

Identification of 14-3-3 ζ as a potential biomarker in gastric cancer by proteomics-based analysis

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Abstract. The identification of tumor biomarkers to support early diagnosis and tumor progression monitoring may potentially reduce the mortality of gastric cancer (GC). The present study aimed to detect novel tumor-associated antigens from the AGS GC cell line, and to identify their associated autoantibodies in sera from patients with GC by proteomics-based approaches. Proteins from AGS cell lysates were isolated using two-dimensional polyacrylamide gel electrophoresis, and western blotting was subsequently performed, to determine autoantibody responses in sera derived from patients with GC and healthy individuals. Positive protein spots were removed from gels stained with Coomassie blue, and were then evaluated by liquid chromatography-tandem mass spectrometry. Sera from patients with GC produced numerous spots, one of which was identified as 14-3-3 ζ . Autoantibody frequency to 14-3-3 ζ was 17.6% (15/85) in patients with GC, which was significantly higher than that in healthy control individuals (2.4%; 2/85; $P < 0.01$). These results suggested that the autoantibody against 14-3-3 ζ may be a potential serological biomarker for the detection and diagnosis of GC.

Introduction

Gastric cancer (GC) is the fourth most prevalent malignancy, and is the second most common cause of cancer-associated mortality worldwide (1,2). In addition, GC is one of the most common malignancies in East Asian countries (3). The leading cause of GC-associated mortality is the late detection of advanced stage GC (IIIA-IV), due to the absence of

early diagnostic biomarkers (4). A previous study revealed that the 5-year survival rate of patients with stage IV GC is only 7-10.1%; however, the 5-year survival rate of patients with stage IA is 78-93.3% (5,6). Early diagnosis of GC is essential for effective therapy; therefore, the detection of sensitive and specific biomarkers for GC diagnosis may significantly improve treatment and decrease mortality rates (7).

Autoimmunity refers to the production of antibodies to autologous cellular antigens and is increasingly being associated with malignancy, as autoantibodies against tumor-associated antigens (TAA) have been discovered in the sera of patients with various cancers and may be used as novel biomarkers (8). Due to the general absence of particular autoantibodies in normal conditions or non-cancer individuals, it is feasible to use autoantibodies as serum biomarkers for cancer diagnosis. In addition, autoantibodies exhibit various properties that make them attractive early cancer biomarkers (9-11). Firstly, autoantibodies may be identified in the asymptomatic stage of cancer, and may be measured as early as 5 years prior to the onset of malignancy (12). Secondly, autoantibodies against TAAs have been detected in the sera of patients with cancer. In addition, autoantibodies may be inherently persistent and stable in the sera for relatively long periods of time. Serum biomarkers may therefore be used to recognize tumorigenesis (13).

Generally, proteomics-based methods are available for recognizing protein biomarkers in the sera of patients with cancer (14). Using this approach, we previously identified novel serological biomarkers, including the anti-heat shock protein (HSP) 60 autoantibody for hepatocellular carcinoma (15), the anti-HSP70 autoantibody for esophageal squamous cell cancer (16) and the anti- α -enolase autoantibody for liver fibrosis (17). Measurement of serum autoantibodies against TAAs may not only facilitate diagnosis of GC, but also aid the advancement of molecular targeted therapy. The present study aimed to verify novel TAAs in the AGS GC cell line and identify associated autoantibodies in patient sera with GC using a proteomics method.

Materials and methods

Clinical materials and sample preparations. All clinical samples (85 sera samples from patients with GC and 85 sera

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samples from healthy control individuals) were obtained from the sera bank at the Cancer Autoimmunity Research Laboratory at the University of Texas (UTEP; El Paso, TX, USA). The patients with GC were histologically diagnosed and confirmed in accordance with the American Joint Committee on Cancer (18). Due to regulations regarding studies on human subjects, the names of the patients were not revealed to investigators, and some clinical information concerning sera was not available. The present study was approved by the Institutional Review Board of UTEP.

