determine how some circulating tumor cells are able to create metastatic lesions in distant organs. Signaling proteins that stimulate the metastatic niche in target organs have been discovered and may serve as independent indicators of metastatic potential.

Traditional proteomic methodologies used for discovery, quantification, and validation of serum biomarkers include two-dimensional electrophoresis, mass spectrometry (MS), Enzyme-linked immunosorbent assay (ELISA) analysis, and bioinformatics approaches. MS is employed to find and identify protein biomarkers. With increasing use of proteomics to find serum biomarkers, several improvements to methodology have overcome specific hurdles. 2D-polyacrylamide gel electrophoresis (PAGE) is not suited for smaller samples or development into a diagnostic test and has a limited dynamic range (2, 3). Matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF MS) MALDI is used for the rapid identification of proteins by peptide mass fingerprinting (4). Yet MALDI still requires considerable sample preparation and can have high background signaling because of contaminants (2). Surface-enhanced laser desorption ionization time-of-flight MS (SELDI-TOF MS) improves on these limitations and allows protein expression to be quickly profiled from many different biological and clinical samples (5). However, SELDI-TOF MS has been criticized as biased towards finding proteins that are present in higher quantities and that are inflammatory markers, rather than tumor-derived (6). Fan *et al*. solved this problem by first capturing low molecular weight proteins and peptides from serum using nanoporous silica chips (7). The bottom-up or "shot-gun" proteomic strategy involves digestion of protein mixtures, liquid chromatography to separate the proteins, and tandem MS to analyze the data (3).

Often if a protein is only present in a relatively small amount compared to several very abundant proteins, it can be difficult to detect. To circumvent this problem, many have tried to remove high abundance proteins prior to performing proteomic analyses. Another approach is to focus on the secretome, a term coined to describe all proteins that are released in any manner from a cell, tissue or organism. Xue *et al*. used cultured cells and selected liquid chromatography-mass spectrophotometry (LC-MS) with labeling methods like isotope coding affinity tag (ICAT) and stable isotope labeling by amino acids in cell culture (SILAC) because ICAT and SILAC can be expensive and time-consuming (8). LC-MS, protein fractionation prior to MS allows for better resolution of the secretome. Data from LC-MS can be quantified using peak intensities and spectral counts. Secretome analysis has also been used to find novel biomarkers in combination with SILAC in pancreatic cancer (9), as well as with MALDI-TOF MS in oral cancer (10) and in lung cancer (11). Ghosh *et al*. used isobaric tags for relative and absolute quantitation (iTRAQ) along with LC-MS, which has the advantage of identifying a large number of protein biomarkers compared with other methods (12).

ELISAs are used to quantitate the levels of specific proteins that are involved in the process of metastasis and determine whether it can be used as a biomarker. Antibodies against proteins of interest are used to probe serum samples from patients with (1) non-metastatic and (2) metastatic cancer as well as (3) non-cancer controls and the results between the three categories are analyzed for statistical significance. Although many biomarker methods have become mainstream and standardized, unique challenges associated with different applications of proteomics have led to new strategies. Because many novel biomarkers do not yet have highly specific antibodies, targeted quantitative proteomics and MS-based absolute quantification strategies have emerged (13).

Colorectal cancer. In colorectal cancer, the serum biomarkers trefoil factor 3 (TFF3), growth/differentiation factor 15 (GDF15), anterior gradient homologue 2 (AGR2), protein-glutamine gamma-glutamyltransferase 2 (TGM2), lipocalin-2/neutrophil gelatinase-associated lipocalin (LCN2), and insulin-like growth factor-binding protein 7(IGFBP7) have been identified as biomarkers of metastasis (8). Calcyclin binding protein (CacyBP) was identified as a potential biomarker of colorectal metastasis and selected for further validation with western blotting and quantitative immuno-fluorescence (12). LC-MS was also used with two-dimensional differential in-gel electrophoresis (2D-DIGE) to find nine potential proteins, out of which alpha-enolase and triosephosphate isomerase were further investigated *in vivo* and were found to be associated with the metastatic process (14). Zhao *et al*. used MALDI-TOF MS to discover heat shock protein-27 (HSP27) as a potential biomarker of metastasis and progression (15).

