Application of Proteomics in the Study of Tumor Metastasis

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Tumor metastasis is the dominant cause of death in cancer patients. However, the molecular and cellular mechanisms underlying tumor metastasis are still elusive. The identification of protein molecules with their expressions correlated to the metastatic process would help to understand the metastatic mechanisms and thus facilitate the development of strategies for the therapeutic interventions and clinical management of cancer. Proteomics is a systematic research approach aiming to provide the global characterization of protein expression and function under given conditions. Proteomic technology has been widely used in biomarker discovery and pathogenetic studies including tumor metastasis. This article provides a brief review of the application of proteomics in identifying molecular factors in tumor metastasis process. The combination of proteomics with other experimental approaches in biochemistry, cell biology, molecular genetics and chemistry, together with the development of new technologies and improvements in existing methodologies will continue to extend its application in studying cancer metastasis.

Key words: proteomics, metastasis, tumor mechanism, protein identification, MALDI-TOF, SELDI-ProteinChip

Tumor Metastasis

Although diagnostic technology and therapeutic treatment have made vast progress during the last decades, cancer mortality remains high, accounting for approximately 25% of the deaths in the developed world (*1*). Metastasis is by far the leading cause of death in cancer patients, responsible for more than 90% of all cancer mortality (*2*). Tumor metastasis is the spread of cancer cells from the original site to other parts of the body. It is a very complex and multi-step process and often referred to as a cascade. The process of metastasis formation begins with some tumor cells breaking adhesions with neighboring cells and detaching from the primary tumor. Those cells then dissolve the extracellular matrix, migrate and invade surrounding tissues, and/or travel via the circulatory system, invade, survive and proliferate at distant new sites (*3*).

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Questions to be answered in metastasis

Histological evidence suggests that metastatic tumor is made up of the same type of cells as the primary tumor. It is obvious that those successful metastatic cells must have a set of particular characteristics that are different from non-metastatic tumor cells, enabling them to complete each step in the metastatic sequence. Perhaps it is these particular characteristics that make metastatic cancer more resistant to treatment than primary tumor even using the most aggressive chemotherapy or radiotherapy (*4*). However, how do metastases arise from primary tumors? What are the underlying molecular and cellular mechanisms in cancer progression? What characteristics does the metastatic tumor possess? Answers to these questions are clearly of considerable importance not only for the understanding of the tumor behavior but also for the clinical treatment of cancer. Aiming at those questions, researchers have been looking for events and factors that may influence tumor dissemination and hoping to provide useful clues for developing more effective approaches to counter the disseminated cancer.

Molecular factors involved in metastatic process

Many molecular factors have been reported to be related to the formation of detectable metastases. Cell adhesion molecules, members of the cadherin families, integrins, and several other cell adhesion molecules including CD44 and the 67–69 kDa laminin-elastin binding protein, have been identified as modulators of metastatic growth (*3*). The proteolytic enzymes including metalloproteinases, uPA/uPAR system as well as the cysteine proteases appear to be crucial for the development of distant organ metastasis (*3*). The emerging role of angiogenesis and lymphangiogenesis in invasion and metastasis has also been recognized to be important for tumor cell dissemination (*5*).

In addition, a number of genes have been found involved in each stage of the metastatic cascade. Oncogenes are a group of factors that have been implicated in the metastatic process (*6*). For example, activating mutations in the Ras and the activation of the Raf-mitogen activated protein kinase 1/2 pathway can result in a metastatic phenotype in a variety of cell types (*7*, *8*). The ectopic expression of other oncogenes including the MET, serine/threonine kinases Mos and Raf, tyrosine kinases Src, Fms and Fes also induces the metastatic phenotype in recipient cells (*9*, *10*).

In contrast, some genes have been described as suppressors of metastasis, bearing abilities to inhibit metastatic cascade at different stages. Most of these genes consistently suppress metastasis without affecting proliferation *in vitro* or primary tumor size *in vivo* (*11*). Among these genes Nm23 is the most interesting one. Steeg compared the expression level of the genes in seven cell lines derived from the same parental melanoma cell line K-1735 with widely varied metastatic potentials (*12*). The expression of Nm23 cDNA was found quantitatively reduced in five highly metastatic cell lines as compared to two related less metastatic cell lines. So far, twelve metastasis-suppressor genes have been described (*12*). In a recent *Cell* paper, Yang and colleagues showed that TWIST—a master gene that controls epithelial–mesenchymal transition in embryogenesis—was required for metastasis in epithelial-derived breast tumors (*13*). TWIST is a gene regulator, instructing genes when to turn on and turn off. Researchers compared the levels of TWIST expression in metastatic and non-metastatic human breast cancer cell lines and showed that only metastatic lines expressed TWIST. As pointed out by the authors, "TWIST is probably the first gene regulator that has been tied so definitively to human cancer metastasis" (*13*). By looking at TWIST gene's on or off, the potential of tumor invasion and metastasis can be estimated.

