

Searching for Gastric Cancer Biomarkers Through Proteomic Approaches

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ABSTRACT

Gastric cancer is the fourth most common cancer worldwide and the second leading cause of cancer death. Researchers are increasingly trying to identify molecular biomarkers that can improve gastric cancer diagnosis, be prognostic indicators, or facilitate better decision-making for therapy. High-throughput technologies, or "OMICS", are particularly interesting biomarker discovery tools. Studies of gastric cancer proteomics are in their infancy, but it is clearly important to discover biomarkers with potential clinical use. The present editorial summarizes the main findings of gastric cancer proteomics and highlights critical points that need to be considered in a study aiming to identify disease biomarkers.

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INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer worldwide and the second leading cause of cancer death. The incidence and mortality rate of GC vary in different countries, with most GC cases occurring in Asia^[1]. Currently, chemotherapy presents only a minimal survival advantage, and curative gastrectomy is considered a standard treatment for GC in several countries. Therefore, the development of new anticancer treatments is necessary to improve the prognosis of GC.

Diagnosis of GC at an advanced stage is one reason for the lower overall five-year survival rate, particularly in developing countries. Early GC (EGC) is defined as a neoplasm confined to the mucosa and/or submucosa regardless of the presence of lymph node metastasis (LNM). The average five-year survival rates of patients with EGC is over 90%, far better than the observed five-year survival rate for advanced GC^[2]. Therefore, the diagnosis of GC in an early stage is critical to improve the prognosis and reduce the mortality of GC.

Researchers are increasingly trying to identify molecular biomarkers that can improve the diagnosis of GC in an early disease stage, be prognostic indicators, facilitate better decision-making for therapy, and help to better understand the molecular heterogeneity and biology of GC concerning its development, progression and propensity for aggressive behavior.

GC, similar to other neoplasias, results from a combination of environmental factors and the accumulation of generalized and specific genetic and epigenetic alterations, which affect oncogenes, tumor suppressor genes and genomic instability. A complex set of molecular alterations occurs during the early stages of the disease, and the complexity increases with the progression of the neoplasia. This complexity of gastric carcinogenesis makes the search for tumor biomarkers difficult.

Several genes/proteins have been proposed as GC biomarkers. In multistage gastric carcinogenesis, alterations of the oncogenes *MYC*, *KRAS2*, *CTNNB1*, *ERBB2*, *FGFR2*, *CCNE1* and *HGFR*, and of the tumor suppressors *TP53*, *APC*, *RB*, *DCC*, *RUNX3* and *CDH1* have been reported so far (see reviews^[3-5]). Although the deregulation

of these genes/proteins has been intensively studied in GC, more complete profiling is necessary to understand the carcinogenesis process.

Proteomics is a set of analytical approaches that allow the identification, characterization and quantification of virtually all proteins expressed by a genome in a cell, tissue or organism. These proteins are believed to be the responsible for the cellular phenotype. The idea of “one gene – one protein” is simplistic and anachronistic. A single gene can encode several protein isoforms. In addition, protein activity depends not only on the translation process but also on its location and post-translational modifications. Moreover, the activities of some proteins depend on their appropriate interactions with other proteins. Protein analyses may provide more information regarding cellular function or dysfunction than genetic analyses. Therefore, the proteomic analysis of clinical GC samples may help to better understand the underlying mechanisms involved in cancer initiation, development and progression.

Proteomic studies of GC are in their infancy compared with studies using genomic and transcriptomic approaches. However, proteomic analysis may facilitate biomarker discovery and help in the diagnosis, disease stratification, prognosis and prediction of the treatment response of GC.

PROTEOMIC ANALYSIS OF GASTRIC TUMORS

Several gastric tumor proteomic studies have been published (see review^[6]). Most of the GC proteomic studies with tissue samples used two-dimensional electrophoresis (2-DE) approaches followed by mass spectrometry (MS) analysis to screen markers of GC^[7-26]. In 2-DE approaches, proteins are separated in two steps: (1) isoelectric focusing, in which proteins are separated by isoelectric points, usually in strips with a pH gradient immobilized in a homogeneous polyacrylamide gel; (2) SDS-polyacrylamide gel electrophoresis (SDS-PAGE), in which proteins are separated by their molecular weights. The result is an SDS-PAGE gel with several small spots, each representing a protein. The size (area and intensity) of a protein spot is directly correlated with the protein expression level, so 2-DE can be used for a quantitative comparison between groups of samples: for example, tumor and non-tumor samples. Typically, differentially expressed proteins are identified by mass spectrometry. MS is the basis of most proteomic studies. A mass spectrometer can be used to determine the mass of a peptide or protein. In addition, tandem MS (MS/MS) can provide the amino acid sequence and may allow the characterization of post-translation modifications. In most GC studies, proteins were identified by MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) and ESI (electrospray ionization) mass spectrometry.

Proteomic studies based on the 2-DE approach reported different proteins possibly deregulated in gastric tumors. However, some potential GC biomarkers were consistently reported in several GC proteomic studies, such as the up-regulated Heat shock 27 kDa (HSP27)^[9,10,12,21,22,24-26], Enolase-alpha (ENOA)^[16,19,24-26], Nicotinamide N-methyltransferase (NNMT)^[19,22,23,26] and Transgelin (TGLN)^[9,10,12,24,26] proteins and the down-regulated Gastrophilin-1 (GKN1)^[10,16,19,25,26] and Carbonic anhydrase 2 (CA2)^[16,18,21,26] proteins. Conversely, down-regulation of ENOA^[21] and HSP27^[8] was also observed, highlighting that a panel of GC biomarkers is necessary to help in disease diagnosis.

The heterogeneity among gastric tumors is in part responsible for differences in the GC proteomic studies. However, variations in the pre-analytical, analytical and post-analytical steps also clearly

contribute to differences in the proteome profiles and, therefore, their findings. Pre-analytical and analytical variables contribute to variance in the resolution of 2-DE maps. Different methods of protein extraction may change the proteome profile because proteins are largely heterogeneous, with variations in size, charge, hydrophobicity and biospecific interactions. Unfortunately, there is no single method to extract all proteins from a tissue sample. In addition, these studies applied different parameters in their isoelectric focusing steps, including differences in the selection of the strips (size and pH range), with some studies using strips with narrow pH ranges^[8,12,14,17,19,22,23] and others using strips with large pH ranges^[7,9,10,16,24-26]. Different staining methods were also applied, including the Coomassie blue method, which has poor sensitivity^[12,18,24], silver staining^[7,9,10,14,16,17,19,21], the fluorescent dye SYPRO[®] Ruby gel stain^[22,26], and two-dimensional difference gel electrophoresis (2D-DIGE)^[8], in which two samples (for example, a tumor and a non-tumor sample) are labeled with different fluorescent dyes, mixed together and run on the same gel, eliminating the problem of gel-to-gel variability between the paired samples.

Concerning post-analytical variables, no standard approach is described for the analysis of proteome data. Most of these 2-DE studies identified differentially expressed proteins based only on fold change between two conditions^[9,10,12,17,18,22-24]. Other GC proteomic studies performed statistical analyses to compare protein expression between groups, but without controlling for Type I (false positive) error^[7,8,16,19,21,25], and our group compared the protein profiling of tumors and non-neoplastic samples using parametric tests with bootstrapping, a resampling method, for differentially expressed protein identification^[26].

Some studies have applied proteomic approaches to screen possible GC prognosis biomarkers^[11,26-28]. The presence of LNM is associated with a poor prognosis. Jung *et al.*^[27] used isotope-coded affinity tag (ICAT) labeling followed by liquid chromatography and MS analysis to identify proteins involved in the metastasis process by comparing differences in their expression profiles between LNM-positive and LNM-negative GCs. After validation, the authors observed that low expression of galectin-2 was significantly associated with the presence of LNM and advanced clinical stage. Our group recently evaluated the membrane proteome of GC cell lines and, after further investigation, observed that galectin-3 was associated with a higher invasive phenotype *in vitro* (unpublished data). Galectins are members of a highly conserved family of β -galactoside-binding lectins with a possible role in the regulation of immune cell homeostasis and tumor cell adhesion. Although some previous studies have reported that positive immunoreactivity of galectins is frequently observed in GC^[29-31], the proteomic studies suggest that the down-regulation of the family of galectins might be involved in the aggressiveness of GC. In addition, our group also previously compared the proteome profile of GC with and without LNM by 2-DE followed by MS^[26]. Consistent with Jung *et al.*^[27], we observed that the deregulation of several proteins related to cell death may be involved in the development of LNM.

It is important to highlight that most of the proteomic studies were performed with tissue samples from individuals from Asian populations, with the exception of the studies developed by Ebert *et al.*^[17] (German population), Kočevar *et al.*^[19] (Slovenian population) and Leal *et al.*^[26] (Brazilian population). The effect of population in gastric carcinogenesis still needs to be fully evaluated because GC is marked by global variation in incidence, etiology, natural course and management^[32].

The analysis of the proteome of tumor and non-tumor samples is a

promising tool for the identification of GC diagnostic and prognostic biomarkers. In addition, the study of the protein profiles of gastric tumors may help to understand the underlying mechanisms and molecules that drive GC malignancy. In our previous proteomic study^[26], we undertook a comprehensive computational analysis of tissue proteomic data to discover pathways and networks involved in gastric oncogenesis and progression. Several networks of molecular interactions and functions were described, including networks of deregulated proteins involved in cellular assembly and organization and in inflammatory processes. Moreover, our group^[26] and Cai *et al.*^[25] reported the deregulation of several metabolic proteins, particularly the down-regulation of enzymes of the citrate cycle (Krebs cycle) and oxidative phosphorylation in distal and proximal GC, respectively. These findings indicated that GC cells present a different metabolite profile compared with non-neoplastic gastric cells. The results of both studies suggested the Warburg effect^[33] in GC. Even under normal oxygen concentrations, tumor cells may shift from ATP generation through oxidative phosphorylation to ATP generation through glycolysis, converting most incoming glucose to lactate^[33]. It has been proposed that highly active glycolysis provides a biosynthetic advantage for tumor cells. Glycolysis provides enough metabolic intermediates by avoiding the oxidation of glucose, which is essential for the synthesis of macromolecules, such as lipids, proteins and nucleic acids, during cell division^[34-36]. Large-scale protein analyses may help in the understanding of gastric carcinogenesis and, therefore, contribute to the development of new anticancer treatments targeting specific oncogenic pathways and the identification of patients who present variable treatment responses.

SEARCHING FOR NON-INVASIVE BIOMARKERS

Non-invasive proteomic biomarkers obtained from serum or gastric fluid might have a future role in the diagnosis of GC in its early stages. A non-invasive test would be applied in the clinical routine for GC screening in high-risk populations. However, the available tumor antigens either in the sera (CEA, CA19.9, CA72-4 and CA50) or in the gastric juice (CEA, CA19.9 and fetalsulfoglycoprotein) are not sufficiently sensitive or specific for GC diagnosis, particularly in the early stage when the levels of these antigens are not too elevated.

The serum^[37-41] and gastric fluid^[42] proteome profiles of GC patients have been evaluated by laser desorption-ionization time-of-flight MS (SELDI-TOF), a technique based on protein chips with different chromatographic surfaces (hydrophobic, ionic, hydrophilic) to capture a specific set of proteins for analysis by mass spectrometry. These studies reported different peaks that would assist in GC diagnosis with high sensitivity and specificity. However, the discriminatory peaks are not consistent among the researcher groups. Although the SELDI-TOF methodology is fast and easy to perform, the peaks are characterized only by their masses. Several questions have been raised regarding the reproducibility of SELDI-TOF spectra. Some studies have demonstrated that sources of pre-analytical and analytical bias can have a pervasive effect across many or all peaks in the spectrum^[43,44]. Therefore, validation with sequence-base or antibody approaches will be important before the direct use of SELDI-TOF in clinical practice.

