

Recent advances in mass spectrometry-based proteomics of gastric cancer

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Abstract

The last decade has witnessed remarkable technological advances in mass spectrometry-based proteomics. The development of proteomics techniques has enabled the reliable analysis of complex proteomes, leading to the identification and quantification of thousands of proteins in gastric cancer cells, tissues, and sera. This quantitative information has been used to profile the anomalies in gastric cancer and provide insights into the pathogenic mechanism of the disease. In this review, we mainly focus on the advances in mass spectrometry and quantitative proteomics that were achieved in the last five years and how these up-and-coming technologies are employed to track biochemical changes in gastric cancer cells. We conclude by presenting a perspective on quantitative proteomics and its future applications in the clinic and translational gastric cancer research.

Key words: Gastric cancer; Mass spectrometry; Protein identification; Proteomics; Protein quantification

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Core tip: Protein identification and quantification by mass spectrometry represent powerful techniques for deciphering the mechanisms underlying the biochemical anomalies that cause human diseases. Due to innovations in mass spectrometry and labeling techniques, cellular protein levels can be monitored routinely with great accuracy. This review provides a brief overview of these technological advances and their applications in gastric cancer biology.

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INTRODUCTION

Functional interpretations of the genes that are associated or linked with cancer by various genomics approaches often require other orthogonal approaches that could provide information beyond the data obtained from sequence analysis of those genes. Since Wilkins and Williams^[1] first proposed the concept of the proteome in 1994, the field of proteomics has recently experienced a dramatic development that has largely been driven by technological advances in the field of mass spectrometry.

The introduction of advanced mass spectrometric instruments and bioinformatics tools has enabled the high-resolution and high-specificity analyses of thousands of proteins and their post-translational modification states in cultured cells, primary tissues, and body fluids^[2]. Despite the inherent low sensitivity and undersampling suffered by mass spectrometry^[3,4], researchers have started to design strategies that capitalize on this emerging technology for the elucidation of disease pathobiology^[5-7].

Gastric cancer is one of the leading causes of cancer-related deaths worldwide^[8,9]. With approximately one million cases diagnosed each year, gastric cancer is also one of the most common cancers, particularly in East Asia^[8]. Despite a modest decline in newly diagnosed cases worldwide, the mortality rate of gastric cancer remains higher than other malignancies, mainly due to the lack of noninvasive handy diagnostics of early gastric cancer^[8]. Moreover, the pathogenic mechanism underlying gastric tumorigenesis is still unknown, and the only curative treatment for gastric cancer remains surgery.

Aimed at obtaining a full understanding of the molecular determinants that drive gastric cancer, many studies have increasingly adopted advanced proteomic technologies that can help identify protein biomarkers and elucidate the molecular mechanisms of gastric cancer. This review discusses the technological breakthroughs in mass spectrometry-based quantitative proteomics and their applications in gastric cancer research.

MASS SPECTROMETRY

Basics and instrumentation

Table 1 summarizes the mass spectrometric technologies that have been adopted to study gastric cancer. Mass spectrometric measurements detect and identify the chemical composition of ionized analytes based on the mass-to-charge ratio, m/z . A typical mass spectrometer is composed of an ion source that ionizes the analytes, mass analyzer(s) for the detection

of m/z , and detector(s) for counting the intensities of the ions^[10]. Ionization is commonly achieved by soft ionization techniques, such as matrix-assisted laser desorption/ionization (MALDI)^[11] and electrospray ionization (ESI)^[12], to measure the masses of proteins and peptides. In MALDI, the analytes are ionized with a crystalline matrix *via* laser pulses, whereas in ESI, the analytes are directly ionized from a solution that is typically eluted from liquid chromatography (LC) columns. MALDI is usually adopted to analyze simple samples, whereas LC-ESI is used to analyze complex mixtures.

Two major types of mass analyzers are used in current proteomics technology. In time-of-flight (TOF) mass spectrometers, the flight times of ions are measured over a fixed distance to match a specific m/z , and the intensity of a measurement is correlated with the amount of the ion. MALDI ionization coupled with TOF technology allows the MALDI-TOF mass analyzer to analyze proteins and peptides with a wide range of molecular weights^[10]. Due to its simplicity, excellent mass accuracy, high resolution, and great sensitivity, MALDI-TOF has been widely adopted to identify proteins associated with diseases, including gastric cancer.

Using MALDI-TOF, Hu *et al.*^[13] have shown that overproduction of the C-X-C chemokine receptor type 1 (CXCR1) was linked with late-stage gastric cancer. The authors compared the protein abundance profiles of the MKN45 gastric cancer cell line in the presence and absence of *CXCR1* overexpression and found that the cellular levels of 29 proteins differed. As these proteins were known to participate in cell adhesion, cellular metabolism, and the cell cycle, CXCR1 was inferred to play a role in the proliferation, metastasis, and invasion of gastric cancer.