Up to date, most metastasis studies have been focusing on single genes, or at most, small groups of genes in an experimental model. For instance, researches using genetically engineered mice usually involve the over-expression or mutation of a single gene. Although vast amounts of information have been gleaned from these studies, they rarely accurately represent the complex milieu of genetic interactions in cancer metastasis in a global sense. In addition, it is widely accepted that the invasion and metastasis formation not only depend on the metastatic capacity of tumor cells but also tightly correlate with surrounding stroma and infiltrating tissues. The recently emerging systematic technologies enable us to investigate thousands of RNA expression levels simultaneously and to identify patterns associated with the biological characteristics of metastasis.

Studies of metastasis by DNA microarray

Several recent analyses of human tumor metastasis using DNA microarrays have obtained exciting information that strongly challenge classic metastasis hypothesis. Studies have shown that a gene set was highly correlated with patient's outcome, and primary tumors can be classified into those with "good" or "poor" prognoses based on their patterns of gene expression (*14*, *15*). Van't Veer *et al* used DNA microarray to analyze 117 young patients with primary breast tumor and identified a set of 70 genes strongly predicting metastatic potential, which are called "poor prognosis signature" (*14*). More recently, a small set of 17 genes was reported to predict metastatic potential for a variety of solid tumors (*16*). These results are in conflict with generally accepted progression model that predicts a series of random mutations within a primary tumor to generate a small subpopulation that acquires full metastatic capability. However, both the conflicting hypotheses can have their supporting and opponent evidence (*11*, *17*, *18*). A research article published in *Cancer Cell* may help to reconcile these two models: evidence showed that highly metastatic variants do exist in the bulk tumor population and these highly metastatic variants do have a "metastatic signature" (*19*). Based on these results, the metastasis paradox may be resolved by

combining the two hypotheses: metastatic potential is determined early in oncogenesis but primarily by host genetic background, on which specific mutations occur to promote metastasis (*20*).

The cDNA microarray analysis has been fruitful in identifying genes that are altered in expression (*6*) and a set of genes that may be the "metastatic signature" (*14*, *16*) in some kinds of tumors. However, it is clear that the functional endpoint of the genetic blueprint lies at the protein level, in the forms of signaling networks and protein interaction maps that make up the circuitry within the cell since cell function is directly regulated through proteins but not through genes or messenger RNA (*21*). Cellular behaviors such as normal growth and differentiation are influenced by a large number of protein molecules and depend on the presence of appropriate proteins in the right context. Through signal transduction cascades and transcriptional networks, alterations of proteins can affect a large number of cellular pathways and result in global effects on cell behaviors. Regulation of translation and post-translational modification of proteins such as proteolytic processing, phosphorlyation, or glycosylation clearly play significant roles in determining cellular functions. Therefore, gene findings in cancer progression and invasion still need to be validated at protein levels. Furthermore, proteins are primary targets and therapeutic molecules in pharmaceuticals. Thus, it is of vital importance to globally identify changes in protein expression in disease states including metastasis.

Proteomics

Traditionally, analysis of the proteins coded by genes was performed on a single protein at a time. The technologies to identify and quantitate proteins on a global scale are not as robust as those available for genomics. As biology enters the post-genomic era, the near completion of human genome sequencing coupled with the technical progress made in protein identification has created new opportunities for proteomic analysis, characterizing and investigating proteins in a systematic manner.

Principle of proteomics

The term "proteome" was coined in 1994 and defined as the entire protein complement expressed by a cell line, tissue, or organism. Proteomics is the study of the proteome and a natural successor to genomics (*22*). Collectively, proteomics can be defined as the study of all proteins, including their relative abundance, distribution, post-translational modifications, functions, and interactions with other macromolecules, in a given cell or organism within a given environment and at a specific stage in the cell cycle. Proteomics can be classified into several different sub-disciplines. Recent studies on tumor metastasis mainly concentrated on "profiling/expression" and "functional" proteomics (*23*). Profiling or expression proteomics focuses on the description of the whole proteome in a given tissue, body fluid or cell, including organelle mapping and differential measurement of protein expression levels in cells and/or under different conditions. In a review by MacBeath *et al* (*24*), profiling proteomics was descried as "unbiased" or "discovery-oriented proteomics" because investigators cannot impose their knowledge of biology on the experimental design and both known and unknown proteins may be identified in such an experiment. Functional proteomics can be thought as "focused" or "system-oriented proteomics" (*24*). It is a research approach that directly analyzes a subset of proteins, such as a family of sequence- or function-related proteins, and characterizes the protein biological functions, protein-protein or protein-DNA/RNA interactions, and protein post-translational modifications.

Methodology of proteomics

A number of complimentary technologies have been developed to analyze proteins on a global scale. Currently, the most commonly used proteomic platforms include protein separation coupled with mass spectrometry (MS) and protein microarray methods (*23*, *25*).

MS analysis in proteomics

MS is an analytical technique that can be used to identify unknown compounds. In the past ten years, this technology matured rapidly due to the invention of two ionization techniques—electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) that enable proteins and peptides to be ionized at high sensitivity without excessive fragmentation. This invention led John Fenn and Koichi Tanaka to be awarded the 2002 Nobel Prize in Chemistry (*26*). Sample ionization by a laser and detection as

ion mass to charge (m/z) ratio with a mass analyzer are two basic steps in MS. MALDI ion sources are most commonly coupled with time of flight (TOF) mass analyzer, whereas ESI is most often coupled with ion-trap or triple-quadrupole MS/MS spectrometer.

In MALDI-TOF MS, peptides derived from proteolytic digested proteins are ionized from a plate into the spectrometer, and the m/z ratios of peptides are measured based on the length of time for the peptides to move in a vacuum tube to reach a detector, and then a list of mass spectra are produced. By applying database search algorithms, MS spectra are then matched to calculate masses in a sequence database, resulting in identification of target proteins. This type of protein identification method is known as peptide mass fingerprinting (PMF; ref. *27*). MALDI-TOF and PMF are usually coupled with two-dimensional gel electrophoresis (2DE) techniques in proteomics.

Tandem mass spectrometry (MS/MS) can be thought as a two-stage MS experiment. At the first stage, peptides are ionized and separated by m/z. A peptide of interest is selected and allowed to pass into a collision cell for further fragmentation. Resulting sequential m/z values are determined in a second MS analyzer to generate a MS/MS spectrum, representing a series of ion fragments of the specific peptide (*28*). A partial amino acid sequence is then constructed according to the MS/MS spectrum. As compared with PMF, the specificity of MS/MS-based protein identification is often higher because it provides information not only about peptide masses but also peptide sequences.

Protein separation

Protein separation methods can be classified as gel-based and non-gel based approaches. 2DE is a classical protein separation technology. In fact, for many years it has been considered synonymous with "proteomics" because of its ability to separate thousands of proteins at a time. In 2DE, proteins extracted from cells or tissues are separated according to their isoelectric points (pIs) in the first dimension and their molecular weights in the second dimension (*28*). After visualizing by Coomassie blue or silver staining or other fluorescent dye, each observed protein spot is quantified by its staining intensity. It has been reported that up to 2,000 spots can be detected in a single gel with advanced visualizing methods. Besides its high resolution, 2DE can resolve post-transcriptionally or proteolytically modified proteins from their "parent" molecules. It has been demonstrated that in a 2D-gel up to a quarter of the spots visualized are modified proteins (*29*, *30*). The ability of 2DE to directly determine abundance of proteins in analyzed samples enables 2DE to become a major tool in expression proteomics. Actually, since 2DE was first described in 1969 and refined by Klose and O'Farrell (*28*), its application in protein separation and comparative analysis has occurred long before the global differential analysis of mRNA, and many of the 2DE principles are commonly used in the systematic quantitative analysis of gene expression patterns (*27*). However, it was not until the technological advances in MS and bioinformatics for protein identification that 2DE became a major technique in proteomics.

Liquid chromatography (LC) is another usually used protein separation approach. It has been shown that this method can successfully resolve the mixture of proteins and allow the isolation of individual proteins based on a particular biochemical property. Some commonly used LC columns include size exclusion LC, ion exchange LC, and reverse-phase LC. For more complete analysis or analysis of very complex protein mixtures, 2D chromatography strategies can be employed (*31*). In the first dimension, proteins are separated by size-exclusion or cation exchange chromatography and in the second dimension by reverse-phase HPLC. Followed by MS measurement and database searching, the fractionated proteins can be directly analyzed. This is often referred as "shotgun" proteomics strategy (*32*). The "shotgun" method shows advantages over gel-based techniques in speed, sensitivity, scope of analysis and dynamic range and it could be amenable to automation. Unfortunately, LC/MS is not capable of determining protein abundance. The emergency of isotope-coded affinity tagging approach (ICAT) adds a quantitative dimension to MS/MS (*27*). The cysteine residues in a pair of samples are labeled with d0- and d8-ICAT reagents respectively. The samples are then combined and analyzed by LC-MS/MS. Each cysteinyl peptide appears as a pair of signals differing by the mass differentially encoded in the mass tag. The ratio of these signal intensities precisely indicates the ratio of abundance of the protein from which the peptide originates and the MS/MS spectrum of the peptide allows the protein to be identified.

Protein microarray

Protein microarray can be considered to be analogous

to DNA microarrays. Affinity reagents such as antibodies, nucleic acids and lipids are arrayed at high spatial density on a solid support and then probed with compounds of interest (*24*). Currently there are two types of protein microarrays: abundance-based microarray and function-based microarray (*33*), enabling researchers to interrogate simultaneously the abundance and function of many different proteins with minimal sample consumption (*34*). Antibody microarrays have been used to screen for potential biomarkers in cancer tissues (*35*) and human blood serum (*36*) in parallel because of their relatively high specificity and extreme sensitivity. Depending on the individual affinities of the immobilized antibodies, antigens can be detected in picomolar (pM) concentrations. In reverse phase protein microarray, sample proteins or peptides are immobilized in a miniaturized format and are used for probing with known binding molecules. This method allows the parallel analysis of multiple biological samples and thus is best suited to clinical practice. Protein microarray also provides a well-controlled *in vitro* way to study protein function, including protein-protein, protein-lipid and protein-nucleic acid interactions on a genome-wide basis (*37–40*). This approach has demonstrated a huge application potential in "focused proteomics". However, to obtain highly specific and stable capture molecules is a major challenge in the array-based proteomics. For example, considering the specificity, affinity and cross-reactivity, only about 5% (*24*)–30% (*41*) of commercial antibodies are suitable for microarray-based analyses. So far, the most successful protein microarray assays are those directed towards cytokines because there are many antibodies to cytokines for choices (*42*).

