

Identification of Serum Biomarkers for Gastric Cancer Diagnosis Using a Human Proteome Microarray*[§]

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We aimed to globally discover serum biomarkers for diagnosis of gastric cancer (GC). GC serum autoantibodies were discovered and validated using serum samples from independent patient cohorts encompassing 1,401 participants divided into three groups, *i.e.* healthy, GC patients, and GC-related disease group. To discover biomarkers for GC, the human proteome microarray was first applied to screen specific autoantibodies in a total of 87 serum samples from GC patients and healthy controls. Potential biomarkers were identified via a statistical analysis protocol. Targeted protein microarrays with only the potential biomarkers were constructed and used to validate the candidate biomarkers using 914 samples. To provide further validation, the abundance of autoantibodies specific to the biomarker candidates was analyzed using enzyme-linked immunosorbent assays. Receiver operating char-

acteristic curves were generated to evaluate the diagnostic accuracy of the serum biomarkers. Finally, the efficacy of prognosis efficacy of the final four biomarkers was evaluated by analyzing the clinical records. The final panel of biomarkers consisting of COPS2, CTSF, NT5E, and TERF1 provides high diagnostic power, with 95% sensitivity and 92% specificity to differentiate GC patients from healthy individuals. Prognosis analysis showed that the panel could also serve as independent predictors of the overall GC patient survival. The panel of four serum biomarkers (COPS2, CTSF, NT5E, and TERF1) could serve as a noninvasive diagnostic index for GC, and the combination of them could potentially be used as a predictor of the overall GC survival rate. *Molecular & Cellular Proteomics* 15: 10.1074/mcp.M115.051250, 614–623, 2016.

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Gastric cancer (GC)¹ is the second leading cause of cancer-related deaths. A total of 952,000 new GC cases (6.8% of the total of the new cancer case) and 723,000 deaths (8.8% of the total new cancer case) occurred in 2012 (1). The highest mortality rates have been reported in East Asia, including China, Japan, and Korea (2–4), and ~60% of new GC cases and deaths worldwide occur in this region. As GC has a 5-year survival rate of less than 15%, accurate diagnosis and prognostic assessment of patients are essential for optimizing therapeutic strategies, predicting the outcome of treatment, extending the survival period of patients, and potentially healing to reduce cancer mortality (5).

A variety of serum antigen biomarkers has been used for GC diagnosis and prognosis, *e.g.* carcinoembryonic antigen, carbohydrate antibody 19-9 (CA19-9), carbohydrate antibody 72-4 (CA72-4), and carbohydrate antibody 50 (CA50); the protein levels of these antigens in serum are usually used as signatures indicating cancer risk. However, generally, these serum antigen biomarkers lack sufficient sensitivity and specificity (6–8).

¹ The abbreviations used are: GC, gastric cancer; CTSF, cathepsins F; GP, gastric polyp; GU, gastric ulcer; CAG, chronic atrophic gastritis; AUC, areas under the curve.

Autoantibodies against proteins that result from abnormal gene expression and gene mutation in patients with various tumors represent another type of serum biomarker (9–12). The corresponding antigens of the autoantibodies are usually recognized as tumor-specific antigens or tumor-associated antigens (13–16). There is particular interest in these autoantibodies due to the potential diagnostic and prognostic applications of the autoantibodies and their corresponding antigens. Indeed, there is a need to identify novel autoantibody-based biomarkers to improve the sensitivity and specificity for the diagnosis of GC.

In this study, we used a human proteome microarray containing 16,368 proteins to discover and independently validate serum autoantibodies with potential for diagnosis and prognosis of GC in a set of 1,401 serum samples. The samples were from 537 GC patients, 550 healthy controls, and 314 individuals of GC-related diseases. Four autoantigen serum biomarkers, COP9 constitutive photomorphogenic homolog subunit 2 (COPS2), CTSF, ecto-5'-nucleotidase (NT5E), and telomeric repeat binding factor 1 (TERF1), were identified with a combined diagnostic sensitivity of 95% and specificity of 92%. Furthermore, our data suggested COPS2, CTSF, NT5E, and TERF1 could also serve as potential predictors for prognostic assessment.

EXPERIMENTAL PROCEDURES

Serum Sample Collection—The cohort was composed of 1,401 serum samples from 537 GC patients, 550 healthy volunteers, and 314 people with gastric ulcer (GU)/gastric polyp (GP)/chronic atrophic gastritis (Tables I and II and Fig. 3A) who had given informed consent and met the eligibility criteria (supplemental Table S1). GC samples were collected from multiple centers and annotated with detailed clinical information, including seven hospitals, *i.e.* Ruijin Hospital, the First Affiliated Hospital of Fujian Medical University, Shanghai East Hospital, Tongren Hospital, Shanghai Putuo Center Hospital, Shanghai Pudong Gongli Hospital, and the Shanghai Fifth People's Hospital, between August 2008 and June 2013 (supplemental Table S2). The sera were prepared according to standard protocol. Briefly, 5 ml of whole blood was collected from each individual and placed in a Vacutainer (BD Biosciences) without anti-coagulant. The whole blood was then left undisturbed at room temperature for 30 min. After centrifugation at $2,000 \times g$ for 10 min in a refrigerated centrifuge, the sera were then transferred as a 0.5-ml aliquot immediately to clean Eppendorf tubes and stored at -70°C .

Protein Microarray Fabrication—A human proteome microarray was constructed as described previously (17). Briefly, the purified fusion proteins were printed onto protein slides (Full Moon BioSystems, Sunnyvale, CA), using a ChipWriter Pro 48-pin contact microarray (Bio-Rad). Each protein was spotted in duplicate. Control proteins, including histone H3, histone H4, BSA, and biotinylated BSA, were also spotted in duplicate. The targeted protein microarrays used for the training/testing phase were constructed following the same procedure as for the human proteome microarray, except that the proteins were printed onto three-dimensional protein slides (CapitalBio Inc., Beijing, China) with 12 identical subarrays per slide. Controls for the targeted protein microarrays included GST, anti-IgG, and elution buffer.

Serum Profiling on Proteome Microarray—Serum samples were diluted 1:100 in Tris-buffered saline containing 0.1% Tween 20 de-

tergent (TBS-T). A total of 5 ml of the diluted serum sample was overlaid onto a protein microarray that was then incubated at room temperature for 1 h. The array was washed with TBS-T, and bound autoantibodies were detected by incubating with Alexa Fluor 532 goat anti-human IgG (H+L) (Jackson ImmunoResearch, West Grove, PA) diluted 1:1,000 in TBS-T at room temperature for 1 h. Arrays were washed with TBS-T and dried in a SlideWasher (CapitalBio Inc., Beijing, China) at room temperature. Microarrays were scanned in a GenePix 4200A (Molecular Devices, Sunnyvale, CA), and fluorescence data were analyzed with GenePix Pro 6.0 software (Molecular Devices). Targeted protein microarrays used in the training/testing phases were probed following the same procedure as that for the human proteome microarrays, except that 50 μl of diluted serum was loaded per subarray.

Protein Microarray Data Analysis—The median foreground and background intensities for each spot in the protein microarrays were obtained with GenePix Pro 6.0 software. The raw intensity for each spot was defined as the ratio of the foreground to the background median intensity. To remove the negative effects of nonspecific binding and spatial heterogeneity across the protein microarray, “norm-exp” (18), a background processing approach, was implemented in the R 2.15.1 package for background correction. To assess the quality of the protein microarrays, Array Quality Metrics in the Bioconductor R package (19) was employed to determine the quality and variability of the protein microarray experiment. Distance between Arrays and Boxplots functions were used to detect outlier microarrays, defined as those that failed both quality assessment methods using default settings in the Array Quality Metrics package. Outlier microarrays were excluded from further analysis.

Identifying Proteins Differentially Recognized by Serum Autoantibodies—After excluding outlier microarrays, the normalization was done twice during microarray data processing. The first round of normalization was performed by using the BackgroundCorrect package implemented in limma. The second round normalization was used to normalize between all the microarrays using Normalize between Arrays package in limma (20). Raw intensities were transformed to log₂ values. Differentially expressed autoantibodies were identified by “limma” and “MASS” packages. *p* values were calculated for each protein using Fisher's test and were adjusted for multiple testing using the Benjamini and Hochberg approach to control for false discovery rates. Proteins with adjusted *p* values of no more than 0.05 were identified as candidates. The Daim package implemented in R 2.15.1 was used to generate receiver operating characteristic curves.

MASS package in R contains a powerful environment for the statistical and graphical analysis of data, which is also called the S environment. Many statistical methods, such as linear, nonlinear, and smooth regression models, tree-based methods, multivariate analysis, pattern recognition, survival analysis, time series, and spatial statistics, are included in the MASS package. Modern techniques such as robust methods, non-parametric smoothing, and bootstrapping can also be found in MASS package.

ELISA—EIA/RIA Stripwell™ 96-well plates were obtained from Corning Glass. Plates were coated with 50 $\mu\text{g}/\text{ml}$ antigens in PBS, 100 $\mu\text{l}/\text{well}$, and kept sealed with parafilm at 4°C overnight. They were subsequently washed once with PBS-T, blocked for 1 h at room temperature with blocking buffer (0.2% bovine serum albumin (BSA), 0.1% gelatin, 0.02% thymersal in PBS-T), and then washed again. Residual buffer was vacuum-aspirated from each well. Each serum sample was diluted 1:100 in blocking buffer, loaded into the wells, and incubated for 1 h at 37°C , with duplicate wells being used for each serum sample. A 1:100 dilution was chosen because it gave the most reproducible results. The plates were then washed six times with PBS-T, and excess buffer was aspirated after the last wash. Incubation with anti-human IgG-peroxidase (Sangon Biotech, Shang-

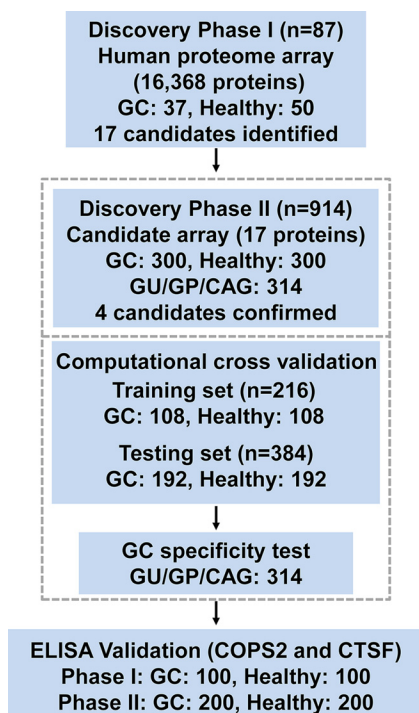


FIG. 1. Study design. This study is composed of three major phases, *i.e.* discovery phase I, discovery phase II, and ELISA validation phase. For a better chance of discovering novel serum biomarkers, the human proteome microarray of 16,368 proteins was applied for comparing a small set of samples. A targeted protein microarray with the 17 candidates identified from discovery phase I was fabricated and applied for the discovery phase II. The final four candidates were then computationally validated by 100 times random sampling. The specificity of the final candidates was also tested by samples of other gastric diseases. Finally, the candidates were tested for the possibility of ELISA application. *GU*, gastric ulcer; *GP*, gastric polyp; *CAG*, chronic atrophic gastritis.

hai, China) was performed for 1 h at 37 °C. After washing, the plates were developed using 100 μ l/well TMB (Tetramethylbenzidine) substrate from Sigma, and the reaction was stopped with 50 μ l/well of 1 M HCl. Optical density was read at 450 nm using a Behring EL311 ELISA microplate reader (Dade Behring Marburg GmbH, Berlin, Germany). A typical four-parameter logistic nonlinear regression model was used for standard curve fitting for the ELISA, which was further used to estimate sample content (units/ml) from the absorbance measurement data. Outliers were detected using the Boxplots function in the Bioconductor package.

Clinical Data Analysis—The differences of the clinical characteristics in each groups were evaluated by Fisher's test or χ^2 test. Survival time was calculated from the data of gastric cancer diagnosis to the date of death or the last follow up. The association between overall survival and protein expression levels was estimated by the Kaplan-Meier method and log-rank test.

RESULTS

Study Design—The whole study was composed of three phases as follows: discovery phase I, discovery phase II, and ELISA validation (Fig. 1). To ensure the reliability of the identified final serum autoantibody biomarkers, only serum samples with detailed clinical records and also meeting the eligi-

TABLE I
Characteristics of study participants in the discovery phase

Variable	GC count (n = 37)		Healthy count (n = 50)		p^a
	No.	Mean	No.	Mean	
Age, years					0.40
Mean		63.2		65.3	
S.D.		13.3		11.8	
Sex					0.013
Male	26	70	21	42	
Female	11	30	29	58	
TNM stage					
I	12	32			
II	8	22			
III	8	22			
IV	9	24			

^a Fisher's test.

bility criteria (supplemental Table S1) were collected. In each study phase, serum samples from GC patients with all four classical tumor stages (according to American Joint Committee on Cancer and Union for International Cancer Control 2010) were included. For global discovery and throughout the validation process, sera from 537 GC patients and 550 healthy volunteers were included for this study (supplemental Table S2). To avoid possible bias, these samples were collected from seven hospitals with similar cases for gastric cancer patients and healthy individuals from each hospital. To test the specificity of the identified serum autoantibody biomarker for GC diagnosis, sera from 314 people with other gastric diseases, *e.g.* GU, GP, CAG, and others, were also collected. In total, there were 1,401 samples included in this study, which is much larger than many other proteome microarray-based serum autoantibody biomarker studies, and thus a higher probability of identifying novel GC serum autoantibody biomarkers is expected. Because of the high cost of the human proteome microarray, it is neither economical nor efficient to carry out the whole study with the proteome microarray. In discovery phase II, the majority of samples probed on targeted protein microarray carries only the serum autoantibody biomarker candidates identified in discovery phase I. Using the targeted protein microarray, 12 samples could be assayed on a single microarray; the cost was significantly reduced, and the efficiency was highly improved. In the ELISA validation phase, the levels of autoantibodies specific to two candidates, *i.e.* COPS2 and CTSF, were assessed to use serum samples from 300 GC patients and 300 healthy controls using ELISA. This study was conducted with the approval of the ethics committee of Ruijin Hospital.

Serum Autoantibody Screening in Discovery Phase I—In discovery phase I, matched serum samples from 37 GC patients and 50 healthy controls were involved. Statistically, there is no bias of age and a slight difference of sex between the GC patient group and the healthy group (Table I). A microarray containing 16,368 human proteins (21) was initially used to screen for proteins that were differentially recognized

TABLE II

Characteristics of the study participants in the training/testing phase

The abbreviations used are as follow: S.D., standard deviation; SCC, squamous cell carcinoma; SRCC, signet ring cell carcinoma; CEA, carcinoembryonic antigen; AFP, alpha feto protein; TNM, tumor node metastasis.

Variable	No.	Mean	%
GC count	300		
Age, years			
Mean		59.55	
S.D.		11.43	
Sex			
Male	222		74
Female	78		26
CEA			
≤5 ng/ml	250		83
>5 ng/ml	50		17
CA 12-5			
≤35 units/ml	282		94
>35 units/ml	18		6
CA 19-9			
≤37 units/ml	256		85
>37 units/ml	44		15
CA 72-4			
≤6 units/ml	237		79
>6 units/ml	63		21
AFP			
≤30 units/ml	295		98
>30 units/ml	5		2
Tumor size			
≤3 cm	156		52
>3 cm	144		48
Differentiation			
Poor	144		48
Moderate	81		27
High	7		2
Others	68		23
Infiltration depth (T)			
T1+T2	126		42
T3+T4	174		58
Positive lymph nodes (N)			
≤6	227		76
>6	73		24
Metastasis (M)			
M1	13		4%
M0	293		98
TNM stage			
I	95		32
II	61		20
III	131		44
IV	13		4
Pathological type			
Adenocarcinoma	231		77
SCC	4		1
SRCC	18		6
Others	47		16
Patients' status			
Survival	67		22
Death	233		78
Median survival time (months)		27.22	

TABLE II—continued

Variable	No.	Mean	%
Healthy count			
Age, years	300		
Mean		54.89	
S.D.		10.45	
Sex			
Male	143		48
Female	157		52
CEA	160		
≤5 ng/ml	155		97
>5 ng/ml	5		3
CA 12-5	90		
≤5 units/ml	90		100
>35 units/ml	0		0
CA 19-9	88		
≤37 units/ml	87		99
>37 units/ml	1		1
CA 72-4	80		
≤6 units/ml	71		89
>6 units/ml	9		11
AFP	151		
≤0 units/ml	151		100
>30 units/ml	0		0

by serum autoantibodies in the serum samples. The Bioconductor package limma (19) and MASS were applied to analyze the protein microarray data and to identify the differences in the response level of the serum autoantibodies between the GC patients and healthy controls. Seventeen proteins that were more significantly bound by the autoantibodies of the GC patient group than by those of the healthy group were identified using a *p* value of <0.01 and fold change > = 1.40 as cutoff (supplemental Table S3). The functions of these proteins were diverse and involved in a variety of biological pathways. This is the initial screening, and some of the candidates from this step may not be a true positive, such as OR5BU1, an olfactory receptor protein. As evidence, in GPMDB Global Proteome Machine Database and PeptideAtlas, the large number of olfactory receptor proteins reported from mass spectrometry studies falls to a very nearly null set upon reanalysis.

Set of Four Serum Autoantibody Biomarkers Were Determined in Discovery Phase II—The most reliable way of biomarker discovery is to test the candidates with a large cohort of samples, which have clear and sufficient clinical records. With this in mind, sera from 300 GC patients and 300 healthy controls with detailed clinical records were collected (Table II). To test these samples in an economical and efficient way, the 17 candidates from discovery phase I were purified and printed with a 12-well format to make the targeted protein microarray, and 12 samples could then be assayed on a single microarray. The Bioconductor package limma (19) and MASS were applied to analyze the protein microarray data and to identify the differences in the response level of the serum autoantibodies between the GC patients and healthy controls.

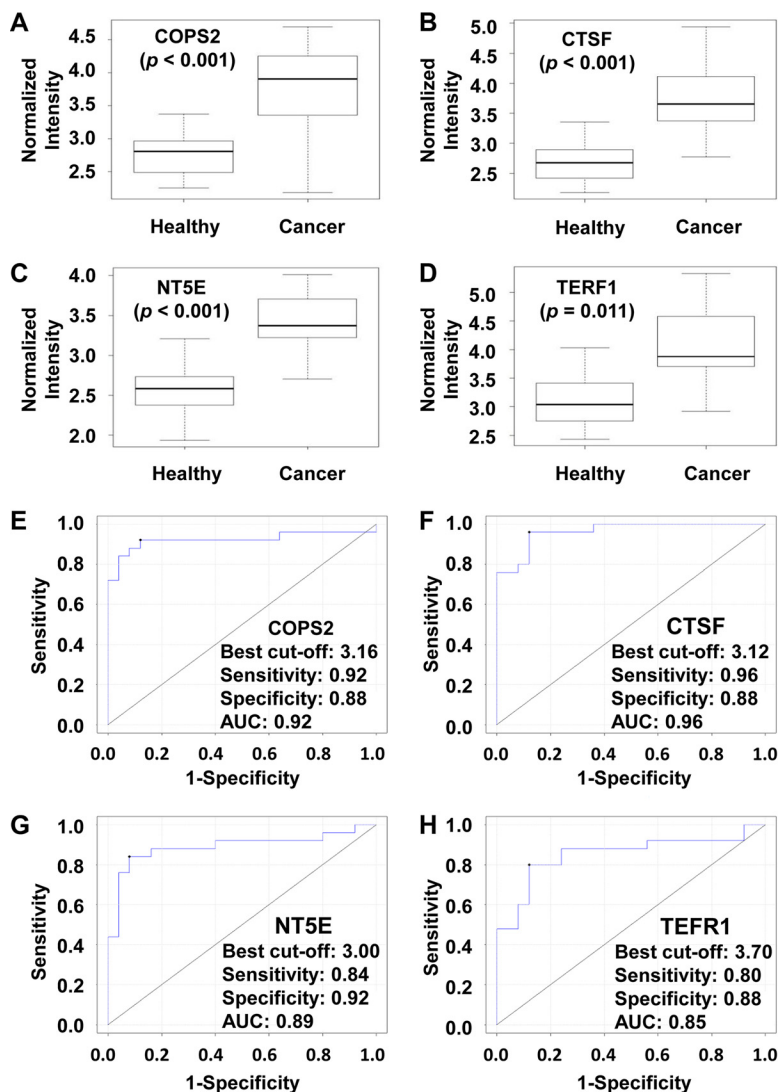


FIG. 2. Four candidates of GC serum biomarker were confirmed in discovery phase II. Reactivity of the four serum biomarkers with sera of healthy people and GC patients on protein microarray is as follows: A, COPS2; B, CTSF; C, NT5E; and D, TERF1. (The y axis is log10 scale.) Receiver operating characteristic curve analysis of the four serum biomarkers for gastric cancer diagnosis is as follows: E, COPS2; F, CTSF; G, NT5E; and H, TERF1. The data presented here are based the 300 GC samples and 300 healthy people from discovery phase II.

Four proteins, COPS2, CTSF, NT5E, and TERF1, that were more significantly bound by the autoantibodies of the GC patient group than by those of the healthy group were determined. To test the four candidates, 100× computational cross-validations were conducted. For each cross-validation procedure, 108 healthy samples and 108 GC samples were randomly selected as the training phase. The rest of the samples were treated as the testing phase (192 healthy samples and 192 GC samples). The results of the computational cross-validation clearly showed that the four candidates alone could serve as potential autoantibody biomarkers for gastric cancer diagnosis based on the protein microarray format (Fig. 2, A–D). To further show the diagnosis accuracy of the four candidates, receiver operating characteristic analyses were also performed; an AUC of ~0.9 was obtained for an individ-

ual candidate (Fig. 2, E–H) and as a combination (supplemental Fig. S1A). A classifier was also generated based on the best model (supplemental Fig. S1B). Surprisingly, the AUCs of known GC antigen biomarkers, such as CA12-5, CA19-9, CA72-4, and carcinoembryonic antigen, were all in the range of 0.5–0.6 (supplemental Fig. S2), which was significantly lower than that of the four candidates we identified on protein microarray.

It is possible that the final four autoantibody biomarkers may also react with the sera from other gastric diseases. To rule out this possibility, an additional set of sera from 314 people diagnosed with GU, GP, CAG, and other gastric diseases was also included in discovery phase II to test the GC specificity of the autoantibody biomarker candidates (Fig. 3A). For all four candidates, *i.e.* COPS2, CTSF, NT5E, and TERF1,

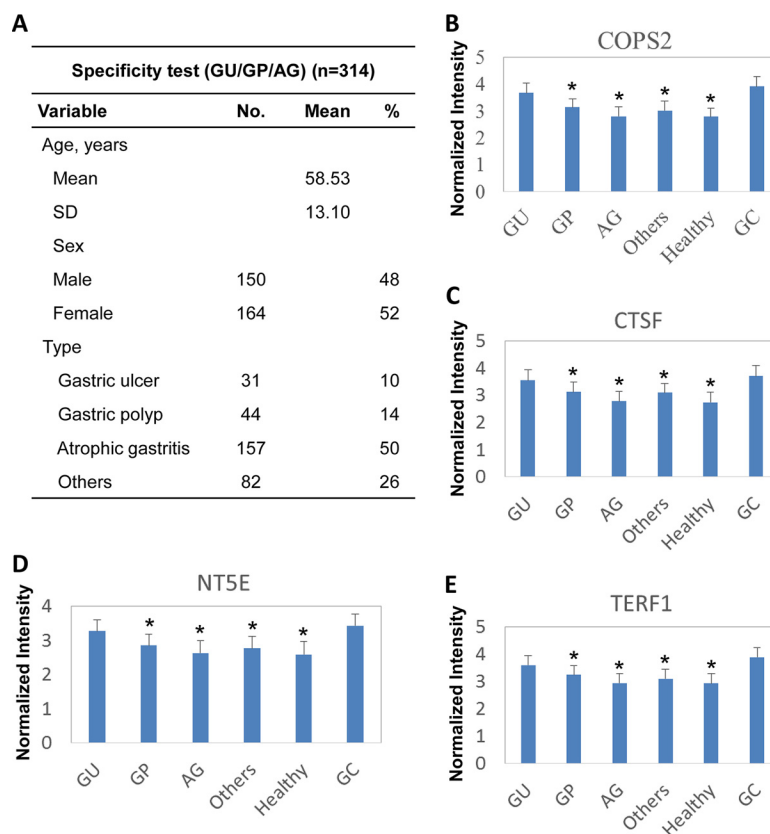


FIG. 3. Set of four serum biomarkers, COPS2, CTSF, NT5E, and TERF1, were specific for gastric cancer but not for other gastric diseases. A, characteristics of study participants of GU/GP/chronic atrophic gastritis group. B–E, comparison of the GU/GP/CAG group, healthy people, and GC patient. All 314 cases were classified into the GU, GP, CAG, or other diseases group on the basis of clinical information. The signals of each of the four candidates are as follows: B, COPS2; C, CTSF; D, NT5E; and E, TERF1 reacting with the sera in each case are shown. (The y axis is log₁₀ scale.) Asterisks indicate statistical difference as compared to the GC group ($p < 0.05$).

the reactions to GC are significantly higher than that to GP, CAG, other gastric diseases, except the GU group, and healthy people. Additionally, there is no significant difference among healthy people and those with GP, CAG, and other gastric diseases (Fig. 3, B–E). These results indicate that all four autoantibody biomarker candidates are generally specific to GC, and they have potential serum autoantibody biomarkers for gastric cancer diagnostics. Clinically, gastric ulcer is a major precancerous condition of gastric cancer, and because the reactions of the four candidates to GU and GC sera are similar, an interesting possibility is that the four serum autoantibody biomarkers may potentially be applied for GC early diagnostics.

Association between Levels of Serum Autoantibodies and Patient Survival—We also tested the ability of the four autoantibody biomarker candidates for predicting GC survival in 300 GC patients. The low serum autoantibody levels against COPS2, CTSF, NT5E, and TERF1 were associated with unfavorable survival. As shown in Table III and supplemental Fig. S3, significant differences were observed for both median survival time and survival rate. Interestingly, patients with high autoantibody levels of COPS2, CTSF, NT5E, and TERF1 had a lower probability of cancer death and live longer. One plau-

sible explanation is that CTSF and COPS2 are tumor-associated antigen/tumor-specific antigens for gastric cancer and also the possible drivers of progression, invasion, and metastasis. The immune system generates autoantibodies to block these antigens thus protects the patients from the harmful effects of gastric cancer. The autoantibody levels among individuals may be proportional to the robustness of their immune system against gastric cancer; thus, a higher level of autoantibody may indicate better protection and eventually longer survival.

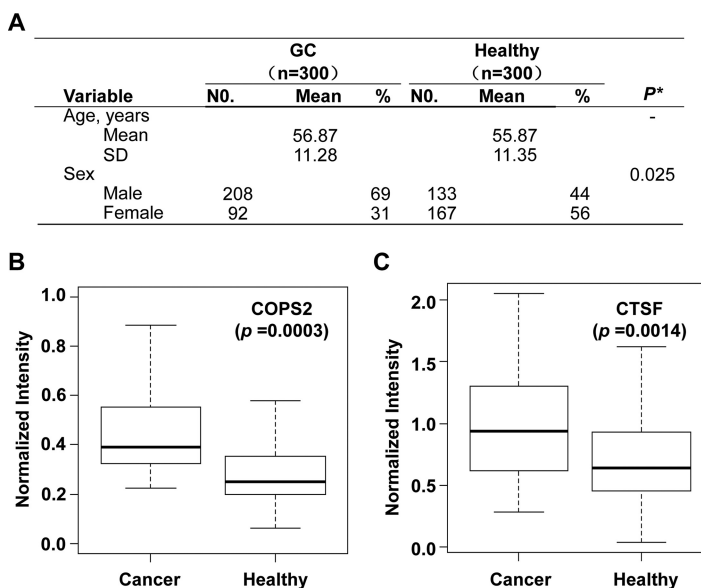
Validation of the Four Candidates Using ELISA and Tissue Microarray—ELISA is commonly used in clinical settings, and to test the probability of using the newly identified autoantibody biomarkers for GC diagnostics, we developed an ELISA with COPS2 and CTSF. These two candidates were chosen because they had the highest AUCs (Fig. 2) and could be easily purified. The sera for ELISA validation included samples from a set of 300 GC patients and 300 healthy controls; specifically, sera from 100 GC patients and healthy controls were randomly selected from the sample set used for microarray analysis (ELISA validation phase I); additional sera from 200 GC patients and healthy controls were freshly collected (ELISA validation phase II) (Fig. 4A). The 96-well plates were

TABLE III
Prognosis possibility of the final four candidates

The correlation of the reactivities of the final four autoantibodies with the survival data of GC patients with follow-up records is shown. The median survival time (MST) was estimated based on the Kaplan-Meier estimates, and the *p* values were calculated using log-rank test.

Candidate	Patients (<i>n</i> = 300)	Deaths (<i>n</i> = 233)	MST (months)	Survival rate	Log-rank <i>p</i> value
COPS2					0.01
Low, ≤3.16	112	95	15.93	15.18%	
High, >3.16	188	138	34.46	26.60%	
CTSF					0.02
Low, ≤3.12	126	105	16.76	16.67%	
High, >3.12	174	128	34.61	26.44%	
NT5E					0.01
Low, ≤3.00	114	99	16.21	13.16%	
High, >3.00	186	134	34.67	27.96%	
TERF1					0.01
Low, ≤3.70	121	102	16.83	15.70%	
High, >3.70	179	131	34.75	26.82%	

FIG. 4. Two candidates of GC serum biomarker, *i.e.* COPS2 and CTSF, were tested by ELISA. *A*, characteristics of the study participants (300 GC sera and 300 healthy sera) in the ELISA validation phase. Statistical analysis showed that there is no bias for both age and gender between the GC group and healthy control. *B* and *C*, reactivity of the two serum biomarkers to the independently collected cohort of sera (200 healthy people and 200 GC patients) by ELISA. *B*, COPS2 and *C*, CTSF. (The *y* axis is log₁₀ scale.)



coated with COPS2 or CTSF and incubated with diluted serum samples, and readout of signals was with HRP-conjugated anti-human IgG. Results showed that for ELISA validation phase I and II, the GC group had significantly higher COPS2 and CTSF signals than that of the healthy control group (supplemental Fig. S4 and Fig. 4, B and C). As expected, the results of ELISA validation phase I are consistent with the same set of samples on the microarray. The correlation coefficient *r*² was 0.87 for COPS2 and 0.85 for CTSF, respectively. To examine the potential clinical relevance of the observed relationship between serum autoantibody levels against COPS2 and CTSF, and the protein levels in tissues, we employed a tissue microarray specific for gastric cancer containing 31 different paired samples (gastric cancer and healthy). The TMAs (Tissue microarrays) were incubated with antibodies for COPS2 and CTSF. As shown in supplemental Fig. S5, interestingly, lower levels of COPS2 and CTSF were observed in the gastric cancer group. To test whether the

potential biomarkers are gastric cancer-specific, a tissue microarray of digestive tract tumor containing 30 samples was applied. There are six types of tumors, five samples for each, *i.e.* gastric cancer, esophagus cancer, colon cancer, rectal cancer, liver cancer, and pancreatic cancer. The results clearly demonstrated that CTSF is specific for gastric cancer, whereas COPS2 is not that specific (supplemental Fig. S6).

DISCUSSION

Our study shows that COPS2, CTSF, NT5E, and TERF1 are recognized by GC-specific serum autoantibodies and that these four proteins have potential as biomarkers for GC diagnosis. Both the individual biomarkers and the combination of the four biomarkers have significantly higher sensitivity and specificity than that of routinely used serum autoantigen biomarkers such as carcinoembryonic antigen, CA19-9, CA72-4 and CA50. In addition to their potential for diagnosis, we found that these four autoantibody biomarkers, *i.e.* COPS2,

CTSF, NT5E, and TERF1, are also associated with the overall survival of GC patients and could serve as a noninvasive predictor of GC prognosis.

In discovery phase II, the efficacy of the four candidates was successfully validated by microarray experiment and computational cross-validation with 100 times random sampling; these validations confirmed the reliability of the four serum autoantibody biomarkers that we identified. Because these serum autoantibody biomarkers are noninvasive and easy to detect in clinical settings, they may also be used to investigate the drug response and chemosensitivity of GC patients by observing changes in expression levels and profiles before and after treatment.

According to the data that we obtained, two forms of test might be used for the clinical application of the identified autoantibody biomarkers, *i.e.* ELISA and microarray. However, the performance of the four autoantibody biomarkers was not as significant as that of protein microarray in the current form of ELISA; this could be improved when developing automatic ELISAs in future studies.

Our study is unique compared with studies on the discovery of circulating serum autoantibodies for GC diagnosis and prognosis (22–26), for the following reasons. First, we screened serum autoantibodies using a protein microarray, which included 16,368 human proteins, enabling us to have a better chance of globally identifying potential diagnostic and prognostic biomarkers. Second, a large cohort with 1,401 samples was tested for this study, which covers most of the stages of GC tumorigenesis. It is well known that the pathogenesis of GC is heterogeneous and that multiple mechanisms of tumorigenesis (such as tumor suppressor genes, oncogenes, viral effects, and angiogenesis) may be involved. It is well known that many types of cancer, including GC are heterogeneous, and some of them have clearly defined subgroups and some do not. It will be interesting to see the differences between/among cancer subgroups for biomarker purposes. However, as a first step, the aim of this study was set to identify serum biomarkers for gastric cancer in general. According to the significance and availability of the clinical resource, it will be interesting to try to identify subgroup-specific serum biomarkers for gastric cancer in future studies. Nonetheless, we hypothesized that similar to other types of cancer, most of GC cases fall into three stages as follows: healthy, gastritis, and GC. Because of its long development process, changes in protein expression may occur during any of the stages (gastritis or GC I, II, IV, and IV) before GC is clinically/pathophysiologically manifested. Thus, we included GC patients at four TNM stages of disease development in our study, and we also included 314 samples of other GC diseases during the training/testing phase. This design may give us an overall picture of the associated immune responses of GC progression and also strengthen the reliability of the identified biomarkers through cross-validations. In addition, our panel of four candidate autoantibody biomarkers was

validated using a large and independent cohort from five medical centers.

All four autoantibody biomarkers identified in this study were related to key signaling pathways and tumorigenesis. COPS2 is a transcriptional co-repressor and was originally identified in a yeast two-hybrid screen as a thyroid hormone receptor-interacting protein that binds in a ligand-dependent manner (27) and participates in various signaling pathways. COPS2 interacts with a subset of nuclear hormone receptors such as the ecdysone receptor DAX-1 and thyroid hormone receptors (28). The COPS complex is composed of eight subunits, is localized in the cytoplasm and nucleus, and possesses an associated unidentified kinase activity that specifically phosphorylates transcriptional regulators such as p53, P105, and I κ B α (29). These facts indicate that the COPS complex participates in various signal transduction pathways and in cell cycle progression. Cathepsins are cysteine proteases from the papain family that are responsible for protein breakdown in lysosomes (30). Human CTSF is a member of the cathepsin family and is composed of cathepsins B, C, H, K, L, O, S, V, W, and X (31). CTSF has been shown to be ubiquitously expressed in several tissues with higher levels of expression in skeletal muscle and the testis. CTSF transcripts have also been found in HeLa cells, suggesting that this enzyme could be involved in degradative processes during tumor progression (32). NT5E, known as CD73, is a glycosylphosphatidylinositol-linked 70-kDa cell surface enzyme found in most tissues (33, 34). CD73, originally identified as a lymphocyte differentiation antigen, is expressed in many cell types, including subsets of lymphocytes (35) and endothelial and epithelial cells (36). Recently, it has been shown that biological actions of CD73 are a consequence (at least in large part) of the regulated enzymatic phosphohydrolytic activity of extracellular nucleotides. This ectoenzymatic cascade, in tandem with CD39 (ecto-ATPase), generates adenosine from the ATP/AMP often released from damaged or inflamed target cells into the extracellular environment (37, 38) and mediates immune suppression (39, 40). Interestingly, CD73 is highly expressed in numerous human solid tumors (41–46), and its overexpression and elevated activity are associated with tumor invasiveness and metastasis (47, 48) and with shorter patient survival time (49), indicating that CD73 is closely involved in cancer progression. Telomeric repeat binding factor 1 (TERF1) is a double-stranded telomere DNA-binding protein (50). It forms a homodimer and nucleates protein complexes called shelterin at the telomeric TTAGGG repeats (51). As the DNA polymerase-dependent replication machinery cannot replicate the very ends of linear DNA, telomeres gradually shorten after every replication cycle. Critically shortened telomeres cannot protect the chromosome ends and thus trigger a DNA damage response, resulting in replicative senescence (52). Most immortal cells escape from replicative senescence by activating telomerase (53–55). Thus, this mechanism may be involved in tumor growth.

This study has some limitations. First, there is no overlap between the autoantibodies identified here and those from other related studies (56–58). This may be due to the complex heterogeneity of GC. The proteome microarray-based strategy has the power of global and unbiased screening. It is quite different from the strategies/technologies used for the previous GC biomarker studies. It is possible that there are more biomarkers to be discovered, and each strategy could only reveal part of them. One plausible explanation for the observation of no overlap is that there are more existing biomarkers, and by using a variety of different strategies, each study, including ours, could only reveal part of the existing biomarkers. However, the autoantibody biomarkers that we have identified could be combined with those from other studies, thus providing higher sensitivity and specificity for both diagnosis and prognosis. Second, it was difficult for us to identify the autoantibodies to tumor antigens bearing structural changes and post-translational modification aberrance, as all proteins on the protein microarray were homogeneously expressed from normal human coding genes. However, our strategy is global and unbiased. On the protein microarray used here, there are 16,368 affinity-purified human proteins, representing ~80% of human genome-encoded proteins. Thus, using human protein microarrays greatly increased our chances of discovering novel biomarkers.

In summary, we have identified a panel of four serum autoantibody biomarkers by using a large number of participants. These autoantibody-based biomarkers could differentiate GC patients from healthy individuals with a high degree of sensitivity and specificity. Our study demonstrates that this panel of serum biomarkers has considerable clinical value for the diagnosis of GC, in prospective studies of cancer outcome, and in therapy response evaluations.

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☐ This article contains [supplemental Materials and Methods, Tables S1 to S3, and Figs. S1 to S6](#).

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REFERENCES

- Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E., and Forman, D. (2011) Global cancer statistics. *CA Cancer J. Clin.* **61**, 69–90
- Chen, W. Q., Zhang, S. W., Zou, X. N., and Zhao, P. (2011) Cancer incidence and mortality in China. *Chin. J. Cancer Res.* **23**, 3–9
- Siegel, R., Naishadham, D., and Jemal, A. (2013) Cancer statistics, 2013. *CA Cancer J. Clin.* **63**, 11–30
- Yang, L., Parkin, D. M., Ferlay, J., Li, L., and Chen, Y. (2005) Estimates of cancer incidence in China for 2000 and projections for 2005. *Cancer Epidemiol. Biomarkers Prev.* **14**, 243–250
- Kang, K. J., Kim, K. M., Min, B. H., Lee, J. H., and Kim, J. J. (2011) Endoscopic submucosal dissection of early gastric cancer. *Gut Liver* **5**, 418–426
- Emoto, S., Ishigami, H., Yamashita, H., Yamaguchi, H., Kaisaki, S., and Kitayama, J. (2012) Clinical significance of CA125 and CA72-4 in gastric cancer with peritoneal dissemination. *Gastric Cancer* **15**, 154–161
- Li, Y., Yang, Y., Lu, M., and Shen, L. (2011) Predictive value of serum CEA, CA19-9 and CA72.4 in early diagnosis of recurrence after radical resection of gastric cancer. *Hepatogastroenterology* **58**, 2166–2170
- Ucar, E., Semerci, E., Ustun, H., Yetim, T., Huzmeli, C., and Gullu, M. (2008) Prognostic value of preoperative CEA, CA 19-9, CA 72-4, and AFP levels in gastric cancer. *Adv. Ther.* **25**, 1075–1084
- Anderson, K. S., Wong, J., D'Souza, G., Riemer, A. B., Lorch, J., Haddad, R., Pai, S. I., Longtine, J., McClean, M., LaBaer, J., Kelsey, K. T., and Posner, M. (2011) Serum antibodies to the HPV16 proteome as biomarkers for head and neck cancer. *Br. J. Cancer* **104**, 1896–1905
- Burford, B., Gentry-Maharaj, A., Graham, R., Allen, D., Pedersen, J. W., Nudelman, A. S., Blixt, O., Fourkala, E. O., Bueti, D., Dawnay, A., Ford, J., Desai, R., David, L., Trinder, P., Acres, B., Schwiertek, T., Gammernan, A., Reis, C. A., Silva, L., Osório, H., Hallett, R., Wandall, H. H., Mandel, U., Hollingsworth, M. A., Jacobs, I., Fentiman, I., Clausen, H., Taylor-Papadimitriou, J., Menon, U., and Burchell, J. M. (2013) Autoantibodies to MUC1 glycopeptides cannot be used as a screening assay for early detection of breast, ovarian, lung or pancreatic cancer. *Br. J. Cancer* **108**, 2045–2055
- Hudson, M. E., Pozdnyakova, I., Haines, K., Mor, G., and Snyder, M. (2007) Identification of differentially expressed proteins in ovarian cancer using high-density protein microarrays. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 17494–17499
- Wang, X., Yu, J., Sreekumar, A., Varambally, S., Shen, R., Giacherio, D., Mehra, R., Montie, J. E., Pienta, K. J., Sanda, M. G., Kantoff, P. W., Rubin, M. A., Wei, J. T., Ghosh, D., and Chinnaiyan, A. M. (2005) Autoantibody signatures in prostate cancer. *N. Engl. J. Med.* **353**, 1224–1235
- Chuang, H. Y., Ren, C. T., Chao, C. A., Wu, C. Y., Shivatare, S. S., Cheng, T. J., Wu, C. Y., and Wong, C. H. (2013) Synthesis and vaccine evaluation of the tumor-associated carbohydrate antigen RM2 from prostate cancer. *J. Am. Chem. Soc.* **135**, 11140–11150
- Dorvillius, M., Garambois, V., Pourquier, D., Gutowski, M., Rouanet, P., Mani, J. C., Pugnère, M., Hynes, N. E., and Pèlegri, A. (2002) Targeting of human breast cancer by a bispecific antibody directed against two tumour-associated antigens: ErbB-2 and carcinoembryonic antigen. *Tumour Biol.* **23**, 337–347
- Jaramillo, A., Majumder, K., Manna, P. P., Fleming, T. P., Doherty, G., Dipersio, J. F., and Mohanakumar, T. (2002) Identification of HLA-A3-restricted CD8+ T cell epitopes derived from mammaglobin-A, a tumor-associated antigen of human breast cancer. *Int. J. Cancer* **102**, 499–506
- Li, Y., Dong, X., Yin, Y., Su, Y., Xu, Q., Zhang, Y., Pang, X., Zhang, Y., and Chen, W. (2005) BJ-TSA-9, a novel human tumor-specific gene, has potential as a biomarker of lung cancer. *Neoplasia* **7**, 1073–1080
- Jeong, J. S., Jiang, L., Albino, E., Marrero, J., Rho, H. S., Hu, J., Hu, S., Vera, C., Bayron-Poueymiroy, D., Rivera-Pacheco, Z. A., Ramos, L., Torres-Castro, C., Qian, J., Bonaventura, J., Boeke, J. D., Yap, W. Y., Pino, I., Eichinger, D. J., Zhu, H., and Blackshaw, S. (2012) Rapid identification of monospecific monoclonal antibodies using a human proteome microarray. *Mol. Cell. Proteomics* **11**, O111.016253
- Ritchie, M. E., Silver, J., Oshlack, A., Holmes, M., Diyagama, D., Holloway, A., and Smyth, G. K. (2007) A comparison of background correction methods for two-colour microarrays. *Bioinformatics* **23**, 2700–2707
- Kauffmann, A., and Huber, W. (2010) Microarray data quality control improves the detection of differentially expressed genes. *Genomics* **95**, 138–142
- Bolstad, B. M., Irizarry, R. A., Astrand, M., and Speed, T. P. (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* **19**, 185–193
- Chen, Y., Yang, L. N., Cheng, L., Tu, S., Guo, S. J., Le, H. Y., Xiong, Q., Mo,

- R., Li, C. Y., Jeong, J. S., Jiang, L., Blackshaw, S., Bi, L. J., Zhu, H., Tao, S. C., and Ge, F. (2013) Bcl2-associated athanogene 3 interactome analysis reveals a new role in modulating proteasome activity. *Mol. Cell. Proteomics* **12**, 2804–2819
22. Hao, Y., Yu, Y., Wang, L., Yan, M., Ji, J., