Molecular Pathological Signatures of Gastric Cancer in Koreans Revealed by Label-Free Proteomics

Hee Jung Sul1, Jong Bok Seo2, Sung-Hwa Sohn1, Ji Woong Cho3, Kab Choong Kim3, A-Young Kim4, Boram Han5, Hyeong Su Kim5, Hyun Lim6, Ho Suk Kang7, Jae Seung Soh7, Jinwon Seo8, Young Ho Koh4,9* and Dae Young Zang1,5*

1Hallym Translational Research Institute, Hallym University Sacred Heart Hospital, Anyang, Korea
2Korea Basic Science Institute Seoul Center, Korea
3Department of Surgery, Hallym University Sacred Heart Hospital, Korea
4Ilsong Institute of Life Science, Hallym University, Korea
5Division of Hematology-Oncology, Hallym University Sacred Heart Hospital, Korea
6Division of Hematology-Oncology, Hallym University Kangnam Sacred Heart Hospital, Korea
7Department of Internal Medicine, Hallym University Sacred Heart Hospital, Korea
8Division of Pathology, Hallym University Sacred Heart Hospital, Korea
9Department of Bio-Medical Gerontology, Graduate School, Hallym University, Korea

*These authors contributed equally to the work

Abstract

Background: Recent studies in Gastric Cancer (GC) suggested that it could be a heterogeneous disease caused by various genetic defects in combination with environmental risk factors.

Objective: In this study, a quantitative label-free proteomic analysis were performed to detect differentially expressed proteins and fusion proteins that are only expressed in GC tissues and to identify the signal transduction pathways involved in the tumorigenesis of GC in Korean patients.

Methods: We identified 72 up-and 29 down-regulated proteins in at least 5 out of 9 GC tissues compared with paired normal tissues.

Results: These proteins were divided into 6 highly interacting clusters and 5 relatively related groups for which no interactions have been reported to date but cellular and molecular functions may be shared. Interestingly, the clusters and groups harbored targets for cancer drugs and prognostic markers for several cancers. In addition to these similarities to other cancers, the expression levels of ACTN4, ARG2 and ARG3 were significantly decreased in GC, suggesting differences in signaling in Korean GC compared with other cancers. Furthermore, we identified 2 fusion proteins that were expressed only in GC tissues and not in normal gastric tissues: TPM4-ALK and hnRNPA2B1-FAM96A.

Conclusion: Our label-free proteomic and biochemical analysis revealed that Korean GC showed tumorigenesis signaling characteristics shared with other cancers in addition to presenting novel defects, such as fusion proteins and altered expression.

Keywords: Stomach; Neoplasm; Label-free; Proteomics; Fusion proteins; TPM4-ALK; hnRNPA2B1-FAM96A

Introduction

Gastric Cancer (GC) is the third most prevalent cause of cancer-related death in the world [1]. In addition, GC was the second most common cancer in Korea in 2013, followed by thyroid cancer. The incidence rate of GC was 13.4% among total reported cancer cases. The mortality caused by GC in Korea was 11.6% in 2014, followed by lung cancer and liver cancer (http://www.cancer.go.kr/mbs/cancer/index.jsp). Recent changes in dietary habits and lifestyles in Korea may contribute to the progressive reduction of age-standardized GC mortality rates (http://www.cancer.go.kr/mbs/cancer/index.jsp). However, the increase in the elderly population (above 65 years of age) and the discovery of early GC according to the popularization of endoscopic examination have resulted in increased overall incidence rates. This trend is a common phenomenon worldwide according
to reports from GLOBOCAN 2012 (http://globocan.iarc.fr/Default.aspx) [2].

The genetic basis of GC has recently been extensively investigated, revealing that GC may not be a homogenous disease but rather a complex of diseases caused by a combination of numerous genetic mutations and environmental risk factors [3,4]. Several genomic studies in GC have suggested that mutations identified in GC tissues are diverse according to the type of GC, age of onset, race, and carcinogenic stomach regions. Numerous mutations, including both germline and somatic mutations, are differentially present in individual GC tissues. In contrast to genomic studies, proteomic studies on GC have not been extensively conducted to date, despite the fact that signal transduction pathways are mostly regulated by proteins expressed in cells and tissues.

One of the major goals in cancer proteomic research is to identify prognostic or predictive biomarkers for cancer patients by identifying differentially expressed proteins in cancer tissues [5]. Compared with other cancers, prognostic or predictive biomarkers for GC have not been extensively defined to date. One clinically applicable prognostic marker for GC is human epidermal Growth Factor Receptor 2 (HER2), which was initially identified in breast cancer and is observed in ~20% of recurrent or metastatic cancer patients [6].

Among cancer-causative mutations, fusion genes resulting from chromosomal translocations and rearrangements are well known driver mutations for numerous types of cancers [7]. The first and best-investigated fusion gene was identified in Chronic Myelogenous Leukemia (CML) and arose through fusion of the Breakpoint Cluster Region (BCR) gene and the Abelson murine leukemia viral oncopogene homolog 1 (ABL1) gene [8]. The identification of BCR- ABL1 fusion genes in CML led to the development of the targeted cancer drug imatinib, a tyrosine kinase inhibitor [9]. Fusion genes have also been found in solid tumors. For example, fusion of the Echinoderm Microtubule-associated protein-Like 4 (EML4) gene and the anaplastic lymphoma receptor tyrosine kinase (ALK) gene was reported to occur 4% of non-small cell lung cancer patients [10]. EML4-ALK-positive patients showed significant clinical benefits when they were prescribed the ALK inhibitor crizotinib (PF02341066).

Recent studies addressing the therapeutic effect of cancer treatments have suggested that genetic differences among cancer patients might be the most important factor determining the efficacy of treatments [11]. Thus, a recent trend in the treatment of cancer patients is the prescription of targeted drugs specific for certain mutations, after identifying genetic defects in cancer patients. One example of a targeted GC drug is Herceptin, a monoclonal antibody that specifically binds and inhibits the activity of HER2. Herceptin could have great clinical beneficial effect in ~20% of advanced HER2-positive GC patients when combined with chemotherapy [6,12]. Additional targets for developing targeted GC therapies include vascular endothelial growth factor receptor 2, Human Epidermal Growth Factor Receptor 3, Phosphatidylinositol 4, 5-bisphosphate 3-kinase catalytic subunit-α, MET and the fibroblast growth factor receptors [13].

For completely personalized medicine, additional genetic defects present in GCs must be elucidated. In this study, we employed quantitative and qualitative label-free proteomics to identify Differentially Expressed Proteins (DEPs) and fusion proteins that are exclusively present in GC tissues and not in normal tissues. The identified DEPs and fusion genes could be used as prognostic and predictive markers and targets for developing tools for precision medicines.

Materials and Methods

Clinical tissue samples

A total of nine pairs of gastric cancer and adjacent normal tissues were collected from informed and consented GC patients who underwent gastric resection at Hallym University Sacred Hospital in 2011. Core areas of the tumors, avoiding necrotic tissues and adjacent normal tissues, as confirmed by a pathologist, were used in this study. This research was approved by the clinical trial committee of Hallym University Sacred Hospital (Approval No.: 2011-1057). In addition, all experiments in this study performed accordance with the relevant guidelines and regulations. Experimental protocols used in this study were approved by a named institutional/licensing committee.

Protein extraction

For the label-free proteomic analysis, proteins were extracted from gastric cancer and adjacent normal control tissues with the T-PER Tissue Protein Extraction kit (Pierce Biotechnology, Rockford, IL). Briefly, 20 mg to 30 mg of tissue was ground with glass beads in 200 μL of the T-PER reagent containing protease inhibitors (Roche Diagnostics, Basel, Switzerland), followed by sonication using 6 bursts of 30 s. The protein extracts were subsequently centrifuged for 10 min at 15,000 g to obtain soluble fractions. All steps were performed on ice. Total proteins in the soluble fractions were quantified using the BCA assay. The supernatant was mixed with the loading buffer (Tris 40 mM pH 7.5, 2% SDS, 10% glycerol, 25 mM DTT), and the mixture was boiled for 5 min at 95°C. Then, 30 μg of protein per lane was loaded into a 12% SDS-PAGE gel. The gels were subsequently cut to separate each sample lane, and each lane was divided into 5 pieces. Finally, each piece was digested with trypsin gold, and the peptides were extracted and completely dried.

Label-free quantitative proteomics

Tryptic peptides were analyzed in triplicate using a nanoACQUITY UPLC (Waters, Milford, MA) coupled to a Synapt G1 HDMS mass spectrometer (Waters). Peptides were separated using a BEH 130 C18 75 μm×250 mm column with a particle size of 1.7 μm (Waters) and enriched on a Symmetry C18 RP (180 μm×20 mm, particle size 5 μm). For each experiment, 2 μL of tryptic-digested peptides was loaded onto the enrichment column with mobile phase A (water with 0.1% formic acid). A step gradient was employed at a flow rate of 280 nL/min, which included 5% to 45% mobile phase B (0.1% formic acid with acetonitrile) over 55 min, followed by a sharp increase to 90% B within 10 min. The eluted peptides were analyzed in positive ionization mode using the data-independent MS² mode. The MS/MS peaks of [Glu1]-fibrinopeptide (400 fmol/μL) were employed to calibrate the time-of-flight analyzer in the range of m/z 50 to 1990, and a doubly charged [Glu1]-fibrinopeptide ion (m/z 785.8426) was employed for lock mass correction. During data acquisition, the capillary voltage was set at 3.2 kV, and the source temperature was set at 100°C. The collision energies for low-energy MS mode (intact peptide ions) and elevated-energy mode were set to the 6 eV and 15 eV to 40 eV energy ramping modes (peptide product ions); respectively. The scan time was set to 1.0 s.

LC-MS² raw data files were processed, and protein identification and relative quantitative analyses were all performed using the ProteinLynx Global Server (PLGS 2.5.1, Waters). The processing parameters included automatic tolerance for precursor and product...
Western blot analysis

Protein extracts from normal and cancer tissues were separated via 12% SDS-PAGE and then transferred to nitrocellulose membranes. After blocking with 5% non-fat dried milk in TBST, mouse anti-TPM3 and anti-TPM4 (Developmental Study Hybridism Bank, University of Iowa, Ames, IO) antibodies were used to detect proteins from 5 pairs of GC samples and normal controls. Monoclonal anti-β-actin antibodies (Sigma-Aldrich, St. Louis. MO) were used as a loading control.

Results

Differentially expressed proteins in Korean GC patients

To identify DEPs in GC tissues, we performed label-free proteomics using 9 pairs of cancer and matched normal stomach tissues. We found that 72 and 29 proteins were commonly up-or down-regulated, respectively, in at least 5 GC tissues compared with normal control tissues (Figure 1).

To investigate the Gene Ontology categories of the DEPs, up- or down-regulated proteins were loaded into the Panther database (www.pantherdb.org) for categorization according to biological processes. Among the 72 up-regulated proteins identified in GCs, 42, 34, 23, and 21 proteins were allocated to the metabolic process, cellular process, localization, and cellular component organization or biogenesis categories, respectively (Figure 1a). In contrast, among the 29 down-regulated proteins, 13, 11, 10, and 9 proteins were allocated to the multicellular organismal process, metabolic process, developmental process, and cellular process categories, respectively (Figure 1b).

To further investigate the molecular and cellular etiology underlying the pathogenesis of Korean GC, the interactions between DEPs in Korean GC tissues were investigated using the Search Tool for Recurring Instances of Neighboring Genes (STRING) database (www.STRING-db.org). Figure 2 presents the results of STRING analysis. Red nodes indicate up-regulated proteins, and blue represents down-regulated proteins in cancer tissues. The colored lines between nodes represent the various types of interaction evidence [14]. The DEPs were further divided into 6 clusters that contained most of the highly interacting DEPs and 5 groups that did not interact within groups but had similar known or predicted functions.

Altered expression of proteins regulating the actin cytoskeleton and motor activity

Cluster I contained components of two major pathways: Regulation of the actin cytoskeleton and motor activities. Interestingly, Actin-binding proteins and cross-linkers, such as Actinin α-4 (ACTN4), Actinin α-1, Actinin α-1 skeletal muscle, Moesin (MSN), Vinculin, and Transgelin, were consistently down-regulated in GCs. The expression levels of ACTN4 in pancreatic, ovarian, lung, and salivary gland cancer [15] and MSN in breast cancer [16] were altered. In contrast, among the levels of proteins regulating actin polymerization, such as Actin-related protein 3 Homolog, IQ motif-containing GTPase Activating Protein 1 (IQGAPI), Adenylate cyclase-associated protein 1, GDP dissociation inhibitor 2, and Rho GTPase activating protein 1, were significantly increased in GCs. Among these proteins, up-regulation of IQGAPI1 is known to be involved in tumorogenesis in lung, ovarian, stomach, and colon cancers [17].

Motor activity regulatory components, such as Myosin light chain 9, regulatory (MYL9), Myosin light chain 6, alkali, smooth muscle and non-muscle, Tropomyosin 1-α (TPM1), Tropomyosin 2-α, and Tropomyosin 3 (TPM3), were down-regulated in GCs. However, Myosin Heavy Chain 9, non-muscle (MYH9) was up-regulated. Among these proteins, MYL9 [18], TPM1 [19] and MYH9 [20] are known to be involved in tumorogenesis in various cancers. Other down-regulated components included intermediate filament components such as Vimentin and Desmin (Figure 2I), which are highly associated with hepatocellular carcinoma [21] and colorectal cancer [22], respectively.
Significantly up-regulated major components of microtubules

Among the diverse Tubulins present in humans, 6 α-Tubulins [Tubulin α (TUBA)-1A, TUBA-1B, TUBA-1G, TUBA-3E, TUBA-4A, and TUBA-8], 7 β Tubulins [Tubulin β (TUBB), TUBB-2A, TUBB-2B, TUBB-3, TUBB-4A, TUBB-4B, TUBB-6, and TUBB-8], and two regulatory components [Filamin A (FLNA), Chaperonin containing TCP1 subunit 6A] were significantly up-regulated in GCs (Figure 2 II). β Tubulins are established targets for anti-cancer drugs [23], and FLNA was reported to be up-regulated in breast cancer tissues [24].

Altered ion-binding proteins in GCs

Among the DEPs, iron- and oxygen-binding proteins, such as Hemoglobin (HB) subunit-β, HB subunit-γ2, HB subunit-δ, HB subunit-ε1, and HB subunit-γ1 were significantly down-regulated in GCs. In addition, the binding of Albumin to Ca²⁺, Na⁺, and K⁺ was down-regulated in GCs. In contrast, another iron-binding protein, Transferrin, and the zinc-containing Carbonic anhydrase enzymes were significantly up-regulated in GCs (Figure 2 III).

Up-regulated glycolysis metabolism-associated proteins in GCs

Proteins in Cluster IV are involved in various metabolic processes. Among these proteins, Glyceraldehyde-3-phosphate dehydrogenase, Enolase 1 (ENO1), ENO2, Glucose-6-Phosphate Isomerase (GPI), Pyruvate Kinase Muscle (PKM), and Phosphoglycerate Kinase 1
(PGK1) are highly associated with glycolysis. These genes were up-regulated and closely associated each other. This cluster also included the down-regulated Malate Dehydrogenase 1, ATP synthase H transporting mitochondrial F1 complex α-subunit and cardiac muscle and the up-regulated Citrate Synthase found in mitochondria (Figure 2 IV).

**Most molecular chaperone-related proteins were up-regulated**

Cluster V (Figure 2V) included two hub molecular chaperones, Heat Shock Protein (HSP) 90 kDa cytosolic-α class A member 1 and HSP90 α-family class b member 1, which strongly interacted with HSP family A member 1 like, HSP family A member 2, HSP family A member 5, HSP family A member 6, HSP family A member 8, HSP family A member 9, HSP family D member 1, Hypoxia up-regulated 1, and HSP B member 1 (HSPB1). With the exception of HSPB1, all HSPs were up-regulated in GCs.

**Up-regulated proteins with protein folding and trafficking activities**

Cluster VI (Figure 2VI) included up-regulated proteins that localized to the Golgi Apparatus (GA) or Endoplasmic Reticulum (ER) and regulated protein folding and trafficking. Valosin-containing protein, Major histocompatibility complex class I (HLA)-B, HLA-C, Ribophorin II (RPN2), Protein Disulfide Isomerase family A (PDIA) member-3, PDIA member-6, Prolyl 4-hydroxylase subunit-β, Calnexin (CANX), Calreticulin (CALR), a domain containing 5, and Glucosidase IIα-subunit were significantly up-regulated proteins.

**Up-regulated proteins involved in protein synthesis**

Three proteins involved in protein synthesis: Eukaryotic translation elongation factor (EEF)-2, EEF-1A1, and EEF-1A2, were up-regulated and interacted with components in other clusters. Among these proteins, EEF1A2 is involved in tumorigenesis in ovarian cancer (Figure 2a) [25].

**Down-regulated proto-oncogenes and up-regulated peptidase inhibitors in GCs**

Proto-oncogenes, such as Anterior gradient 2 [26,27], Anterior gradient 3 [27], Ras suppressor-1 [28], SET nuclear proto-oncogene [29], Tryptophanyl-tRNA synthetase [30], and Ubiquitin-like modifier activating enzyme 1 [31], were significantly down-regulated in GCs (Figure 2b). In addition, Serpin peptidase inhibitor clade (SERPIN)- A member 1 (SPERPA1), which is involved in tumor progression in GCs [32] and colorectal cancer [33], and SERPIN-H member 1, which has been identified as a potential biomarker for early-stage hepatocellular carcinoma [34], were found to be significantly increased in GCs in the present study (Figure 2c).

**Altered proteins involved in energy metabolism and cellular structural components**

Creatine kinase B, which regulates energy homeostasis in tissues and is decreased in cervical cancers [35], was down-regulated in GCs (Figure 2d). In contrast, ATPase Na+/K+-transporting subunit α-1, which maintains energy homeostasis; mitochondrial Aldehyde dehydrogenase 2 family, which oxidizes aldehydes to generate carboxylic acids; and Gastric type Lipase F, an enzyme involved in the digestion of dietary triglycerides, were significantly up-regulated in GCs.

Other proteins with multiple domains that are important for organizing cellular structures also exhibited alterations. For example, POTE Ankyrin domain family member (POTE)-I and POTEI were down and up regulated in GCs, respectively (Figure 2e). Lumican, a member of the small leucine-rich proteoglycan family that regulates collagen fibril organization and is involved in prostate cancers [36], was significantly down-regulated in GCs. Major Vault Protein is highly overexpressed in drug-resistant cancer [37]. Karyopherin subunit β-1, Junction Plakoglobin, Lamin A/C, Multiple PDZ domain crumbs cell polarity complex component, Clathrin heavy chain, and Leucine-rich pentatricopeptide repeat-containing were up-regulated.
Nevertheless, GCs remain one of the most common cancers in Korea contributed to remarkably decreased mortality worldwide [13].

Discussion

To gain further insight into the possible roles of the 2 fusion proteins in tumorigenesis, the 3-D structures of the two fusion proteins were predicted by performing homology modeling. The 3-D structure of the TPM4-ALK fusion protein showed that it contains an ALK kinase domain and a TPM that primarily consists of an alpha helix and may assemble into parallel dimeric coiled-coils with normal TPM4 or other TPM4-ALK fusion proteins (Figure 3a). The 3-D modeling of the hnRNPA2B1-FAM96A fusion protein revealed that the fusion protein presented as a dimer, given that FAM96A is known to form dimers (Figure 3b) [38]. The ability of the fusion proteins to dimerize suggested that the localization of the fusion proteins could be different from that of the normal proteins and might alter cellular and molecular processes.

Increased size of bands recognized by monoclonal TPM4 antibodies in GC tissues

To confirm the presence of TPM4-ALK fusion proteins, we performed Western blot analysis using monoclonal antibodies specific to TPM3 (Figure 4B) or TPM4 (Figure 4A). Anti-TPM4 antibodies recognized bands of increased sizes that were not detected in normal gastric tissues. However, anti-TPM3 antibodies did not detect any additional bands. Only one band from GC tissues showed the same migration pattern observed in normal gastric tissues (Figure 4).