Cell culture and cell extracts. The AGS GC cell line was purchased from American Type Culture Collection (Manassas, VA, USA) and was cultured in 1640-RPMI medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 U/ml streptomycin. AGS cells were allowed to reach 90% confluence in 75-cm² Falcon tissue culture flasks. Cells were rinsed once with PBS, incubated with 1640-RPMI medium containing 25% trypsin-EDTA (Gibco; Thermo Fisher Scientific, Inc.), and finally placed into a 15 ml centrifuge tube.

Two-dimensional gel electrophoresis (2-DE) analysis. AGS cells were lysed in rehydration buffer (50 nM dithiothreitol, 8 M urea, 0.2% bio-lyte 3/10 ampholyte, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate and 0.001% bromophenol blue) purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA), and agitated using a vortex at room temperature for 90 min. Insoluble substances were discarded by centrifugation at 2,922 x g at 4°C for 30 min. The resulting supernatants were harvested, and the protein concentration was determined using the Bradford assay (Bio-Rad Laboratories, Inc.). For the first dimensional gel electrophoresis assay, 150 µg protein was mixed in rehydration buffer containing bromophenol blue reconstituted in proteomics-grade water, and electrophoresis was performed on a pH 3-10, 7-cm isoelectric focusing (IEF) strip (Bio-Rad Laboratories, Inc., Hercules, CA, USA). IEF was conducted at 50 mA per gel, 250 V for 30 min, followed by 4,000 V for 1.5 h, and 4,000 V for 5 h. Strips were instantly stored at -80°C until required. In the second dimensional gel electrophoresis assay, 12% SDS-PAGE gels for strips were used. Proteins from AGS cell lysates were isolated with 2-DE and visualized by Coomassie blue (Bio-Rad Laboratories, Inc.). To determine autoantibodies against antigens from AGS, proteins were isolated by 2-DE, transferred onto NC membranes for western blotting and then probed with sera from 10 patients with GC or 10 healthy controls. The protein spots were visualized using PDQuest 2-DE analysis software version 8.0.1 (Bio-Rad Laboratories, Inc.) (19).

One- and two-dimensional western blotting. To screen the autoantibody-positive sera, AGS cells were lysed in rehydration buffer directly and then boiled for 10 min. After the removing of the insoluble fraction by centrifuge, samples were loaded onto 12% SDS-PAGE gel, which is subsequently transferred onto nitrocellulose membrane (Bio-Rad Laboratories, Inc.) for western blotting. Following blocking with 5% non-fat

milk prepared in Tris-buffered saline (TBS), containing 0.05% Tween-20 (TBST), for 1 h at room temperature, the membrane was incubated with sera at a solution of 1:200. Horseradish peroxidase-conjugated goat antihuman IgG (cat. no. 31410; Invitrogen; Thermo Fisher Scientific, Inc.) was used as secondary antibody with a dilution of 1:5,000 for 1 h at room temperature. The positive bands were detected with an ECL kit (Thermo Fisher Scientific, Inc.). For 2D-Western blotting, the proteins on 2D gel were directly transferred onto nitrocellulose membrane and following the same protocol as described above.

In-gel digestion. Excised gel fragments were destained with 40 mM NH₄HCO₃ in 50% acetonitrile. Reduction was performed with 5 mM tris-2-carboxyethylphosphine hydrochloride (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at room temperature for 1 h, followed by alkylation with 50 mM iodoacetamide (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature in the dark. The dehydrated gel slices were digested with trypsin in 10 mM NH₄HCO₃ for 18 h and peptide digests were extracted using extraction buffer, which was performed in a linear gradient from 5 to 40% solvent A to B (Solvent A: 5% acetonitrile/0.1% formic acid, Solvent B: 80% acetonitrile/0.1% formic acid) over 60 min at a flow rate of 30 nl/min.