A special case of the protein array is the surface enhanced laser desorption ionization (SELDI)-ProteinChip. SELDI-ProteinChip system is an evolving proteomics platform that allows rapid and sensitive analysis of complex protein mixtures stemmed from body fluids, cells, and/or tissues at the femtomole level (*43*). The chip surfaces are chemically or biochemically modified to be able to capture a certain group of proteins based on their specific physical and chemical properties. The surface properties of the protein chips include weak cation exchange, strong anion exchange, immobilized metal affinity chromatography, reverse phase and normal phase, as well as biochemical affinities allowing proteins or antibodies to be bound directly to the chip (*28*). Samples such as serum or protein extracts are applied to the surface, incubated, and washed to remove non-bound proteins and contaminants. The retained proteins are then analyzed in a mass spectrometry to generate proteomic profiles. The great advantages of this approach are its sensitivity to analyze small amounts of raw protein samples and its ability to detect proteins with molecular weights lower than 6 kDa. These characteristics make SELDI-ProteinChip technology very suitable for biomarker discovery. However, SELDI is not a quantitative approach and protein identification cannot be directly determined through this method (*44*). Nevertheless, protein microarrays and chips probably constitute the most promising technologies for the future development of cancer proteomics.

Application of Proteomics in Studying Tumor Metastasis

There are two main expected outcomes from proteomic analysis of tumor metastasis. The first is to discover new molecular markers from the profiling of metastatic tumors. The second is to decipher the intracellular signaling pathways that lead cancer cells to be metastatic. Expression and functional proteomics are respectively suited for the purposes of the metastasis studies. The resulting data would provide knowledge bases for the early detection and prediction of metastasis and for the identification of novel targets for drug development and therapeutic intervention.

Expression proteomics

Considering that cell behavior is ultimately reflected by actual protein contents within cells, protein expression patterns of certain tissues or cells in a given disease state will have complicated changes that should contain important information about the pathologic process taking place in the cells. The presence and/or the amount alteration of a particular protein may not only serve as a potential biomarker but also provide insights into the basic etiology of disease. Most of the proteomic analyses for tumor metastasis use expression proteomics (Table 1). 2DE followed by protein identification using MS and PMF is the most mature platform for large-scale analysis of proteins directly from biological samples (*45*). By comparing protein expression profiles of clinical specimens obtained from primary and metastatic tumors or cell lines originated from the same parental cell line but with different metastatic potency, researchers may discover and

Table 1 Summary of Representative Proteomic Studies in Cancer Metastasis

	Tumor	Samples	Methods	Ref.
Expression proteomics	Breast cancer	Tumor interstitial fluid;	2DE, MS	*92*
		Highly metastatic MDA-MB-435 cells and metastasis-suppressed BRMS1-transfected MDA-MB-435 cells;	2DE, MS	*72*
		A pair of monoclonal cell lines from the human breast carcinoma cell line MDA-MB-435 that have different metastatic phenotypes;	LC/LC-MS	*67*
		Plasma membrane proteome of the metastatic MDA-MB-435 cells.	2DE, MS	*93*
	Colorectal carcinomas	Primary and metastatic tumor tissues;	2DE, MS	*46*
		Laser-microdissected cells from normal and malignant colonic epithelium and stroma;	LCM, SELDI	*61*
		Normal tissue, adenoma, carcinoma and metastasis.	2DE, MS	*50*
	prostate cancer	Androgen-stimulated prostate cancer cells;	2DE, MS	*94*
		Dunning prostate cancer cell lines with variable metastatic potential;	SELDI	*95*
		Conditioned medium of PC3 cells.	2DE, MS	*96*
	Gastric cancer	Gastrointestinal stromal tumor tissue;	2DE, MS	*51*
		Normal, cancer and metastases tissues.	2DE, MS	*49*
	Lung cancer	Highly and poorly metastatic sublines (PLA801D and PLA801C);	2DE, MS	*48*
		Bronchial epithelial immortalized cells and malignant transformation cells.	2DE, MS	*97*
	Head and neck tumor	UMSCC10A and UMSCC10B cell lines derived from primary tumor and a metastatic lymph node of the same patient respectively;	2DE, MS, SELDI	*55*
		Laser-microdissected cryostat sections from tumors and adjacent mucosa.	LCM, SELDI, 2DE	*60*
	human salivary gland adenoid cystic carcinoma	Poorly metastatic Acc-2 cell line and highly metastatic Acc-M cell line.	2DE, MS	*52*
	Hepatocellular carcinoma	Hepatocellular carcinoma cell lines Hep3B, MHCC97L, MHCC97H with different metastasic potential.	2DE, LC-ESI-MS/MS	*47*
Functional proteomics	Colon cancer	Antisense uPAR cDNA transfected and mock-transfected HCT116 cells.	2DE, MS	*68*
	Breast cancer	Adriamycin resistant cell line MCF-7/ADR and metastatic cell line MDA-MB;	2DE, MS	*53*
		Mammey cells transfected and/or stimulated by growth factors.	2DE, MS	*56*
	Ovarian cancer	Laser-microdissected cells from ovarian tumor tissues.	LCM, reverse-phase protein array technology	*76*
	Lymphosarcoma	Canine lymphosarcoma tissue.	RPLC, MS	*98*
	Enzyme activity	A panel of Breast cancer cell lines and melanoma cancer cell lines.	Activity-based protein profiling	*37*
	Fibrosarcoma	HT-1080 fibrosarcoma cells.	Functional proteomic screens	*99*

identify differentially expressed proteins that may be associated with cancer progression and metastasis formation.