Figure 4: Monoclonal TPM4 antibodies recognized large fusion proteins in protein extracts from GC tissues, but not those from normal stomach tissues. a) When monoclonal anti-TPM4 antibodies were used, two TPM4 bands were detected in GC tissues but not in normal control stomach tissues. The above bands were predicted to be fusion proteins. b) Monoclonal-β-actin antibodies were used as loading controls. c) Monoclonal TPM3 antibodies only detected single bands in GC tissues. d) The amount of protein loaded in each lane was confirmed with monoclonal β-actin antibodies. Monoclonal-β-actin antibodies were used as loading controls.

Label-free proteomic analysis identified 2 fusion proteins in GC

We searched MS data obtained from 9 pairs of GC and normal tissues using the COSMIC fusion gene database. Twenty-six candidate fusion proteins were obtained. We manually examined whether peptides spanning the junction of two proteins were identified by mass spectrometry. Only 2 fusion proteins were confirmed to have a corresponding peptide spanning two proteins: TPM4-ALK and hnRNPA2B1-FAM96A (Figure 3).

Interestingly, the 6 clusters identified in this study harbored targets for currently prescribed cancer drugs or their homologs as well as proteins known to play pivotal roles in tumorigenesis and metastasis. For example, various βTubulins are known targets for Paclitaxel and Vinca alkaloids that inhibit chromosome mitosis and angiogenesis in tumor cells [23], and these targets formed a cluster (Figure 2II). In addition, the levels of heat shock molecular chaperones, which are increased in most cancers [40], were found to be significantly increased in the present work as well and formed a cluster (Figure 2V). Furthermore, ER- and GA-resident components, which regulate protein folding, modifications, and trafficking, were also significantly increased and formed a cluster. Among these proteins, RPN2, a component of an N-oligosaccharyl transferase complex that is involved in anti-cancer drug resistance in breast cancer [41] and non-small cell lung cancer [42], was significantly increased, suggesting possible conservation of drug resistance and metastasis mechanisms in GC. In addition, the significant increases in HSPA5, CALR, and CANX in GC (Figure 2V and 2VI) suggested that the Unfolded Protein Response (UPR) might be activated in GC, similar to the activation of the UPR observed in Helicobacter-induced GC [43], to overcome hypoxia and a low nutrient supply, which are two initial stress conditions observed in cancer cells that induce aggressiveness in a murine model of melanoma [44]. Interestingly, we also identified a cluster harboring significantly up-regulated glycolytic enzymes, such as PKM, PGK1, ENO1, ENO2, and GPI (Figure 2 IV). Recent studies on cancer metabolism have suggested that up-regulation of glycolytic enzymes is associated with tumorigenesis and drug resistance [45,46] and could be used as prognostic bio-markers [47]. Considering these findings together, most of the DEPs identified in this study are already known to be linked to tumorigenesis, metastasis, and drug resistance.
Another interesting finding of this study was the two fusion proteins identified in GCs (Figure 3 and Additional file 1). The use of targeted cancer drugs has several advantages, such as high responsive rates with lower side effects in treatments [39]. However, only one targeted GC drug, Herceptin, is currently used to treat GC, and Herceptin is only effective in ~20% of HER2-positive GC patients [6,12]. Thus, to realize precision medicine in GC, new target genes or proteins should be elucidated. The two fusion proteins identified in this study are very intriguing given that ALK fusion genes are driver mutations in several cancers and targets for developing targeted cancer drugs [48]. In addition, hnrRNA2B1 also regulates the expression of tumor suppressor genes [49] and down-regulation of FAM96A has been reported in Gastrointestinal Stromal Tumors (GISTs) [50]. Furthermore, 3-homology modeling of fusion proteins suggested ALK fusion genes are driver mutations in several cancers and targets for developing targeted cancer drugs [48]. In addition, hnrRNA2B1 also regulates the expression of tumor suppressor genes [49] and down-regulation of FAM96A has been reported in Gastrointestinal Stromal Tumors (GISTs) [50].

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