Prostate cancer. Prostate-specific antigen (PSA) is one of the best known serum biomarkers for prostate cancer, however recent controversy has called into question the use of this test for the detection of prostate cancer because (1) the biomarker cannot distinguish aggressive disease (needing treatment) from indolent disease (needing surveillance, but not treatment), (2) the sensitivity and specificity of the assay may preclude cost-effectiveness, and (3) the target cohort of older males frequently die of competing causes (16, 17). Thus, alternatives for PSA testing have been a priority for this common disease and biomarkers that can alert the clinician to metastatic growth would be particularly useful. Progression of prostate cancer from early to advanced stages can be monitored using the insulin-like growth factor-binding protein 2 (IGFBP2). Serum levels of this protein were found to be inversely proportional to the advancement of prostate cancer (18, 19). Skeletal metastasis in particular is indicated by serum levels of insulin-like growth factor-binding protein 3 (IGFBP3), presenting also with an inverse relationship (18,

19). In addition to ELISA testing, radioimmunoassay can also be used to detect protein. Increased transforming growth factor (TGF)-β1 is a biomarker of metastatic spread in prostate cancer. It can indicate that the tumor is organ-confined or that it is an aggressive tumor with extra-prostatic extension, seminal vesicle involvement, and/or regional lymph node metastases. Elevated serum levels of IL6 and its soluble receptor (IL6sR) indicate metastatic potential. It does not appear that serum levels indicate presence of metastasis, but rather that the tumor is capable of metastasizing. TGF-β1, IL6, IL6sR were found by quantitative immunoassay of patient samples (20).

Nasopharyngeal cancer. Although very treatable, nasopharyngeal cancer has a high probability of recurrence. Serum Amyloid A (SAA) was found to be a biomarker of recurrence of nasopharyngeal cancer (21). SAA levels were not elevated with primary tumor or in cases with local metastatic spread to lymph nodes. Instead, SAA appears to be a biomarker of distant metastatic spread to lung, liver or bone (21). Diamandis suggests that SAA is not useful as a cancer biomarker because it is an acute-phase reactant and not specific to any type of cancer (22). However, Li *et al.* implicated SAA as a marker of advanced stage of multiple cancers and augmented the SELDI-TOF MS methodology with ZipTip desalting, acetonitrile precipitation, high-performance liquid chromatography (HPLC) separation and MALDI-TOF-MS in order to increase resolution and mass accuracy (23). Interestingly, a similar debate existed in the 1980s. SAA was discovered as early as 1979 as a marker of cancer metastasis (24). Certain reports concluded serum SAA levels to be a prognostic indicator that correlates with disease stage, with levels being highest at metastatic disease (25). Other reports were more skeptical, finding that SAA concentrations were unable to distinguish between metastatic and non-metastatic tumors (26). SAA has also been shown to correlate with C-reactive protein (CRP), which is used clinically as a non-specific marker of inflammation; however, SAA may be elevated in the absence of increased CRP levels (26, 27). It has been recently suggested that despite the fact that SAA is not tumor-derived in all cases, it may be useful as a biomarker of metastasis in several cancers, including renal (28), breast, colorectal, lung (29), prostate (30, 31), gastric (32), and ovarian (33). Inclusion of SAA with other biomarkers increases the accuracy of some malignancy tests currently used in clinical practice (33) and could be used in combination with other biomarkers to indicate worsening physical conditions in advanced-stage cancers of many types (23).

Ovarian cancer. Often single biomarkers are not as powerful as a panel of biomarkers; this may be because proteins are interrelated. Thus, a combination of biomarkers can lead to increased sensitivity in some settings. In addition, it is possible to couple biomarkers with imaging to increase the accuracy of both tests. For example, the most commonly used test for ovarian cancer is the biomarker cancer antigen 125 (CA 125). However, by itself, CA 125 has a positive predictive value of less than 10%. The positive predictive value is increased to about 20% with use of ultrasound imaging, but use of additional blood-based biomarkers with CA 125 can increase the positive predictive value by three-fold. Anderson *et al.* identified CA 125, human epididymis protein 4 (HE4), and mesothelin (MSLN) as biomarkers of ovarian cancer by fitting Lowess curves to biomarker levels in patients *versus* control subjects; levels of these biomarkers showed a trend (visually) of increasing, in cancer patients starting about three years prior to diagnosis, but were not detectable in the abnormal range until one year before diagnosis (34). The 5-biomarker panel (CA 125, CRP, SAA, IL6 and IL8) was also found to improve accuracy by comparing the area under the receiver operator characteristic curve, which is a plot of the true positive rate *versus* the false positive rate (91.9 to 94.9, $p=0.007$) (33, 35). CA 125 has also been associated with poor prognosis in ovarian, non-small cell lung, and cervical cancers (36) and is a biomarker of lung invasion and survival for breast cancer (36, 37).