Colon cancer is one of the highly metastatic tumors. To find out proteins over-expressed in metastatic colonic adenocarcinomas, Tachibana *et al* compared the protein profiles of primary and metastatic tumors by 2DE and found a number of protein spots that appeared in metastatic tumor in liver but not in primary tumor (*46*). One of these spots was identified by MS as apolipoprotein A1, suggesting that the expression of apolipoprotein A1 may be associated with colonic adenocarcinoma progression and the protein may be a potential marker for the aggression of the cancer. Cui and colleagues performed the comparative study to differentiate the protein expression profiles of several hepatocellular carcinoma cell lines with various metastatic potentials and found that there were 16 proteins over-expressed and 10 proteins under-expressed in metastatic cell lines (*47*). Two proteins, annexin-1 and S100A4, were believed to be the key molecules related to hepatocellular carcinoma metastasis and recurrence. Other studies also showed that proteins including annexin-1 were significantly up-regulated in a highly metastatic lung cancer cell line (PLA810D) compared to the poorly metastatic cell line (PLA810C) (*48*). These results suggested that annexin-1 may play a role in tumor metastasis. Similar comparative studies were also carried out with other kinds of tumor tissues (*49–51*) and cancer cell lines (*52–55*). These proteomic data are valuable and informative for understanding tumor metastasis.

However, due to the limitation of 2DE technology, it is impossible to survey all of the changes in protein expression for a given tissue in a certain disease state. This is because a typical 2DE gel can only separate and detect about 1,000–2,000 proteins, much less than the number of proteins expressed in cells (*56*). Some proteins, such as high hydrophobic membrane proteins, low soluble proteins, and proteins with extreme molecular sizes and *p*Is, may not be detectable by 2DE. Low abundance proteins are lost in 2DE analysis. A study by Gygi *et al* indicated that up to 75% of cell proteins were below the protein detection limit of 2DE (*57*). In addition, there are problems associated with reproducibility and visualization methods (*58*). Refinements in the technique are currently being developed to address each of these problems. For example, the use of narrow-range immobilized pH gradient strips and pre-fractionation can greatly improve the 2DE resolution (*58*). Removal of high abundance proteins before 2DE can increase the chances of identification of low-abundance proteins. For those highly complex and heterogeneous tissue samples, laser capture microdissection (LCM) can be used for the procuring and enriching subpopulations of cells prior to sample preparation for 2DE proteomic analysis (*44*). The development of fluorescent dye labels allowing proteomic comparison of two samples on a single gel, referred as difference gel electrophoresis (DIGE), not only improves the detection sensitivity but provides more accurate quantification and spot statistics (*59*).

As a complementary approach, SELDI-ProteinChip technology has also been utilized for protein profiling in studying tumor metastasis. In comparison, SELDI-ProteinChip has a number of advantages over 2DE, including less amount of sample requirement, better reproducibility, improved identification for proteins at extremes of *p*Is, and greater sensitivity and accuracy for detecting low-molecular-weight proteins. By combining SELDI-ProteinChip technology with LCM and immunohistochemistry, Melle and co-workers have successfully identified and characterized head and neck tumor-related biomarkers (*60*). They firstly detected differentially expressed proteins in head and neck tumors using ProteinChip technology, subsequently enriched and identified the proteins of interest by 2DE and MS, and finally found that proteins S100A8 (calgranulin A) and S100A9 (calgranulin B) may be responsible for invasion and metastasis. Krieg *et al* also used SELDI-TOF-MS to analyze the protein profiles of colonic epithelium and stroma cells and found that three peptides were increased in the colon tumor epithelium and stroma compared to normal colon and stroma in nine patients (*61*). The study demonstrated that 500–1,000 cells were enough to do the comparative protein profiling by SELDI-ProteinChip technology. Serum proteomics analysis has recently been shown to be an effective means to detect early or recurrent malignancy (*62–64*). Hingorani *et al* analyzed 46 PanIN (putative precursor to invasive pancreatic cancer) and 51 control serum samples using SELDI-TOF and obtained a parameter model comprised of 10 specific molecular ions that was able to accurately distinguish PanIN from control samples. Tests on a separate blinded experiment demonstrated that the sensitivity and specificity of this model was 90% and 87% respectively, higher than 67% and 88% of CA19-9, the most commonly used biomarker of invasive pancreatic can-

cer (*65*).

Other strategies were also used in studying tumor metastasis. Wu's research group provided a model system for proteomic studies on selected tissue samples and specific cell types by combining the LCM and reversed phase HPLC (*66*). A number of proteins were identified through this model by using approximately 10,000 breast cancer cells, raising the possibility of such studies on needle biopsy samples. By using the mass-mapping technique, Kreunin *et al* identified some metastasis-associated proteins including osteopontin, annexin I and extracellular matrix protein 1 in a tumor metastasis model by profiling and comparing the component proteome of conditioned media collected from a pair of monoclonal cell lines derived from human breast carcinoma cell line MDA-MB-435 with different metastasis potentials (*67*).