In an attempt to understand the inhibitory function of curcumin, curcumin-treated samples were analyzed with MALDI-TOF, and 75 proteins displayed significant changes in abundance. In this study, Singhal *et al.*^[14] identified putative biomarkers of gastrointestinal tract cancers by analyzing biopsy samples obtained from patients with gastroesophageal malignancies using MALDI-TOF.

Recently, the application of MALDI has been expanded to obtaining mass images of tissues^[15]. In MALDI imaging mass spectrometry, the masses of biomolecules are probed two-dimensionally in a thin tissue section, providing valuable spatial information about the analytes that is lost in typical LC-mass spectrometry experiments. Balluff *et al.*^[16] have utilized MALDI imaging mass spectrometry to identify prognostic biomarkers that can be used to predict disease outcomes after surgical resection. The prognostic value of the three identified proteins (CRIP1, HNP-1, and S100-A6) was validated immunohistochemically with tissue microarrays using an independent validation cohort.

Surface-enhanced laser desorption/ionization

Table 1 Summary of recent proteomic analyses of gastric cancer

Sample	Measurand	Mass spectrometry	Identification/quantification	Ref.
Tissue samples				
GC tissue	Global proteome	MALDI TOF/TOF, LTQ Orbitrap XL	Label-free, Mascot	Balluff <i>et al</i> ^[16] , 2011
GC tissue	Global proteome	Q-TOF	O18/O16, MassLynx (v4.0)	Zhang <i>et al</i> ^[73] , 2013
GC tissue	Global proteome	LTQ Orbitrap	Label-free, ProLuCID (v1.3)	Aquino <i>et al</i> ^[74] , 2014
GC tissue	Global proteome	MALDI TOF/TOF	Label-free, Mascot (v2.2)	Wu <i>et al</i> ^[75] , 2014
GC tissue	Global proteome	LTQ Orbitrap XL	Label-free, Mascot (v2.2)	Ichikawa <i>et al</i> ^[38] , 2015
GC tissue	Global proteome	LTQ Orbitrap XL	Label-free, Bioworks Browser (v3.3.1), Trans-Proteomic Pipeline (v4.0)	Shen <i>et al</i> ^[76] , 2015
GC tissue	Membrane proteome	LTQ Orbitrap Velos	TMT, MaxQuant (v1.2.2.5)	Gao <i>et al</i> ^[31] , 2015
Gastroesophageal malignancy	Global proteome	MALDI TOF/TOF	iTRAQ, Mascot	Singhal <i>et al</i> ^[14] , 2013
Serum samples				
Sera from GC patients	Serum proteome	MALDI-TOF LTQ Orbitrap XL MS/MS	Label-free, Autoflex, Peptide mass fingerprinting	Fan <i>et al</i> ^[57] , 2013
Sera from GC patients	Serum proteome	Triple TOF 5600	Multiple reaction monitoring	Humphries <i>et al</i> ^[59] , 2014
Sera from GC patients	Serum proteome	MALDI-TOF Orbitrap Q-Exactive	Label-free, MaxQuant (v1.4.1.1)	Abramowicz <i>et al</i> ^[50] , 2015
Sera from GC patients	Serum proteome	LTQ Orbitrap Velos	iTRAQ, SEQUEST HT, Mascot (v2.2)	Subbannayya <i>et al</i> ^[29] , 2015
Sera from GC patients	Serum proteome	SELDI TOF MS MALDI TOF/TOF	Label-free, Mascot	Song <i>et al</i> ^[17] , 2016
Cell lines				
BGC823, MKN45, SCG7901	Global proteome	MALDI TOF/TOF	Label-free, Mascot	Cai <i>et al</i> ^[71] , 2013
AGS, AZ521, FU97, MKN7, MKN74, NCI-N87, SNU16, YCC1, YCC2, YCC3, YCC9	Global proteome	MALDI TOF/TOF	iTRAQ, ProteinPilot	Hou <i>et al</i> ^[69] , 2013
AGS	Global proteome	Q-TOF	iTRAQ, Mascot (v2.1.1)	Hu <i>et al</i> ^[72] , 2013
MKN45	Global proteome	MALDI TOF/TOF	Label-free, Mascot	Hu <i>et al</i> ^[13] , 2013
OCUM-2MD3, OCUM-12	Global proteome	Q-TOF	iTRAQ, ProteinPilot	Morisaki <i>et al</i> ^[28] , 2014
AGS, BGC823, MKN45, SGC7901	Global proteome	MALDI TOF/TOF	Label-free, Peptide mass fingerprinting	Yang <i>et al</i> ^[67] , 2014
HGC27, MGC803, SGC7901	Global proteome	LTQ Orbitrap	Label-free, Mascot (v2.3.2), Scaffold (v4.0.5), X! Tandem CYCLONE (v2010.12.01.1)	Qiao <i>et al</i> ^[62] , 2015
AGS	Global proteome	Q-TOF	iTRAQ, Mascot (v2.3.2)	Lin <i>et al</i> ^[61] , 2015
HGC27	Global proteome	Triple TOF 5600	iTRAQ, Mascot (v2.3.2)	Chen <i>et al</i> ^[77] , 2016
AGS, MKN7	Secretome	MALDI TOF/TOF	iTRAQ, ProteinPilot	Loei <i>et al</i> ^[51] , 2011
AGS, KATO III, NCI-N87, SNU1, SNU5, SNU16	Secretome	LTQ Orbitrap Velos	SILAC, Proteome Discoverer (v1.3.0.339), Mascot, SEQUEST	Marimuthu <i>et al</i> ^[33] , 2013
AGS, KATO III, SNU1, SNU5, MKN7, IM95	Membrane proteome	LTQ-FT Ultra	Label-free, Trans-Proteomic Pipeline, Mascot (v2.2.07)	Guo <i>et al</i> ^[68] , 2012
AGS, IM95, KATO3, MKN7, MKN28, MKN45, NUGC3, NUGC4, SCH, SNU1, SNU5, SNU16	Membrane proteome	Q-TOF	iTRAQ, ProteinPilot	Yang <i>et al</i> ^[70] , 2012
AGS, HGC27, KATO III, MKN45, NUGC3, SCH, SGC7901, SNU5, SNU484, TSK1	Membrane proteome	Q-TOF	iTRAQ, ProteinPilot	Goh <i>et al</i> ^[60] , 2015
Multidrug-resistant GC cell lines: GC7901/VCR, SGC7902/ADR	Surface glycoproteome	LTQ Orbitrap XL	Triplex stable isotope dimethyl labeling, Mascot, MSQuant (v2.0a81)	Li <i>et al</i> ^[47] , 2013
AGS	Interactome	LTQ Orbitrap Velos	Label-free, Mascot (v2.2.2)	Yu <i>et al</i> ^[57] , 2013
AGS	Phosphoproteome	MALDI TOF/TOF	SILAC, Mascot (v2.1)	Holland <i>et al</i> ^[43] , 2011
AGS	Phosphoproteome	LTQ Orbitrap XL	SILAC, MaxQuant (v1.3.0.5)	Glowinski <i>et al</i> ^[42] , 2014