Lung and other cancers. Wen *et al.* combined 2D difference gel electrophoresis (2D-DIGE) and nano-LC-MS to test whether glycoproteins in the serum could predict metastasis based on the theory that glycosylation is important to oncogenic transformation and metastasis. The fucosylation index of E-cadherin may be a biomarker of metastatic lung adenocarcinoma (38). E-cadherin was also found to be an indicator of poor prognosis in prostate (39), breast (40), gastric (41), colorectal (42), and other cancers. An ELISA-based approach revealed that cytokeratin fragment (CYFRA) 21-1 is a biomarker of lung cancer. As the cancer progresses, CYFRA21-1 levels increase and high levels can indicate presence of distant metastasis (43, 44). CYFRA21-1, along with four other common pleural fluid biomarkers—carcinoembryonic antigen (CEA), cancer antigen (CA) 15-3, CA 19-9, and CA 125—is also increased in pleural fluid associated with malignant tumor (45).

Tissue biomarkers. Tissue is an excellent source for biomarker discovery to measure diagnostic, prognostic risks and therapeutic parameters. Development of protein biomarkers can be performed on both fresh-frozen and archival paraffin-embedded specimens (46). Traditionally, targeted proteins can be measured by IHC, however non-protein biomarkers can also be measured *via* DNA and RNA quantitative measures. Protein staining patterns can be important, including assessment of cytoplasmic *versus* nuclear staining and tumoral *versus* stromal staining. The use of semi-quantitative IHC of tumor tissues has been

particularly helpful for use in prognostic evaluation for early metastatic disease. Companion diagnostics, the use of biomarker measurement in tumor tissue to guide treatment choices for an individual patient, has become a highly useful tool in clinical oncology (47). For example, tumors that express HER2-neu may be susceptible to Herceptin or HER-2-neu vaccination, while tumors expressing epidermal growth factor receptor (EGFR) may benefit from erlotinib, or EGFR-targeted monoclonal antibodies, such as cetuximab or panitumumab.

Breast cancer. Cabioglu *et al.* examined preferential expression of CXCR4, CCR7, ER, PR and HER2-neu in metastases of breast cancer tumors to bone, brain, lung, liver and omentum using immunohistochemistry (IHC). Both CCR7 and CXCR4 were expressed more often in bone metastases than in visceral metastases (48). Also, CCR7 expression in primary tumors was found to predict lymph node metastases in small (T1) breast cancer tumors. Accuracy of the CCR7 biomarker was improved when it was combined with CXCR4 and HER2-neu. These biomarkers were assessed using IHC staining of tissue samples. For lymph node-positive tumors, there is increased rate of high cytoplasmic CCR7, HER2-neu and cytoplasmic CXCR4 staining; nuclear CXCR4 staining, however, is greater in lymph node-negative tumors (49). Liu *et al.* also found that cytoplasmic CXCR4 and CCR7 indicated an increased probability of lymph node metastases, but found no correlation with nuclear CXCR4. Additionally, they found that EGFR expression is associated with the presence of lymph node metastases and histological grade (50). High CXCR4 expression is potentially a biomarker of isolated tumor cells in the bone marrow, which is characteristic of a poor prognosis (51). Other lymph node metastasis biomarkers include positive IHC staining of TPM4, HSP60 and PDX6. Additionally, TPM4 staining was correlated to clinical stage (52).