Functional proteomics

Deciphering the signaling pathways involved in tumor growth and metastasis is another major application of cancer proteomics. These types of data should greatly improve our understanding of the biological process of tumor development and progression, thus fostering the identification of not only single protein-specific therapeutic targets but also pathway-specific targets. Classical methods to study signaling pathways were based on the use of specific antibodies to purify known signaling proteins followed by SDS-PAGE separation. Such studies can now be performed by using 2DE technology to globally investigate changes in protein expression. Protein microarray technology has a great advantage to study function on a system-wide or genome-wide basis with a well-controlled *in vitro* way.

The uPA/uPAR system has been shown strongly correlated with metastasis process. Tumor cells acquire growth and survival advantages by over-expressing uPA/uPAR. This makes the uPA system an attractive target for the development of cancer drugs that attack cancer by acting at multiple points of the metastatic process. In a study by Ahmed *et al*, a highly metastatic colon cancer cell line, HCT116 was transfected with an expression vector containing a 5′-uPAR cDNA fragment in an antisense orientation (*68*). Using proteomic analysis, they observed the loss of approximate 200 proteins and the quantitative expression differences of 141 proteins in the antisense-clone by comparing to wild-type and mock-transfected controls. Such global changes in protein expression not only illustrate the involvement of uPAR-mediated signaling pathway in tumor metastasis but also provide valuable information for the development of therapeutic approaches to counter colon cancer.

Oncofetal gene H19, which surprisingly encodes an untranslated mRNA, has been shown to greatly stimulate cancer cell growth (*69*). H19-transfected breast epithelial cells appear to grow faster, but no molecular target has been described for H19. Using 2DE approach with ^{35}S-methionine protein labeling, Lottin *et al* applied proteomic profiling to study breast mammary cells transfected with H19 oncofetal gene (*70*). One of the major proteins regulating intracellular redox metabolism, thioredoxin, was identified to be significantly changed in protein synthesis by H19 gene transfection, suggesting that thioredoxin may be associated with the mechanism of H19 action.

Breast cancer metastasis suppressor 1 (BRMS1) was recently identified as a novel breast cancer metastasis suppressor gene. BRMS1 significantly suppresses lung metastases without affecting primary tumor growth when transfected into breast cancer cell lines and melanoma cells (*71*). To understand the biology behind the metastasis suppression via BRMS1, Cicek's group applied 2DE analysis and MS to identify proteins differentially expressed between highly metastatic MDA-MB-435 cells and metastasis-suppressed BRMS1-transfected MDA-MB-435 cells (*72*). Annexin I and alpha B-crystallin were found expressed *in vivo* in lungs containing metastasized MDA-MB-435 cells but not in normal lung tissue of athymic mice, implicating that these two proteins are important cellular factors in BRMS1-mediated metastasis suppression.

Many gastrointestinal stromal tumors contain oncogenic mutations of the KIT receptor tyrosine kinase gene (*73*). In a research combining mutation detection and analyses of 2DE and MS, Choi *et al* found that the over-expression of high mobility group box 1 in gastrointestinal stromal tumors was related to the KIT mutation, which may accelerate tumor growth and invasion.

Post-translational modifications are important to the function of the protein. Cells have taken advantage of over 200 described post-translation modifications (*74*) to regulate the activities of their complement of proteins. In many cases, changes in post-translational modification are the primary regulators of protein function. Phosphoralytion is one of the most obvious modifications that have functional ef-

fects. Reverse-phase protein microarray is an approach suited to simultaneously screen a broad range of pathway targets in large numbers of tumors in a high-throughput manner (*75*). Wulfkuhle *et al* detected the expression alteration of some key kinases and phosphateases in 40 ovarian cancer microdissected samples by using reverse-phase protein array coupled with LCM (*76*). They spotted lysates from 40 cases of ovarian cancer tissues corresponding to tumor histotypes and disease stages and then probed with ERK1/2 and phosphor-ERK1/2, Akt, phosphor-Akt S374 and phosphor-Akt T308 antibodies (*76*). To address the issue of specificity, the authors first validated all antibodies used in this study by Western blotting against microdissected tumor tissue lysates, and then compared protein levels of total ERK1/2 and phosphor-ERK1/2, total Akt and phosphor-Akt between tumor histotypes and disease stages. The results suggested that patterns in signal pathway activation in ovarian tumors might be patient-specific rather than type or stage specific.

Most proteomics technologies are restricted to detecting changes in protein abundance, and therefore offer only indirect evidences in protein activity. Liu *et al* have developed a chemical proteomics strategy referred as activity-based protein profiling (ABPP) that allows significant fractions of an enzyme proteome to be analyzed in an activity-dependent fashion (*77*). In ABPP, chemical probes covalently label the active sites of enzyme superfamilies in a manner that provides a direct readout of changes in catalytic activity, distinguishing functional proteases from their inactive zymogens and/or endogenously inhibited forms (*78*). In the experiments by Jessani *et al*, this strategy was applied to quantitatively compare enzyme activities across a panel of human breast and melanoma cancer cell lines that differ in hormone responsiveness, invasiveness, and metastatic potential (*37*). Proteomes were labeled with serine hydrolases superfamily (the one of the largest and most diverse classes of enzymes in the human proteome) targeted fluorophosphonate, then separated by SDS-PAGE and visualized in-gel by using a laser-induced fluorescence scanner. Strikingly, it was found that nearly all of serine hydrolase activities were down-regulated in the most invasive cancer lines, while the activities of a distinct set of secreted and membrane-associated enzymes including urokinase and enzyme KIAA1363 were up-regulated. These results suggested that invasive cancers may share proteomic signatures that are more reflective of their cellular phenotypes than tissue of origin, and that a common set of enzymes might become attractive prognostic factors or drug targets.

Complementarity of genomics and proteomics

Table 2 summarizes research data of metastasis studies both in genomic and proteomic level. It is obvious that proteomic data may not be positively correlated to the genomic results. Firstly, compared to genomics, proteomics at present can only study a small fraction of total proteins as a result of its limited resolution. Although great improvements in proteome study techniques have been achieved, it is still impossible to survey all proteins at a single experiment due to the low abundance proteins and a variety of post-translational modifications. Secondly, the expression profiles at the RNA and protein levels may not be similar. It has recently been shown that there is not necessarily direct correlation between mRNA and protein expression levels *in vivo* (*79*). Nishizuka *et al* compared these two levels across 60 human cancer cell lines by reverse-phase protein lysate microarray (*80*). The mean cDNA/protein and oligo/protein correlation coefficients for 52 proteins studied in their experiments are from $-0.10/-0.15$ to $+0.87/+0.88$. Among these, structure-related proteins were almost always better correlated with mRNA levels than other protein such as nuclear and cell-cycle proteins across the 60 cell lines. This is actually reasonable given the facts that post-transcriptional regulations and post-translational modifications may occur in protein expression and synthesis. This also emphasizes the complementarity of genomics and proteomics, with genomics looking at the early stages of malignant transformation while proteomics focusing on the resulting events of gene alterations and regulation. In fact, by examining both genomics and proteomics, a biological event such as tumor metastasis can be better understood in a comprehensive and global fashion.

Metastasis models in proteomics

Before research data can be correlated with clinical practice, a fundamental problem is the establishment of an ideal metastastic model (*81*). So far, most of experiments employed cell lines, biopsy or tissue samples as analytic materials. Each of them has strength and weakness.

Cell lines have the advantages of reproducibility, availability in large numbers, and homogeneity in

Table 2 Summary of Differentially Expressed Proteins in Tumor Metastasis

Marker of tumor metastasis	Characteristics of the molecule	Expression level in matastasis	Ref.
Annexin-1	Belongs to a family of closely related calcium- and membrane-binding proteins. Annexin 1 exerts significant effects on several physiological and pathological processes, including cell growth, differentiation, apoptosis, membrane fusion, and was found with expression alterations in different kinds of malignant tumors.	Over expression in non-metastatic cell line Increase	*67* *47, 48, 55, 72*
Metastasis-associated S100A4 (Mts1) protein	An 11-kDa calcium-binding protein strongly linked to the formation of metastatic phenotype via regulation of cell motility, signal transduction and invasiveness.	Increase	*47*
Osteopontin	A glyco-phosphoprotein that is expressed and secreted by numerous human cancers and linked with the regulation of metastatic spread of tumor cells.	Increase	*67*
Extracellular matrix protein 1	A secretory glycoprotein, promotes angiogenesis and is produced by tumor cells.	Increase	*67*
IL-18	An essential interferon-γ (IFN-γ)-inducing factor. IL-18 is a product of macrophages and may play a role in metastasis by altering the microenvironment.	Increase	*48*
Apolipoprotein A1	A protein that is normally expressed in liver, small intestine and colon carcinoma cell lines and might be a potential marker of the tumor aggression.	Increase	*46*
Cytokeratin	An important structural components of the epithelial cytoskeleton, the type of cytokeratins present in the cells is related to their biological function. Resent studies have indicated that cytokeratins play an important role in the regulation of cell migration and invasion.	CK18 – Increase CK19 – Increase	*48* *100*
High mobility group box 1	An intranuclear protein that interacts with several transcription factors and accelerates genes related to tumor growth and invasion.	Increase in 86% of GISTs with KIT mutation	*51*
Galectin-1	Galectins are galactoside-binding proteins that exhibit an important function in tumor progression by promoting cancer cell invasion and metastasis formation.	Associated with the metastatic phenotype	*93, 96*
Metalloproteinase (MMP)	An important modulator of carcinogenesis and contributes to the processes of local invasion and metastasis by controlling the ability of a tumor to transverse tissue boundaries.	MMP1 – Associated with the non-metastatic phenotype MMP2 – Increase	*67* *51*
Heat shock protein (HSP)	Ubiquitously present in many cells. They are induced by heat shock and other environmental and pathophysiologic stresses. Besides their putative role in thermoresistance, these proteins may be involved in the survival and recovery of the cells when exposed to stressful conditions. HSPs act as molecular chaperones to regulate appropriate protein folding and packaging.	Hsp27 – Increase Hsp60 – Decrease Hsp90 – Increase	*49* *48* *54*
Urokinase	A kind of serine protease, which can activate plasminogento fibrinolysin, the latter can degrade most kinds of extracellularmatrix. Thus construct path for metastasis from the extracellular local lysis region.	Increased enzyme activity	*37*