The studies are listed according to sample types (tissues, sera and cell lines) and measurands in the order of publication year and then in alphabetical order by the first author. GC: Gastric cancer; MALDI: Matrix-assisted laser desorption ionization; TOF: Time-of-flight.

(SELDI) is a variation of MALDI, in which the analytes are bound to a surface before the mass analysis. The surface can be modified to allow for specific binding of the analytes of interest. Like MALDI, SELDI is usually coupled with TOF for protein identification. Using SELDI-TOF, Song *et al*^[17] identified 15 proteins that were differentially regulated in the serum samples from 296 gastric cancer patients.

The second type of mass analyzer is the ion trap, in which the ionized analytes are first trapped and then subjected to mass spectrometry. The ion trap is less expensive than the MALDI-TOF analyzer but is still sensitive enough to measure non-abundant analytes. Therefore, until recently, ion traps have been commonly used to obtain a majority of proteomics data, despite their relatively low mass accuracy^[10].

The Fourier transform (FT) mass spectrometer is an advanced ion trap mass analyzer that exploits strong magnetic field to measure the m/z of ions. FT mass spectrometry boasts sensitivity, accuracy, resolution, and dynamic range^[18]. These advantages make FT mass spectrometry suitable for analyzing proteins in a complex mixture. However, its application in proteomics has largely been hindered by its cost and difficulties in operation and maintenance.

The Orbitrap analyzer, a variant of the FT mass spectrometer, made its debut in 2005. Like the FT mass spectrometer, the Orbitrap mass analyzer converts the image currents produced in a trap to mass spectra by Fourier transform^[19]. Orbitrap uses electrostatic forces rather than a magnetic field and, thus, does not require the expensive superconducting magnets used in FT mass spectrometers. Orbitrap is currently widely used in proteomics research and is chosen for complex proteome analysis.

Fragmentation

"Shotgun proteomics" resulted from the coupling of high-performance liquid chromatography with ESI technology^[20]. In this approach, the proteome subject to mass analysis is first digested with a specific protease(s), such as trypsin. The resulting digest composed of proteolytic peptides from the entire proteins in the sample is separated by liquid chromatography before the first mass analysis of the intact peptides (MS1). Additional information about the parent peptide ions is obtained by fragmenting the parent peptides with non-proteolytic methods and measuring the m/z of product ions in the second mass analysis (MS2)^[21].

This non-proteolytic fragmentation step is a key to peptide identification, as the amino acid sequence information is inferred from the mass spectra of the fragmented peptides^[21]. The most common method used to generate fragment ion spectra of the selected precursor ions is collision-induced dissociation (CID)^[22]. Electron transfer dissociation (ETD), an alternative fragmentation technique, has some advantages over CID in accurately assessing post-translational modifications such as glycosylation and phosphorylation, because ETD tends to preserve these modifications when the modified peptide is fragmented^[23].

QUANTIFICATION METHODS

Instead of providing mere lists of proteins, quantitative proteomics can deliver information about the differences in proteomes between two samples, and this information may be more useful for studying biological and biochemical processes. The development of quantitative proteomics owes much to the unique labeling strategies that enables a mass spectrometer to distinguish the same proteins or peptides from

different samples. These labeling strategies are designed such that labeling causes a known mass shift in the labeled protein or peptide in the mass spectrum.

In general, differentially labeled samples are mixed and analyzed in the same mass spectrometric run, where the differences in the peak intensities of the labeled peptide pairs are assumed to reflect the differences in the abundance of the corresponding proteins. For instance, it is possible to compare the proteomes from normal and cancerous tissues using this approach. Quantitative mass spectrometry-based proteomics approaches include stable isotope labeling techniques and label-free strategies.

Stable isotope labeling approaches

Stable isotope labeling entails the incorporation of stable heavy atoms such as ^{13}C and ^{15}N into specific biomolecular entities, as previously reviewed^[24]. In most cases, these labels are chemically or metabolically introduced into peptides or proteins. One of the first chemical labeling strategies adopted for protein quantification by mass spectrometry was the isotope-coded affinity tag (ICAT) technique^[25]. In this approach, the sulfhydryl groups in cysteine residues are covalently modified by the ICAT reagents containing "light" or "heavy" isotopes. The presence of the light or heavy ICAT tags leads to the separation and concomitant quantification of modified peptides during the precursor ion measurements in the first mass spectrometry process (MS1).

Recently, chemical labeling strategies utilizing isobaric tags, *i.e.*, tags with the same molecular weight, developed for relative and absolute quantification (iTRAQ)^[26] and tandem mass tags (TMT)^[27] have gained popularity in proteomics. iTRAQ and TMT reagents differ from ICAT reagents in that the ϵ -amino group of lysine and α -amino group of the N-terminal residue in peptides are modified. The labeled peptides are quantified during the second mass spectrometry process (MS2) when the tags are released upon fragmentation of the peptides (Figure 1). Advantages of the isobaric tags include a multiplexing capacity of up to eight separate samples in a single mass spectrometric run. Additionally, because isobaric tags modify amino groups, which are more abundant than sulfhydryl groups in most proteins, the coverage of quantification by iTRAQ and TMT is also greater than by ICAT.

A large number of studies quantifying the proteomes of gastric cancer using the iTRAQ approach have been reported. Morisaki *et al.*^[28] applied iTRAQ to identify potential biomarkers in gastric cancer stem cells and identified nine proteins that were overproduced in gastric cancer stem cells. Using iTRAQ, Subbannayya *et al.*^[29] defined a set of potential biomarkers in sera from gastric cancer patients. In their study, more than 50 proteins were found to exhibit altered levels in samples from gastric cancer patients.