Lung cancer. E-cadherin is essential to the intracellular junctions in epithelial tissues (40). Thus, decreased E-cadherin may allow for tumor cells to move and metastasize. Low IHC expression of E-cadherin was associated with metastasis to the brain in non-small cell lung cancer (NSCLC) (53). Integrin-linked kinase (ILK) could be responsible for the relationship between E-cadherin and metastasis in NSCLC. Studies have shown that ILK overexpression leads to the epithelial-mesenchymal transition (EMT), which down-regulates E-cadherin. Increased ILK protein expression in tumor tissues was determined to be an indicator of lymph node metastasis as well as being related to TNM (tumor-node-metastasis) stage (54).

Gastric cancer. Tumors cannot grow beyond 1-2 mm without vascularization (55). Thus tumor aggression in some cancers correlates with an increase in angiogenic cytokine expression

levels, like vascular endothelial growth factor (VEGF) (56). VEGF increases vascular permeability and acts to trigger mitosis in endothelial cells, making it a surrogate marker of angiogenesis (57). VEGF protein measurement in cancer tissue can be used for both prognostic and therapeutic purposes, as there are chemotherapeutic agents that can target VEGF. In addition, VEGF is an intravascular protein present in tumor blood supply that can be exploited for molecular imaging diagnostics.

IHC staining demonstrated that VEGF-C and VEGF-D proteins were more likely to be present in the gastric mucosa of gastric adenocarcinoma *versus* healthy tissue. Increased staining of primary tumors for VEGF-C and VEGF-D correlated with lymphatic metastases. Presence of VEGF-D and VEGFR-3 in primary tumors indicated decreased survival (58). VEGF-C, VEGF-D and VEGFR-3 have been studied as biomarkers of multiple other cancers (58, 59).

In addition to the gastric cancer IHC results, tumor *VEGF* mRNA expression levels have been shown to correlate inversely with stage of invasive cervical cancer (60). Although VEGF is released from tumors, it is also released from platelets during clotting. Therefore, use of serum levels of VEGF as an indicator of tumor levels of VEGF is controversial. Perhaps for this reason, researchers have found varied results for whether serum levels of VEGF are a prognostic indicator (61). Serum VEGF was not found to correlate independently with prognosis in renal cell carcinoma (62) and cervical cancer (57). However, Poon *et al*. quantified serum VEGF (by ELISA), tumor VEGF (by ELISA), and tumor mRNA (by RT-PCR) for patients with hepatocellular carcinoma and concluded that serum VEGF level does reflect tumor VEGF expression (61). Similarly, Mitsuhashi *et al*. found that serum levels of VEGF-C (by ELISA) significantly correlate with recurrence of cervical squamous cell carcinoma (63). However, VEGF-C was not associated with lymph node metastases. Interestingly, VEGF-C levels were elevated in patients with cervical squamous cell carcinoma *versus* healthy controls, but this correlation did not hold true for cervical adenocarcinoma, indicating the feasibility of identifying disease-specific biomarkers and perhaps shedding light on the varied results of validation of VEGF as a prognostic marker (63). *VEGF* mRNA expression (by quantitative RT-PCR) was found to be decreased in Papanicolaou (Pap) smears of women with cervical cancer (64). Thus, VEGF could potentially be a biomarker assessed using Pap smears.

Pancreatic cancer. Pancreatic cancer is highly lethal and some headway has been made in the identification of prognostic and therapeutic biomarkers. Winter *et al*. found that Muc-1 and MSLN protein expression in tumor tissue can predict poor prognosis of pancreatic adenocarcinoma (PDAC) with a hazard ratio of 29 (*p*=0.004) and 12 (*p*=0.01),

respectively (65). Such patients may benefit from aggressive, personalized, or alternative therapies. Other biomarkers that could help direct management include hENT1, RRM1, and ERCC1, which can help predict response to standard-of-care chemotherapeutic, gemcitabine (66). Cellular histone levels, as measured by IHC staining of H3K4me2, H3K9me2, or H2K18ac, were predictive of survival in patients with node-negative pancreatic cancer as well as in patients receiving adjuvant chemotherapy with 5-FU, but not gemcitabine (67). In a proteomic study of long-term PDAC survivors (more than 10 years) *versus* short-term survivors (less than 1 year), Chen *et al*. discovered and validated the prognostic value of galectin-1 staining in the stroma of pancreatic cancer tissue. Low galectin-1 expressors predicted long-term survival with a hazard ratio of 4.9 ($p=0.004$) (68). Recent mouse model mutagenesis studies discovered that deubiquitinase USP9X plays a role in pancreatic cancer. USP9X appears to repress neoplastic transformation and facilitates pancreatic cancer cells to enter programmed cell death. Low expression of USP9X protein and mRNA in human pancreatic cancer tissue correlates with shortened survival after surgery and increased metastatic burden (69).