Table 2 ***Continued***

Marker of tumor metastasis	Characteristics of the molecule	Expression level in matastasis	Ref.
Tropomyosin	Tropomyosins (TMs) are ubiquitous actin-binding proteins found in muscle and nonmuscle cells. In nonmuscle cells, they are associated with cytoskeletal actin in microfilaments. Changes of expression level of TMs may lead to a change in cell motility, which is considered to be one of the important characteristics of high-metastatic tumor cells.	TM – Increase TM1 – Increase TM2 – Decrease	*48* *84, 101* *102*

cell lineage. Lots of experiments attempted to identify biomarkers or obtain biological insights of tumor metastatsis by comparing the protein expressions between cell lines that differ in metastatic potential (Table 1). Tang and his co-workers have developed a stepwise metastatic human HCC model system including a low metastatic subclone MHCC97L, a high metastatic subclone MHCC97H, and two even higher metastatic potential cell lines HCCLM3 and HCCLM6 (*82*). With this model system, interesting results were obtained in the search for HCC metastasis-related chromosomes/proteins/genes. However, no cell lines in culture are fully representative of tumor *in vivo*. Researches have proved that without a supporting tumor microenviroment, only cancer cells alone are not enough to confer metastatic status (*19*, *81*) and that tumor-microenviromental interaction has a decisive role in controlling local cancer growth, invasion and distant metastasis (*83*). Jessani *et al* analyzed enzyme activity profiles of human breast cancer cell line MDA-MB-231 when grown in culture and as orthotopic xenograft tumors in nude mice (*84*). Cells isolated from tumors exhibited dramatic posttranslational up-regulation of urokinase plasminogen activator and down-regulation of the glycolytic enzyme phosphofructiokinase. This result indicates that specific host components have a contribution to tumor biology.

Biopsies and tumor blocks are ideal experiment materials in cancer research. The data derived from such samples exhibit the real condition of cancer development. But a major difficulty is the cellular heterogeneity of tumors. Besides cancer cells, tumor tissues also contain other cell types, including myoepithelial cells, fibroblasts and endothelial cells. It is possible that a proteomics analysis may be confounded by opposed protein changes in different cell types and then the sensitivity of the analysis may be greatly reduced. LCM technology may provide help, enabling researchers to collect specific cell types from a tissue sample. Using LCM researchers can obtain more accurate representation of cells and make more accurate comparisons of protein expression between specimens. LCM is extremely useful when only a few tumor cells exist in a solid tissue such as metastaic lymph nodes. LCM technique has been successfully coupled with 2DE (*85*, *86*), LC/MS (*87*, *88*), SELDI (*60*, *61*) and protein microarray (*76*) in proteomic analyses.

Other commonly used metastatic models were formed by inoculating tumor cells or tissue blocks to nude mice to develop visible metastases at secondary sites. Those metastasis-associated molecules then were identified based on these models. These assays are hardly able to clarify which steps in the metastatic process are affected by specific molecules (*89*, *90*) and which are metastatic-related and metastatic-induced factors.

However, endeavors have continually been made for establishing an ideal model for tumor metastasis research. Recently Hingorani and his colleagues developed a mouse model of human pancreatic intraepithelial neoplasias (PanIN) to investigate the development of invasive and metastasis of pancreatic cancer (*65*). It has been reported that activating mutations in the KRAS proto-oncogene were found in over 90% of invasive pancreatic ductal adenocarcinoma and were thought to represent an initiating event (*91*). This raises a hypothesis that physiological levels of oncogenic KRAS would serve to initiate pancreatic cancer. The authors targeted expressed oncogenic KRAS to progenitor cells of mouse pancreas and demonstrated that the endogenous expression of KRAS initiated the development of PanINs identical to all three stages found in the cognate human condition (*65*). Using this model, researchers can investigate the entire progression of pancreatic ductal adenocarcinoma from preinvasive neoplasias to invasive and metastatic disease.

Conclusion

Despite of many molecular factors having been identified as contributing to the formation of detectable metastases (Table 2), the road of unveiling the mechanism of tumor metastasis is still far. The relevance of these data to clinical practice still has to be established. Indeed, none of the potential markers described so far by proteomics is routinely used by clinicians for either diagnosis, treatment choice or prognosis (*56*). This highlights the necessity to use high-throughput systematic approaches to rapidly identify more metastasis-related factors that can be considered in an integrated way.

Nevertheless, current research data demonstrate the value of proteomic analysis for understanding the molecular mechanism involved in metastasis activity. A combined approach is expected to become the basis for the development of the mechanistic studies. Further evaluation and characterization of the genomic and proteomic variations may lead to the identification of biomarkers that can be specifically applied to metastatic assay and diagnosis in clinic. In addition, integration of proteomics with other experimental disciplines, particularly biochemistry, cell biology, molecular genetics and chemistry, will continue to extend the application of proteomics in investigating tumor metastasis. The technological advances in protein detection and identification are surely also critical. Rapidly progressing technologies will promote future development in the field of cancer proteomics.

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