Nano-liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) analysis. The digests were analyzed using an Eksigent nanoLC™-1D-plus (SCIEX, Framingham, MA, USA) coupled to a LTQ XL™ Linear Ion Trap mass spectrometer (Thermo Fisher Scientific, Inc.) as follows: The digests were loaded onto an online dual trap set up (Eksigent Chrom XP nanoLC trap-column C18-CL-3 µm 120 Å, 350 µm x 0.5 mm) at a flow rate of 1.5 µl/min using channel 1A solution [98% water, 2% acetonitrile (ACN), 0.5% formic acid (FA)]. Separation was achieved on an Eksigent Chrom XP nano-LC C18-reverse phase column (3C18-CL-3 µm 120 Å, 0.075x150 mm) using channel 2 mobile phases (solvent 2A: 5% ACN/0.1% FA; solvent 2B: 80% ACN/0.1% FA, on a linear gradient of 5-45% 2B over 60 min at a flow rate of 300 nl/min). The MS system was set to perform one full scan (400-1,700 m/z range) followed by MS/MS scans of the 10 most abundant parent-ions (ESI voltage, 3 kV; isolation width, 3.0 m/z; 35 normalized collision energy). The dynamic exclusion was set to collect each parent-ion twice and then excluded for 120 sec.

Data analysis. The resulting MS/MS spectra (350-5,000 Da, monoisotopic) were searched against a Uniprot protein database (<http://www.uniprot.org/>) downloaded on April 4, 2013 comprising *Homo sapiens*, *Bos Taurus* and porcine trypsin using a SEQUEST® algorithm in Proteome Discoverer 1.4 software (Thermo Fisher Scientific, Inc.). The parameters for database search were: i) 2.0 and 1.0 Da for peptide and fragment mass tolerance, respectively; ii) full digest using trypsin after K/R (cleaving C-terminal of K and R amino acid residues) with up to two missed cleavages allowed; and iii) methionine oxidation as a fixed modification, and cysteine carbamidomethylation and deamidation of asparagine and

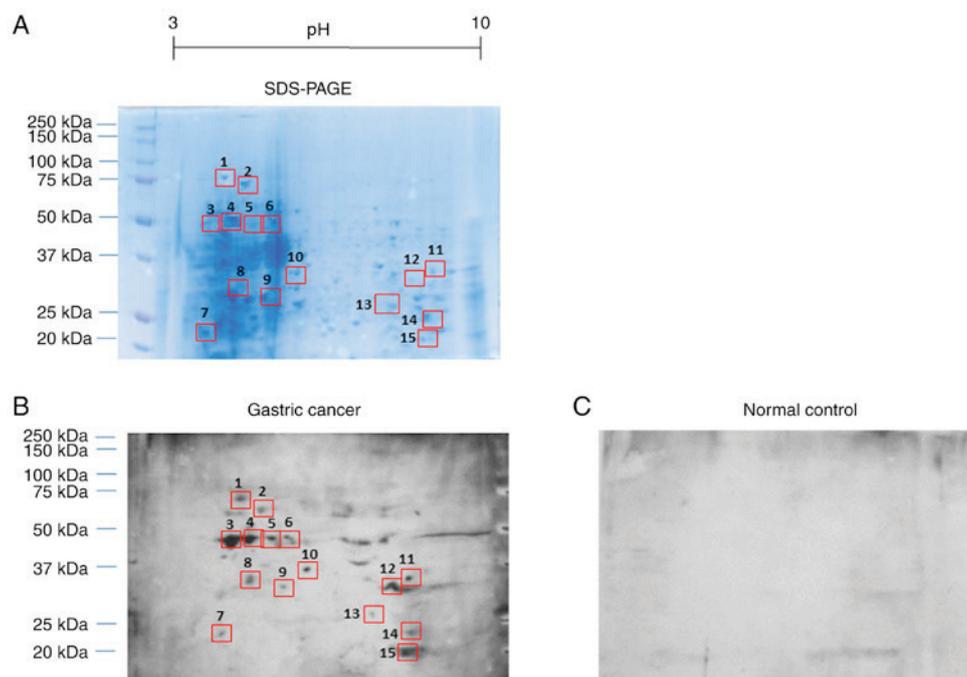


Figure 1. Screening of tumor-associated antigens. (A) Proteins from AGS cell lysates were separated by two-dimensional gel electrophoresis and visualized by Coomassie blue staining. (B) Proteins from AGS cell lysates were transferred to a NC membrane, which was then incubated with diluted sera from patients with gastric cancer. (C) NC membrane cultivated with sera from normal controls. NC, nitrocellulose.

glutamine as variable modifications. At least 2 peptides were used for assignment of proteins and search results were filtered for a false discovery rate of 1%, employing a decoy search strategy utilizing a reverse database.

Recombinant proteins and antibodies. The recombinant 14-3-3 ζ protein were provided by our laboratory (Department of Biology Sciences, UTEP; El Paso, TX, USA) (20). Polyclonal anti-14-3-3 ζ rabbit antibody (cat. no. ab51129) was purchased from Abcam, Inc. (Cambridge, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (cat. no. sc-2004) was from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), and HRP-conjugated goat anti-human IgG (cat. no. 31410) from Invitrogen (Thermo Fisher Scientific, Inc.). The recombinant proteins were for ELISA and antibodies for western blotting analysis.

ELISA. After diluting the 14-3-3 ζ protein in PBS to a final concentration of 0.5 μ g/ml to coat polystyrene 96-well microtiter plates (Thermo Fisher Scientific, Inc.), the plates were blocked with gelatin post-coating solution at room temperature for 2 h. The antigen-coated wells were incubated with human sera diluted at 1:100 with serum diluent (875 ml ddH₂O, 100 ml gelatin at 10 mg/ml, 20 ml 0.5 M phosphate buffer, 8.2 g NaCl, 0.1 g Thimerosal, 5 g bovine gamma globulin, 1 g bovine serum albumin and 5 ml 10%-Tween-20) at room temperature for 2 h. The final reactivity was detected using goat anti-human IgG-HRP and the substrate 2,2'-azino-bis-(3-ethylbenzo-thiazoline-6-sulfonic acid) (Invitrogen; Thermo Fisher Scientific, Inc.) (17). Data were analyzed by measuring the average optical density (OD) value at a wavelength of 405 nm. The cut-off value, which indicated a positive reaction, was the mean OD of 85 normal human sera (NHS) + 3 standard deviations.

Statistical analysis. Statistical analysis was conducted using SPSS software version 21.0 (IBM Corp., Armonk, NY, USA). Data were assessed by χ^2 test and ELISA data are presented as the mean \pm 3 standard deviations. $P < 0.01$ was considered to indicate a statistically significant difference.

Results

Identification of immunoreactive proteins in GC cells by LC-MS/MS. Proteins from AGS cell lysates were isolated with 2-DE and visualized by Coomassie blue staining (Fig. 1A). To determine autoantibodies against antigens from AGS, proteins were isolated by 2-DE, transferred onto NC membranes and then probed with sera from 10 patients with GC or 10 healthy controls. These sera were randomly chosen in both groups. Each NC membrane was incubated with mixed sera samples as the primary antibody, followed by an HRP-conjugated goat anti-human IgG as a secondary antibody. The reactivity of patients' sera with GC resulted in 15 spots (Fig. 1B), whereas no reactive protein spots were observed in the normal control samples (Fig. 1C). Therefore, the sera were considered to have non-specific reactivity. In the subsequent study, 15 immunoreactive protein spots were excised from the SDS-PAGE gels, digested with trypsin and further analyzed by LC-MS/MS. The resulting MS/MS spectra were searched with a Uniprot protein database, which is a comprehensive database for human protein sequences. As demonstrated in Table I, 14 of the 15 protein spots were identified by LC-MS/MS, with the exception of one uncharacteristic protein.

Prevalence and autoantibody titers against 14-3-3 ζ in GC. Sera from patients with GC and normal controls were tested for the response of autoantibodies to 14-3-3 ζ . The sera that

Table I. Summary of identified protein spots by mass spectrometry.

Spot no.	Accession no.	Identified protein	Molecular mass (kDa)	Protein abundance	Protein functions
1	P11021	GRP78	78	56	Interacts with many endoplasmic reticulum protein and monitors transport through the cell
2	B1AHM1	DEAD (Asp-Glu-Ala-Asp) box polypeptide 17	72.5	35	Implicated in a number of cellular processes involving alteration of RNA secondary structure
3	Q53G71	Calreticulin variant	46.9	30	Acts as a major Ca ²⁺ -binding protein in the lumen of the endoplasmic reticulum
4	Q53G71	Calreticulin variant	46.9	17	Acts as a major Ca ²⁺ -binding protein in the lumen of the endoplasmic reticulum
5	Q53G71	Calreticulin variant	46.9	21	Acts as a major Ca ²⁺ -binding protein in the lumen of the endoplasmic reticulum
6	Q53G71	Calreticulin variant	46.9	42	Acts as a major Ca ²⁺ -binding protein in the lumen of the endoplasmic reticulum
7	P62820	Ras-related protein Rab-1A	22.7	61	Controls vesicle traffic from the endoplasmic reticulum to the Golgi apparatus
8	B4E3C4	Uncharacterized protein	33.8	5	-
9	E7EUT4	Glyceraldehyde-3-phosphate dehydrogenase	31.5	59	Serves a role in glycolysis and nuclear functions
10	B4DVQ0	Actin	37.3	14	Involved in various types of cell motility
11	P22626	hnRNP A2/B1	37.4	17	Serves an important role in proliferation
12	Q1WWK5	SEPT9 protein	36.5	9	Is overexpressed in diverse human tumors
13	P63104	14-3-3 protein zeta	27.7	45	Mediates signal transduction by binding to phosphoserine-containing proteins
14	Q06830	Peroxiredoxin-1	23	55	Serves a role in cancer development or progression
15	P30086	PEBP	21	2	Interacts with MAP2K1, c-Raf and MAPK1

GRP78, glucose-regulated protein 78; DDX3X, DEAD-box helicase 3 X-linked; Rab1a, RAB1A member of RAS oncogene family; hnRNP A2/B1, heterogeneous nuclear ribonucleoprotein A2/B1; SEPT9, septin 9; PEBP, phosphatidylethanolamine-binding protein; MAP2K1, mitogen-activated protein kinase kinase 1; MAPK1, mitogen-activated protein kinase 1; c-Raf, proto-oncogene c-RAF.

were detected included 85 from patients with GC, and 85 from normal human individuals. Table II revealed that autoantibody frequency to 14-3-3 ζ was 17.6% (15/85) in GC sera, which was significantly higher than that in NHS (2.4%; 2/85; $P < 0.01$). In addition, as presented in Fig. 2, autoantibody titers against 14-3-3 ζ in GC sera were higher than in NHS ($P < 0.01$). These ELISA results were further confirmed by western blot analysis (Fig. 3), where a positive reaction to 14-3-3 ζ was observed in representative GC sera compared with normal sera.

Discussion

The recognition of TAAs that evoke an antibody response may be utilized for the early diagnosis of cancer, in monitoring prognosis and as immunotherapy targets (21,22). With advancement in protein isolation and identification methods, proteomics-based technologies have acquired increased popularity for the detection and recognition of TAAs and associated autoantibodies. Thus, 2-D/MS remains a key technique in

Table II. Frequency of autoantibodies against 14-3-3 ζ in human sera, as determined by ELISA.

Type of sera	No. tested	Autoantibody to 14-3-3 ζ (%)
GC	85	15 (17.6) ^a
NHS	85	2 (2.4)

Cut-off value: Mean + 3 standard deviations of NHS; ^a $P < 0.01$ vs. NHS. GC, gastric cancer; NHS, normal.

proteomics for global protein profiling and serves a complementary role to LC-MS-based analysis (23). In addition, it enables experiments to be performed with autoantibodies, and therefore improves screening of antigenicity associated with abnormal post-translational alterations of cancer cell proteins.