Ovarian cancer. IHC staining displayed localized expression of VCAM-1 protein in limited patches of mesothelial cells of biopsies from ovarian tumors lacking peritoneal metastasis, while VCAM-1 expression spread to the entire mesothelial cell layer for advanced tumors with peritoneal metastasis. Peritoneal washings had also less VCAM-1 staining in the absence of peritoneal metastasis *versus* presence of metastasis (70). Expression of another ovarian protein biomarker, anterior gradient-3 (AGR3), in a tumor sample was able to differentiate between different subtypes of ovarian carcinoma (serous papillary, endometrioid, mucinous and clear cell). Because the subtypes differ in behavior, AGR3 could be a biomarker of aggression and therapeutic response (71). Despite the correlation found between AGR3 and estrogen-receptor (ER) expression in breast cancer (72), Gray *et al*. found no association between AGR3 and ER (71). AGR3 staining was found to be related to AGR2 staining, a known biomarker for early detection of ovarian cancer (73). King *et al*. also found that AGR3 expression in serous ovarian carcinoma tissue indicates improved prognosis and increased survival, using IHC, immunofluorescence and western blotting in addition to RNA-based techniques (74).

Other Fluid Biomarkers

Although blood is a convenient source for biomarkers and likely to contain metastatic biomarkers because of the involvement of the circulatory system in metastasis, blood is not the only potential source of biomarkers. Creativity in selection of potentially informative biological samples has led

to biomarker discovery in nipple aspirate fluid (75), expired air (76), Pap smear (77), cerebrospinal fluid (78), saliva (79), peritoneal washing fluid (70), tissue samples (51), and pancreatic cyst fluid (80). Use of non-blood biological fluids varies in level of invasiveness and has the potential to yield high therapeutic advantage. Proteomic methods for discovery of biomarkers in other biological fluids are similar to those of serum biomarkers, but many require different purification techniques. Additionally, metastatic biomarkers measured by IHC can also be useful in cytological analysis of biopsies.

Imaging

Imaging biomarkers has advantage over other forms of biomarker detection in that they can identify location of the metastasis in a non-invasive setting allowing (1) detection, and (2) monitoring of response to therapy. The plasma membrane represents a barrier to most agents except small molecules and, therefore, biomarkers for most targeted imaging agents are typically present on the cell surface of the tumor or the tumor microenvironment. Traditional proteomic methodologies do not distinguish cellular location of protein and select against hydrophobic integral membrane proteins. Hoffman *et al*. approached this problem with a series of experiments: continuous free-flow gel electrophoresis, liquid-based isoelectrofocusing, Sodium Dodecyl Sulfate PAGE, and HPLC-MS (81). While this addresses selection against hydrophobic membrane proteins, it does not ensure that proteins will be visible to a circulating imaging agent. McKinney *et al*. added an initial subcellular fractionation step in order to determine the cellular location of each biomarker discovered (82). Phage display-based functional proteomics has the advantage of not only identifying biomarkers that are on the surface of the cell, but also identifying a peptide sequence that interacts with that biomarker. The interacting peptide can be used to develop imaging agents that target the biomarker of metastasis (83).