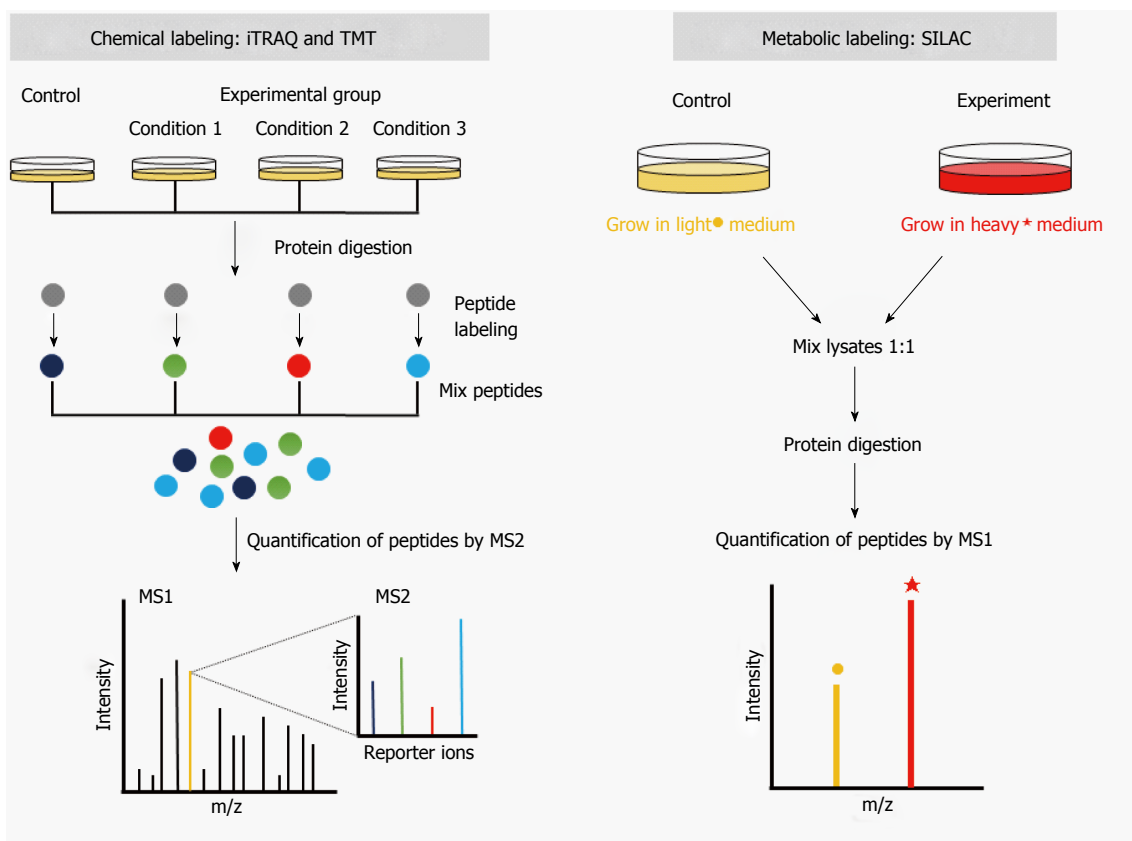


Figure 1 Schematic overview of labeling strategies used in quantitative proteomics. Chemical labeling utilizes the isobaric tags for relative and absolute quantification (iTRAQ) and the tandem mass tags (TMT). In this approach, proteolytic peptides from separate samples are labeled with discrete isobaric tags and pooled. Precursor peptide ions are fragmented (MS2) to generate reporter ions with distinct m/z , whose relative intensities represent the relative abundances of the peptides producing the corresponding reporter ion. Metabolic labeling represented by the stable isotope labeling with amino acids in cell culture (SILAC) strategy takes advantage of the metabolic incorporation of heavy amino acids into mature proteins. In this strategy, the relative peak intensities (MS1) represent the abundances of the precursor peptide ions.

TMT has also been used to quantify gastric cancer proteomes. Gao *et al.*^[30] found that 234 mitochondrial protein genes were differentially expressed in gastric cancer using TMT. In another study employing TMT, Gao *et al.*^[31] revealed that 82 plasma membrane proteins were dysregulated in gastric cancer.

An alternative to stable isotope labeling technique is metabolic labeling. This approach takes advantage of the metabolic incorporation of heavy isotopes in live cells under culture conditions. Quantification by metabolic labeling is less error-prone than chemical labeling because the labels are introduced before the samples are prepared. Stable isotope labeling with amino acids in cell culture (SILAC)^[32] is one of the most popular metabolic labeling techniques (Figure 1). Developed by Ong *et al.*^[32], SILAC labels proteins in the cells by growing them in the medium containing heavy amino acids. The most common heavy amino acids used in SILAC are lysine-4, lysine-8, arginine-6, and arginine-10. Different combinations of heavy lysines and arginines can be used such that up to three simultaneous quantifications are possible as follows: a light sample (Lys-0 and Arg-0), medium sample (Lys-4 and Arg-6), and heavy sample (Lys-8 and Arg-10).

As trypsin is the most popular protease used for

the preparation of peptide mixtures, which cleaves the carboxyl side of lysine or arginine, the use of heavy lysine and arginine in SILAC helps increase the coverage of quantification by ensuring that every peptide analyzed by the mass spectrometer contains at least one heavy amino acid. Like labeling with ICAT, the quantification of proteins labeled with the SILAC approach is carried out by comparing the intensities of precursor peptide ions in the MS1 process. Quantification employing the SILAC method has been applied in gastric cancer proteomics. Marimuthu *et al.*^[33] studied the secretomes from neoplastic and non-neoplastic gastric epithelial cells using SILAC. The authors identified 263 proteins that were upregulated in gastric cancer-derived cells compared to non-neoplastic gastric epithelial cells.

Label-free approaches

Another quantification strategy used in proteomics, the label-free approach, inherently suffers from low reproducibility caused by experimental errors and requires precise optimization of mass spectrometric instrumentations^[34]. Nevertheless, label-free quantification can overcome some of the limitations of stable isotope labeling strategies. For example, the

time-consuming and costly labeling steps can be eliminated, and the sample numbers are not limited by the multiplexing capacity (three for SILAC and eight for iTRAQ or TMT).

Two types of label-free quantification approaches are routinely used. In spectral counting, the relative abundance of a specific protein among the samples is evaluated by the number of tandem mass (MS2) spectra that can be matched to the protein^[35]. Employing this approach, Uen *et al.*^[36] identified biomarker candidates in plasma samples obtained from gastric cancer patients. The authors found 17 proteins with differential expression patterns in gastric cancer.

The second label-free quantitative method requires high-resolution mass spectrometers and quantifies the intensities of the precursor peptide ion, in which the number of possible tryptic peptides from a given protein are often used for normalization^[24]. Using this method, Fan *et al.*^[37] defined novel diagnostic biomarkers for gastric cancer. The authors analyzed serum samples from gastric cancer patients and discovered four deregulated proteins that were differentially expressed in the patients' sera. Another recent label-free quantitative proteomics by Ichikawa *et al.*^[38] showed the prognostic importance of a tumor suppressor *PML* (promyelocytic leukemia) in treating gastrointestinal stromal tumors.