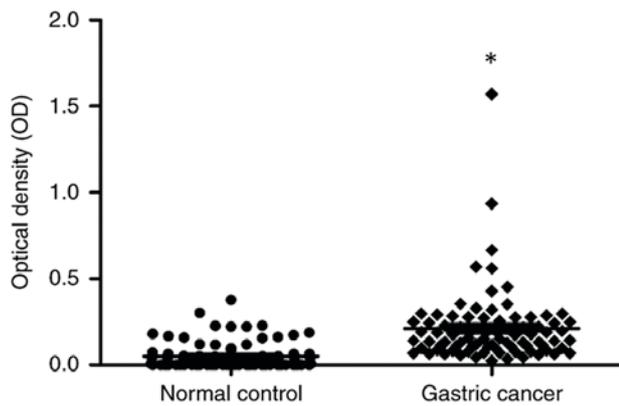


Figure 2. Autoantibody titers to 14-3-3 ζ in sera of patients with GC or normal controls. The range of antibody titers to 14-3-3 ζ were expressed as OD obtained via ELISA. Titers of autoantibody to 14-3-3 ζ were greater in GC sera compared with in normal control sera. * $P < 0.01$. GC, gastric cancer; OD, optical density.

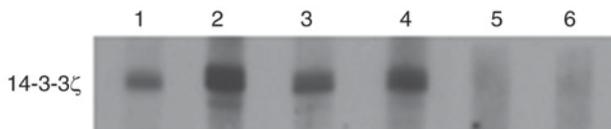


Figure 3. Reactivity of GC or normal control sera to 14-3-3 ζ , as determined by western blotting. The polyclonal anti-14-3-3 ζ antibody served as a positive control (lane 1). Sera from three representative GC patients, which were positive by ELISA, also had strong reactivity with 14-3-3 ζ recombinant protein by western blotting (lane 2, 3 and 4). Randomly selected normal control sera that had negative reactivity (lane 5 and 6). GC, gastric cancer.

Proteomics techniques have been adopted to explore protein expression in various human cancers. The most widely used technique for large-scale protein expression detection is 2-DE combined with MS, which may be an optimal method in the pursuit of reliable immunodiagnostic biomarkers. With this proteomic technique, autoantibodies against annexin-II (24), annexin-I, protein gene product 9.5 (25), peroxiredoxin-I (26), calreticulin (27), peroxiredoxin-VI (28) and RS/DJ-1 (29), have been identified in the sera of patients with pancreatic, lung, esophageal squamous cell and breast cancers.

In the present study, an immunoproteomics-based approach was used to identify biomarkers associated with the humoral immune responses in patients with GC. This approach incorporated SDS-PAGE, 2-DE and western blotting to detect autoantibodies in sera from patients with GC, which reacted to proteins separated by 2-DE and were confirmed by MS. In total, 11 available proteins were identified. One of them, glucose-regulated protein 78 (GRP78), has been regarded as a diagnostic biomarker for GC (30). Two other proteins, actin and GAPDH, are housekeeping proteins. Therefore, the 8 remaining tumor-associated proteins may be considered candidate antigens.

The molecular functions of the identified proteins have been documented in the literature, and the majority of these proteins are associated with various cellular functions, including cell differentiation, proliferation, apoptosis and signaling transduction. To further investigate the association of these identified proteins with cancer, literature searches

were conducted using PubMed (<http://www.ncbi.nlm.nih.gov/pubmed/>). GRP78 is a molecular chaperone in the endoplasmic reticulum (ER), which may be a candidate biomarker in GC (30). DEAD-box helicase 3 X-linked (DDX3X) is a hepatitis C virus core protein-associated cellular factor that belongs to the DEAD box RNA helicase family (31-33). Whole-exome analyses have revealed that mutated DDX3X serves an essential role in oncogenesis (34). Calreticulin is also an ER chaperone, which serves a role as a stress protein. Overexpression of calreticulin occurs in numerous malignancies, including in cancers of the prostate, breast, bladder, liver and lung (35-38). Rab-1A, which is a Ras-associated protein, may be of pathological significance in dealing with glucocorticoid-induced osteoporosis (39). GAPDH and actin have various functions in cells, in particular, they serve a vital role in the control of gene expression. Heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP A2/B1) is a member of the hnRNP A/B family that may be considered a prognostic marker of hepatocellular carcinoma (40). Septin 9, which is a cytoskeletal component, has been recognized as a promising oncogene in breast tumorigenesis (41). 14-3-3 ζ is a member of a family of seven highly conserved proteins that is considered a novel predictive biomarker of tamoxifen therapeutic resistance in breast cancer (42,43). Peroxiredoxin-1 is a member of the peroxiredoxin family of antioxidant enzymes and may be a potential biomarker for screening patients with hepatocellular carcinoma (44). Phosphatidylethanolamine-binding protein is regarded as a signal transduction mediator and a suppression of metastasis in cancer, and is often downregulated in various human malignancies (45), primarily in highly metastatic cancers (46-50). However, the present study focused on the 14-3-3 ζ protein to identify whether this antigen may be considered a tumor biomarker in the immunodiagnosis of GC. As these identified proteins are associated with cancer, future work to evaluate which of these may be attractive TAAs in GC is required.

The 14-3-3 proteins are highly conserved regulatory molecules that are universally expressed in all eukaryotic organisms (51). In humans, seven isoforms of 14-3-3 (β , ϵ , η , γ , τ , ζ and σ) have been verified (52). Although 14-3-3 proteins are short of endogenous enzymatic activity, they function by forming homo- or hetero-dimers and binding to phosphorylated-serine/threonine motifs on their target proteins (53). By modulation of their binding cooperators, 14-3-3 proteins have been implicated to regulate a mass of cellular processes, including mitogenesis, apoptosis, cell cycle progression, stress signaling, metabolism, cytoskeletal integrity and transcription (54,55). Numerous studies have reported that members of the 14-3-3 family, particularly 14-3-3 ζ , serve a pro-oncogenic role in various tumor types, and overexpression of 14-3-3 ζ is primarily associated with poor survival of patients with cancer (56,57). 14-3-3 ζ has been recognized as a clinically relevant prognostic marker for lung cancer, breast cancer, and head and neck cancer (58-60). The present study identified 14-3-3 ζ as a candidate biomarker for GC and determined autoantibodies against 14-3-3 ζ by ELISA and western blot analysis. It was demonstrated that serum autoantibody prevalence to 14-3-3 ζ was significantly stronger in patients with GC (17.6%) than in normal individuals (2.4%). Intensive studies are required to assess the sensitivity and specificity of

autoantibodies against 14-3-3 ζ in a greater number of samples, and to explore the underlying molecular mechanism of autoantibody response to 14-3-3 ζ in the progression of GC.

14-3-3 proteins are generally cytosolic but also bind to several nuclear proteins; therefore, it is hypothesized that they may serve as a cytoplasmic anchor and block nuclear import of target proteins (61). Several proteins whose nuclear trafficking is regulated by 14-3-3 include a bipartite nuclear localization signal (NLS). 14-3-3 binding masks the NLS and displaces the import complex, which leads to the cytoplasmic localization of target proteins (61). 14-3-3 proteins do not have any catalytic activity; however, they exert their effect by modulating subcellular localization or catalytic activity of target proteins and by regulating formation of protein complexes (62). 14-3-3 proteins interact with several proteins that potentially mediate various cellular processes (63,64). Therefore, 14-3-3 ζ may bind several proteins and potentially mediate the protein complex involved in numerous biological functions and tumor progression.

In conclusion, the present study detected autoantibodies against 14-3-3 ζ in sera from patients with GC. 14-3-3 ζ may be attractive not only as a serological tumor biomarker, but also to guide efficient immunological surveillance. With regards to the detection of novel autoantibodies, including 14-3-3 ζ , the proteomics approach employed in the present study may be considered a promising method to identify potential proteins that have clinical utility in malignancy.

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