Endometrial cancer. Epithelial membrane protein-2 (EMP2) expression, as stained by IHC, in endometrial adenocarcinoma indicates poor prognosis and a higher probability of recurrence (84). Fu *et al*. developed a PET imaging agent by conjugating anti-EMP2 antibody fragments to dodecaneN, N',N'',N'''-tetraacetic acid (DOTA) and radiolabeling it with ^{64}Cu (85). This imaging agent is able indicate whether a tumor is EMP2-positive or -negative to assess prognosis. It can also clearly image tumor boundaries and potentially monitor tumor response to treatment. Alternatively, Shimazaki *et al*. used phage display to make a targeted agent to EMP2 and tested the therapeutic potential of these anti-EMP2 diabodies (antibody fragments) on endometrial cells. Cellular growth was decreased with anti-EMP2 diabody-treatment and attributed to apoptosis, using flow cytometry. The apoptotic effect is increased with the

presence of progesterone, which is known to increase EMP2 expression. Anti-EMP2 diabodies did not cause any toxicity in skin or lung, which also express EMP2, in *in vivo* imaging studies. Tumors excised after 30 days of growth showed a four-fold decrease in tumor size between anti-EMP2 diabody-treated mice and control diabody-treated mice (86).

Pancreatic cancer. In vivo imaging of cancer to predict metastatic state could improve the standard-of-care for patients because presence of metastases is often indeterminable preoperatively (87). Plectin was discovered as a biomarker for pancreatic cancer using phage display-based functional proteomic approaches (88). Non-invasive, *in vivo* imaging using targeted SPECT/CT compatible imaging agents demonstrated that plectin imaging was successfully used to image not only the primary tumor, but also peritoneal and liver metastases. In addition to imaging, plectin was assessed with IHC experiments and shown to be up-regulated in advanced precursor lesions of PDAC, pancreatic intraepithelial neoplasms 3 (PanIN 3 or ductal carcinoma *in situ*) as well as in primary and metastatic lesions to the lymph nodes and liver. Although the majority of pancreatic cancer tumors are PDAC, the detection of intraductal papillary mucinous neoplasms (IPMN) has increased with pervasive use of high-resolution CT scanning in medicine. These IMPN lesions can progress to cancer, thus distinguishing between benign and malignant lesions becomes of paramount importance. Recently, plectin presence was shown to indicate malignancy in both cyst fluid and IPMN tissues. The presence of plectin protein in tissue, as measured by IHC, could distinguish malignant *versus* benign IPMN with an overall sensitivity of 84% and specificity of 83%. When cyst fluid was examined, however, pilot studies suggested that all cases of plectin positive (4/4) cyst fluids were malignant while all cases of fluids that were plectin-negative (3/3) were benign (80).

Another example of a PDAC biomarker is integrin $\alpha_v\beta_6$. IHC has shown that $\alpha_v\beta_6$ is strongly up-regulated in PDAC, intestinal-type gastric carcinoma and lung adenocarcinoma, moderately up-regulated in diffuse-type gastric carcinoma, duodenal adenocarcinoma, colorectal adenocarcinoma, and slightly up-regulated in liver cell carcinoma and neuroendocrine tumors (89). However, $\alpha_v\beta_6$ is in low abundance or absent in adult tissues. $\alpha_v\beta_6$ has a role in metastasis (90) and is also a biomarker of poor survival in colon carcinoma (91), cervical squamous cell carcinoma (92) and other cancers. Hausner *et al*. created a PET imaging agent 4-[^{18}F]Fluorobenzoyl A20FMDV2 (93) specific to $\alpha_v\beta_6$ and then improved it by adding PEG: [^{18}F]FBA-PEG$_{28}$-A20FMDV2. The $\alpha_v\beta_6$ targeted radiotracer was tested in a subcutaneous xenograft mouse model of pancreatic cancer and demonstrated a four-fold increase in tumor accumulation when compared with normal pancreas (87).
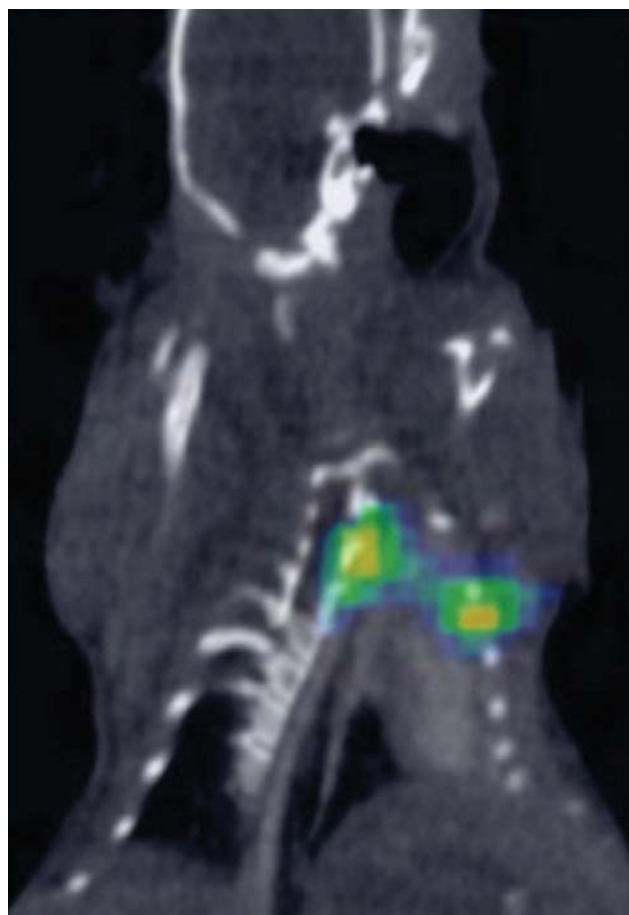

Figure 1. *FMT-CT Fusion. A composite of the CT and FMT images of the lungs shows that SPARC-targeted nanoparticle is able to detect prostate cancer metastasis (97).*

Molecular imaging using intravascular biomarker targets of neoangiogenesis has been developed and represents a promising, inexpensive method that could facilitate earlier detection of cancer and metastasis along with better assessment of therapeutic response (94). Using tiny gas-filled bubbles (microbubbles) bound to neovascular biomarkers VEGFR2 ligand and/or integrin $\alpha_v\beta_6$ ligand, it is possible to detect very early-PDAC and ovarian cancer in mouse models (95). Studies in human phase III trials are soon to follow.

Prostate cancer. Secreted protein and acidic and rich in cysteine (SPARC) is a biomarker correlated with advancing stage in various cancers, including pancreatic, colon, lung, prostate, and ovarian. Increased SPARC expression influences extracellular matrix interactions, possibly promoting metastasis (96) and also promotes key steps that allow tumor cells to gain metastatic potential like destruction of the extracellular matrix and focal adhesions. SPARC expression increases in prostate tumors as the cancer advances and is highest at the metastatic phase (97). SPARC-targeted

nanoparticle (SPARC-NP-680) is a molecular imaging agent developed to monitor SPARC levels in prostate cancer to indicate metastatic potential. SPARC-NP-680 was also able to image bone and lung metastases of xenografted prostate tumors in mice (Figure 1) (97).

Conclusion

Clinically relevant and accurate biomarkers present a minimally invasive clinical tests that physicians can use to personalize therapeutic strategies for cancer patients. By assessing the metastatic potential with biomarkers, physicians can eliminate over-treatment of localized tumors and prevent under-treatment of aggressive tumors. Biomarkers can also be used to monitor recurrence and potentially predict sites of tumor metastasis. Biomarkers are frequently found in biological fluids, but can also be detected in tumor cancer cells or in the tumor microenvironment. Imaging biomarkers can preoperatively stage disease as well as identify the metastatic potential of a primary tumor. Biomarker discovery techniques like phage display allow non-biased discovery of biomarkers and provide a way to target biomarkers for use with imaging agents. Despite the advantages of clinically relevant biomarkers for future application, two important barriers to its practical use remain: (1) better bioinformatics to process the vast amount of data generated in high-throughput screens and (2) more appropriately screened candidates need to progress to clinical trials. Large strides have been made in proteomic screening techniques, creating a demand for faster, more efficient bioinformatics. Especially important is the elimination of false-positives, which are more likely to arise in such large screens. Additionally, new methods have been developed to lessen the bias inherent in certain proteomic methods, for example selection against hydrophobic proteins or low abundance proteins. As methods become less biased and analysis of data improves, more potential biomarkers will fail in early validation steps. Because it is too expensive and time-consuming to put all potential biomarkers through extensive validation, emphasis should be placed on developing techniques for eliminating biomarkers that are unlikely to succeed as well as highlighting biomarkers that are likely to work.

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