MEASURANDS OF MASS SPECTROMETRY

Post-translational modifications

In addition to quantifying the global proteome, the analysis and quantitative assessment of post-translational modifications (PTMs) of a proteome is a powerful approach to understanding the signal flux in disease samples, including gastric cancer cell lines and tissues. Mann *et al.*^[39] have previously reviewed PTM analysis by mass spectrometry. Of the many PTMs, phosphorylation and glycosylation associated with gastric cancer have been studied using quantitative proteomic methodologies. When only a minute fraction of protein is modified at any given time point, the detection and quantification of PTM by mass spectrometry are significant challenges.

Due to these technological limitations, mass spectrometric PTM analysis requires additional sample preparation steps that enrich the modified peptides. For instance, phosphoproteome analyses rely on the enrichment of phosphorylated peptides using an anti-phosphopeptide antibody, immobilized metal affinity chromatography (IMAC), or titanium-dioxide beads^[40,41]. Using an immobilized phosphotyrosine-specific antibody, Glowinski *et al.*^[42] reported 85 different proteins that exhibited altered phosphorylation upon infection with *Helicobacter pylori* (*H. pylori*), an organism strongly linked with gastric cancer. Holland *et*

al.^[43] also found that the phosphorylation of 20 proteins was differentially regulated by *H. pylori* infection using IMAC enrichment approach.

Additionally, protein glycosylation has been mapped in gastric cancer cells. Asparagine, serine, and threonine residues of proteins are glycosylated in the endoplasmic reticulum and Golgi apparatus. Increased glycosylation has been associated with the proliferation and progression of various cancers, and glycans with specific structures have been associated with tumor malignancy^[44-46]. Li *et al.*^[47] performed quantitative proteomic analysis to identify and quantify eleven cell surface N-glycoproteins with differential expression patterns in multidrug-resistant (MDR) gastric cancer and to define the cell surface glycoproteome that is related to resistance to multiple drugs, including vincristine (also known as leurocristine) or doxorubicin (*e.g.*, Adriamycin), which are used to treat gastric cancer.

Secretome and serum proteome

A systematic assessment of the secretome, *i.e.*, all secretory proteins from a cell, may provide crucial insights into cancer biology, as the composition of proteins that are secreted from cancer tissues differs from the proteins that are secreted from normal tissues^[48]. The secretome and serum proteome, *i.e.*, all proteins in the blood serum, are considered a major source of cancer biomarkers, and some important regulatory proteins that are secreted into the serum have been used as tumor biomarkers^[49].

In a study seeking to characterize the serum proteome from local and invasive gastric cancer, Abramowicz *et al.*^[50] analyzed serum samples acquired from patients with locally advanced or metastatic cancers and healthy controls. Several proteins with different abundances were detected in cancer patients, with no evidence of differences between the patients with local and invasive cancers. Loei *et al.*^[51] have compared the secretomes of AGS and MKN7 cells using iTRAQ labeling and found that 43 protein genes were differentially expressed between the two cell lines. Among these proteins, granulin was confirmed by immunohistochemistry to be frequently found in gastric tumor tissues, but it was not found in the normal gastric epithelia.

Interactome

As tumor has been defined as a disease of pathways^[52], interactome analysis may offer some valuable insights into cancer biology by offering information beyond the changes in the abundance of individual proteins^[53]. In practice, characterizing alterations in protein-protein interactions in cancer is becoming more relevant, as many studies have reported that patients affected by the same type of cancer display diverse protein expression patterns and activation of oncogenic kinases^[54-56]. In these cases, classifications based on protein-protein interaction subnetworks