Others authors have applied direct analysis of serum samples from GC patients and healthy controls by MALDI-TOF-MS to generate a comparative peptide profile and thereby discover serum peptides/proteins with potential diagnostic applications^[45-48]. Ebert *et al.*^[45] identified one peptide, fibrinopeptide A, up-regulated in

the serum of GC patients. Increased fibrinopeptide A levels in GC patients and high-risk individuals were validated by enzyme-linked immunosorbent assays (ELISA). However, the increased level of fibrinopeptide A is not specific to GC. Increased fibrinopeptide A serum levels can also be observed in patients with other neoplasia types^[49-52]. In addition, because an overlap of fibrinopeptide A levels between GC and control groups has been reported, the analysis of this isolated peptide may not be useful for GC diagnostic purposes.

Umamura *et al.*^[46] compared the peptide profiles of GC patients and controls and pre- and postoperative sera from GC patients. The authors identified a 2209 Da peptide as a high molecular weight (HMW) kininogen fragment that was expressed at higher levels in preoperative GC patients than in postoperative sera and in the sera of healthy controls. The analysis of this peptide presented greater diagnostic ability than the conventional CEA and CA19.9, particularly in stage I GC. Using the 2D-DIGE methodology and mass spectrometry, Liu *et al.*^[53] also reported that kininogen-I was deregulated in the serum of GC patients and, in combination with other protein analyses, would be a useful diagnostic biomarker. However, before future clinical use, it is necessary to establish sample quality criteria (collection, processing and storage) and to understand changes in the serum proteome because of several factors, such as menstrual cycle, age, nutritional status, drug use and the presence of inflammation.

Gastric fluid can be collected during endoscopic examinations without additional discomfort. Currently, endoscopies yield a high GC detection rate. However, endoscopies have high technical requirements and require trained medical professionals^[54]. In addition, even if endoscopic procedures are performed properly, EGC can be not diagnosed if an endoscopist does not recognize the lesion. Superficial mucosal lesions mimicking gastritis (gastritis-like lesions) are difficult to detect even with optimum preparation and technique^[55]. Therefore, the implementation of new protein-based approaches in gastric fluid samples may be important for the accurate diagnosis of GC, particularly in its early stage.

The findings from gastric fluid proteomic studies have been recently reviewed by Wu & Chung^[56]. Different methodologies for large-scale evaluation of proteins have been applied for GC biomarker discovery in gastric samples. Most of the studies applied 2-DE followed by MS analysis, with some variation in experimental protocols and data analyses. Lee *et al.*^[57] and Hsu *et al.*^[58] reported that the α 1-antitrypsin and its precursor level, respectively, increase in the gastric fluid of GC patients. In a subsequent study, Hsu *et al.*^[59] evaluated α 1-antitrypsin in the gastric fluid of GC patients and controls by immunoassays. The authors reported that gastric fluid α 1-antitrypsin concentrations were markedly higher in GC patients than in healthy subjects, gastric ulcer patients and duodenal ulcer patients. Using a cutoff value of 717 μ g/dL of α 1-antitrypsin in gastric juice, the sensitivity, specificity, and accuracy to detect GC were 96%, 92% and 93%, respectively. In addition, the authors also evaluated the applicability of the gastric juice α 1-antitrypsin test for cancer screening using a string test to obtain gastric juice. The sensitivity and specificity of the gastric fluid α 1-antitrypsin string test at 85% accuracy were 74% and 88%, respectively. Chang *et al.*^[60] directly evaluated the gastric fluid proteome by MALDI-TOF-MS and also described that an α 1-antitrypsin fragment may help in GC diagnosis.

Wu *et al.*^[61] also used 2-DE (with higher resolution in the protein separation step than in the images presented in the previous studies) followed by MS to evaluate 12 patient gastric fluid samples (stages I, III, IV and gastritis), and differentially expressed proteins were validated by Western blots in 60 gastritis and GC patients. The

authors suggested that the combined analysis of S100A9, GIF and AAT from the gastric fluid may assist in GC diagnosis and prognosis, highlighting the idea that a panel of biomarkers is necessary to increase the accuracy of GC detection.

The gastric fluid proteome contains the salivary proteome because saliva is mixed with food before entering the stomach. In addition, blood or other body fluids, such as intestinal fluid and bile, may collect with the gastric fluid as a result of intestinal reflux^[43,44]. The saliva proteome or other fluid contaminants may be a confounding factor in gastric fluid analysis. Therefore, caution in sample handling and standardization of sample collection is necessary for the identification of gastric fluid biomarkers by proteomic approaches and for future clinical use.

In addition, gastric fluid proteomic analysis would reflect all abnormal lesions presented in the patient stomach, including non-neoplastic lesions. However, the analysis of the proteome or of a panel of protein biomarkers in gastric fluid and in serum samples may be used in combination with endoscopy examination and endoscopic follow-up of patients with clinical symptoms or precancerous lesions to detect EGC with high accuracy.

POST-TRANSLATION MODIFICATIONS ARE ALSO IMPORTANT

Most proteins undergo modifications after translation, such as phosphorylation, glycolysation, acetylation, methylation, ubiquitination and SUMOylation. These modifications are crucial for protein heterogeneity and contribute to variation in protein stability, location and function.

Using a proteomic approach to search for differentially expressed proteins in GC samples, our group previously observed that some proteins were reflected by multiple spots, which is most likely because of post-translational modifications resulting in shifts in the 2-DE gel^[26]. Using 2-DE analysis, we identified two spots for the ENOA protein that presented a higher expression in GC compared with non-neoplastic gastric samples. One of the spots displayed higher expression in both tumors with and without LNM compared with non-neoplastic samples. However, the other spot only differed between tumors with LNM and non-neoplastic tissues. In contrast to the 2-DE observation, the protein level of ENOA displayed a 1.5-fold reduction in 35.3% of the GC samples compared with their paired non-neoplastic gastric tissues, and only one protein displayed a 1.5-fold increase by Western blot. In our proteomic study, we only selected the spots differentially expressed by at least 1.5-fold between groups for MS analysis. Thus, the other spots of ENOA may display slightly reduced expression, but with a high effect for the mean protein expression. Our results demonstrated that different spots may be differently regulated inside a heterogeneous gastric sample and that the two differentially expressed spots may be involved in gastric carcinogenesis.

Lim *et al.*^[14] identified one spot of NNMT with elevated expression in GC samples compared with non-tumor specimens by proteomic analysis. The authors selected this protein for further investigation. Using an anti-NNMT antibody in a 2-DE gel, the authors detected a single spot in gastric ulcer tissues, whereas four to five spots were detected in GC tissues. The study suggested that NNMT receives a post-translational modification in a cancer-specific manner.

Post-translational modifications may also be used as markers for diagnosis and prognostic monitoring in GC. Bones *et al.*^[56] performed a glycomic and glycoproteomic analysis of sera from patients with GC and controls. The authors identified 12 differentially expressed

proteins (with different isoforms) in the sera of GC patients. Most of these proteins carried sialyl Lewis X (SLe^x) epitopes on triantennary trisialylated glycans. In addition, the authors observed that the levels of SLe^x present on the leucine rich-R2-glycoprotein, haptoglobin and kininogen-1 increased with tumor stage, whereas the levels of SLe^x present on clusterin decreased. SLe^x structures are recognized by selectin molecules present on the surfaces of platelets, endothelia and innate immune cells. The authors suggested that the processing of SLe^x epitopes on tumor cell surface glycoproteins may help in the interaction with selectins for transport through the vasculature to a secondary site for the propagation of metastases after extravasation. The levels of SLe^x on the differentially expressed proteins could potentially offer clinical utility as markers for monitoring cancer progression when used in conjunction with other diagnostic tools, such as CA19-9 screening^[62,63]. Moreover, the analysis of glycoproteomics, as well glycomics, may also help in the understanding of GC as a systemic disease.

The study of post-translation modifications may provide insights into the regulation of gastric cell function. Guo *et al.*^[57] performed an integrated LC-MS/MS-based and protein antibody array-based proteomic study to investigate the phosphoproteome of GC cell lines and endoscopic gastric biopsies from normal subjects and patients with benign gastritis or GC. The authors identified several phosphorylated proteins potentially implicated in critical roles in gastric carcinogenesis, including the phosphorylation of p53^[64]. In glioma stem cells, the phosphorylation of p53 and other checkpoint proteins was associated with radioresistance^[65]. The phosphorylation of p53 may explain GC resistance against several non-surgical treatments. The analysis of the glycoproteome of GC cell lines has also contributed to the understanding of the resistance of GC cells to chemotherapy. Li *et al.*^[66] compared the cell surface glycoproteome of two multidrug resistant cell lines and their parental drug sensitive GC cell line. The authors identified 11 glycoproteins, such as a glycosylated form of EGFR, that may be possible biomarkers for predicting multidrug resistance or key regulators for targeted therapies.

ADDITIONAL CONSIDERATIONS

It is important to highlight that analysis of the GC proteome remains dependent on the available technologies. Each technology presents distinctive strengths and weaknesses. Currently, a single proteomic technique cannot evaluate the entire diversity of proteins of a complex sample or the large dynamic range in the abundance of proteins in a single sample^[67]. The technologies of MS and sample fractionation have been constantly improving. Such progress will increase the knowledge of protein expression and modification in gastric physiology and pathology and therefore increase the chance to discover sensitive and specific GC biomarkers. However, the validation of proteomic results is necessary before they can be applied in clinical routines.

Proteomics studies have typically investigated a small number of samples. Although approximately 90% of stomach tumors are adenocarcinomas^[68], several factors result in biologically and clinically distinct GC subsets: antecedent tumorigenic conditions, such as *Helicobacter pylori* gastritis and other chronic gastric pathologies; location of the primary tumor (cardia and noncardia region); subtypes of adenocarcinoma (diffuse, intestinal, or mixed^[69]); ethnicity of the afflicted population (differing levels of susceptibility and aggressiveness of the tumors); and a predictive biomarker (ERBB2)^[70]. Thus, the term “gastric cancer” is used to describe several neoplasias that affect the stomach region. Because GC is

a heterogeneous disease and proteins are dynamically regulated, validation of potential biomarkers in large samples is necessary to better understand individual variations.

Moreover, there is not a consensus on the proteomic data and statistical analyses. The amount of information detected by proteomic approaches is vast, and therefore, data analysis is a challenge for the researcher. Typically, two or more biological groups are compared, and the proteins significantly different between groups are reported as potential biomarkers and selected for further investigation. However, it is necessary to highlight that the statistical comparisons currently applied have potential limitations in the understanding of complex diseases, such as GC. Even with the extraction of molecular signatures of biological processes using bioinformatics tools, much information is lost because gastric tumors are heterogeneous, and therefore, distinct pathways appear to be involved in the etiology of this disease.

FUTURE IMPLICATIONS

The large-scale analysis of proteins by proteomic approaches may help in the understanding of gastric carcinogenesis as a local and systemic disease. Proteomic analysis is a promising tool for the identification of biomarkers that (in combination) will help in GC diagnosis, prognosis and patient management and in the development of new anticancer treatments. However, further investigations are still required. Planning these studies considering the pre-analytical, analytical and post-analytical variables that can interfere in the results is important. The development of large studies performed by different research groups utilizing identical analysis parameters is vital to identify and validate the biomarkers with the greatest effects. Although GC proteomic analyses are still in their infancy, they are clearly important approaches for discovering biomarkers with potential clinical use.

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