Table 2 Cell line-based summary of recent proteomic analyses of gastric cancer

Cell line	Ref.	Aim of study	Proteins analyzed	Validation ¹
AGS	Holland <i>et al.</i> ^[43] , 2011	Phosphoproteome upon <i>H. pylori</i> infection	20 altered in abundance by <i>H. pylori</i>	No
	Loei <i>et al.</i> ^[51] , 2011	Secretome of AGS and MKN7	43 differed	IHC/WB
	Guo <i>et al.</i> ^[68] , 2012	Plasma membrane proteome	1473 identified	IHC
	Hou <i>et al.</i> ^[69] , 2013	Biomarkers for GC metastasis	19 increased and 34 decreased in metastasis	IHC/WB
	Hu <i>et al.</i> ^[72] , 2013	Global profile of miR-148a-regulated proteins	55 altered by miR-148a	WB
	Marimuthu <i>et al.</i> ^[33] , 2013	Secretome	263 increased and 45 decreased in GC	IHC
	Yu <i>et al.</i> ^[57] , 2013	Interactome of VCP	288 putative partners, including 18 PI3K/ Akt proteins	WB
	Glowinski <i>et al.</i> ^[42] , 2014	Tyrosine signaling upon <i>H. pylori</i> transfection	85 altered by <i>H. pylori</i>	No
	Goh <i>et al.</i> ^[60] , 2015	Membrane proteome of 11 GC cell lines	882 altered, including 57 increased in ≥ 6 cell lines	WB
	Lin <i>et al.</i> ^[61] , 2015	Tanshinone IIA regulation	102 altered by tanshinone IIA treatment	WB
BGC-823	Cai <i>et al.</i> ^[71] , 2013	Effects of curcumin on viability and apoptosis	75 altered by curcumin treatment	No
HGC-27	Goh <i>et al.</i> ^[60] , 2015	Membrane proteome of 11 GC cell lines	882 altered, including 57 increased in ≥ 6 cell lines	WB
	Qiao <i>et al.</i> ^[62] , 2015	Proteomes of three GC cell lines	9 altered	IHC/WB
	Chen <i>et al.</i> ^[77] , 2016	Proteome with FAF1 or <i>H. pylori</i>	157 altered by FAF1, 500 by <i>H. pylori</i> and 246 by both	WB
MGC-803	Qiao <i>et al.</i> ^[62] , 2015	Proteomes of three GC cell lines	9 altered	IHC/WB
MKN7	Loei <i>et al.</i> ^[51] , 2011	Secretome of AGS and MKN7	43 differed	IHC/WB
	Guo <i>et al.</i> ^[68] , 2012	Plasma membrane proteome	1473 identified	IHC
MKN45	Yang <i>et al.</i> ^[70] , 2012	Membrane proteome	175 altered	IHC/WB
	Hu <i>et al.</i> ^[33] , 2013	Proteome changes following CXCR1 knockdown	16 increased and 13 decreased by CXCR1 knockdown	WB
	Yang <i>et al.</i> ^[67] , 2014	Proteome changes following NAIF1 overexpression	5 increased and 3 decreased by NAIF1 overexpression	WB
	Goh <i>et al.</i> ^[60] , 2015	Membrane proteome of 11 GC cell lines	882 altered, including 57 increased in ≥ 6 cell lines	WB
	Li <i>et al.</i> ^[47] , 2013	Cell surface glycoproteome of MDR	11 altered in MDR cell lines	WB
SGC-7901	Goh <i>et al.</i> ^[60] , 2015	Membrane proteome of 11 GC cell lines	882 altered, including 57 increased in ≥ 6 cell lines	WB
	Qiao <i>et al.</i> ^[62] , 2015	Proteomes of three GC cell lines	9 altered	IHC/WB

¹Validation indicates whether a selected set of altered proteins have been validated using immunohistochemistry (IHC) or Western blot (WB) or have not been (No). The cell line studies are listed in the order of publication year and then in alphabetical order by the first author. CXCR1: C-X-C chemokine receptor type 1; FAF1: Fas-associated factor 1; GC: Gastric cancer; *H. pylori*: *Helicobacter pylori*; MDR: Multidrug resistance; NAIF1: Nuclear apoptosis-inducing factor 1; VCP: Valosin-containing protein.

offered greater accuracy than classifications based on individual marker genes^[55].

For this reason, interactome analysis represents an attractive avenue for understanding gastric cancer biology, although its broad applications remain to be established. The first interactome analysis of gastric cancer was performed with valosin-containing protein (VCP), a protein associated with *H. pylori*-induced gastric cancer^[57]. In seeking the interacting partner proteins for VCP, Yu *et al.*^[57] immunoprecipitated VCP and then performed a quantitative mass spectrometric analysis. The authors identified 288 putative binding partners of VCP in the AGS gastric cancer cell line, providing unexpected new insights into the function of *H. pylori* in gastric cancer.

Targeted proteome

In targeted proteomics, prior knowledge of analytes is necessary, which is an attribute not essential for the aforementioned discovery-based proteomics. In this regard, targeted proteomics is similar to immunoassays,

in which antibodies recognize and identify specific proteins. Targeted proteomics is emerging as an alternative approach to discovery proteomics or immunoassays, particularly when pre-defined analytes are present at low levels and no reliable antibodies are available.

Selected reaction monitoring (SRM) is the most common approach used for targeted proteome measurements and requires a triple-quadrupole mass spectrometer, as previously reviewed by Picotti *et al.*^[58] In SRM, a peptide precursor ion from a specific protein with a particular *m/z* is selected in the first phase of tandem mass spectrometry, and a signature product ion is produced by fragmenting the precursor ion and detected by the second phase of mass spectrometry.

The sensitivity and reproducibility of SRM are greater than conventional discovery-based mass spectrometry because only a set of predefined proteins is programmed to be analyzed by the SRM mass spectrometer. Another advantage of SRM lies in its speed. After SRM assays have been defined, they

are significantly faster than a typical discovery-based mass spectrometry. In addition, the measurements can be multiplexed such that one can measure hundreds or even thousands of peptides in a single mass spectrometric run. The SRM approach has been successfully exploited to test the specificity of afamin, clusterin, haptoglobin, and vitamin D-binding protein as potential serum biomarkers of gastric cancer^[59].

Gastric cancer cell lines

Several gastric cancer cell lines have been subjected to recent mass spectrometric analyses as a model mimicking gastric cancer. These studies are summarized in Table 2. In a recent study aimed at defining the proteomes of gastric cancer, Goh *et al.*^[60] have quantified the membrane proteomes of eleven gastric cancer cell lines, including AGS, HGC-27, MKN45, and SGC-7901 cells. A total of 882 proteins were detected, and 57 proteins were upregulated, with a greater than 1.3-fold change in at least six of the eleven cell lines. Depletion of DLAT, a subunit of the pyruvate dehydrogenase complex that was upregulated, reduced cell proliferation. This study contributed to the recent interest and discussion in cancer energetics and related phenomena, such as the Warburg and reverse Warburg effects.

In another proteomic analysis of AGS cells, the most intensively studied cell line in gastric cancer proteomics, Lin *et al.*^[61] sought to elucidate the mechanism of tanshinone IIA (TIIA) regulation. TIIA is a plant extract used in traditional Chinese herbal medicine that has been reported to have anti-tumor potential against gastric cancer. The authors reported that the cellular levels of the 102 unique proteins were altered upon TIIA treatment.

Other gastric cancer cell lines have also been adopted for proteomic analysis. Qiao *et al.*^[62] have quantified the proteomes from SGC-7901, HGC-27, and MGC-803 cells. The authors have found that filamin c and a large actin-cross-linking protein were significantly downregulated, establishing functional roles for these proteins in gastric cancer.

PROTEOMIC BIOMARKERS

Quantitative proteomic analyses can provide information on proteins that are differentially abundant in cancerous tissues. These proteins, if verified, may serve as biomarkers for the diagnosis and prognosis of cancer and could be extremely useful for clinical purposes. A mass spectrometry-based cancer biomarker study typically starts with the aforementioned discovery-based proteomics by assessing the differences in the proteome profiles in small cohorts or model systems.

Once candidate biomarkers are identified, orthogonal methodologies, such as antibody-based assays, are applied for biomarker validation and verification^[63]. An increasing number of studies have adopted mass spectrometry to identify gastric cancer biomarkers

recently and are reviewed in detail by Tsai *et al.*^[64], Liu *et al.*^[65], and Lin *et al.*^[66] We briefly discuss some cases in which the quantitative proteomic approaches described in this review are applied.

In an effort to identify membrane-originated biomarkers of gastric cancer, Yang *et al.*^[67] compared the relative abundances of membrane proteins from gastric cancer and control samples using the iTRAQ technique. Upregulation of the plasma membrane protein SLC3A2 in gastric cancer cells was validated by immunoblotting of a panel of thirteen gastric cancer cell lines and immunohistochemistry on tissue microarrays comprising 85 matched pairs of normal and tumor tissues.

Plasma membrane proteomes, including cluster of differentiation proteins and receptor tyrosine kinases, have been the subject of another biomarker search. A proteomic investigation by Guo *et al.*^[68] showed that four proteins, MET proto-oncogene receptor tyrosine kinase (MET), ephrin type A receptor 2 (EPHA2), fibroblast growth factor receptor 2 (FGFR2), and integrin beta 4 (ITGB4), were upregulated in tumor tissues from 90% gastric cancer patients. Furthermore, three of them, MET, EPHA2, and FGFR2, were upregulated in all intestinal-type gastric cancers from this cohort.

In another attempt to identify potential biomarkers of gastric cancer, Marimuthu *et al.*^[33] quantified the secretome of gastric cancer by SILAC. The authors were able to identify and validate several gastric cancer biomarkers, including proprotein convertase subtilisin/kexin type 9, lectin mannose binding protein 2, and PDGFA-associated protein 1.

Biomarker candidates for gastric cancer metastasis have also been identified by quantitative proteomics. Hou *et al.*^[69] compared metastatic and non-metastatic gastric cancer cell lines with iTRAQ methods. The authors discovered that caldesmon was downregulated in metastasis-derived cell lines, which was confirmed by a further analysis of seven gastric cancer cell lines. In this study, knockdown of caldesmon in gastric cancer cells lead to an increase in cell migration and invasion, whereas upregulation of caldesmon resulted in a decrease in the phenotype.

CONCLUSION

Due to the progress made in mass spectrometry and quantitative proteomics over the past decade, it is now possible to probe thousands of proteins in a complex proteome. These technological advances include streamlined sample preparation, novel labeling strategies, and improved instrumentation, all of which contribute to the identification of gastric cancer-specific biomarkers, with increasing sensitivity and accuracy. Armed with these advanced proteomics technologies, research endeavors are seeking precise assessments of protein abundance, PTM, and protein-protein interactions that could help define the molecular

signatures of gastric cancer susceptibility.

One remaining question is how these state-of-art technologies can be used in clinics and can make a bigger impact on the real-world management of gastric cancer. In this regard, one could envision the entrance of targeted proteomics into the realm of personalized diagnostics and medicine. Targeted proteomics can provide sensitivity and reproducibility, the core requirements for the technology to be applied to these new and exciting fields. In this scenario, once the key molecular determinants of gastric cancer are defined with time-consuming, discovery-based proteomics, targeted proteomics approaches, such as SRM, could be utilized for the rapid and reproducible monitoring of these key molecules and their networks for individual diagnostics or analyses of treatment responses.

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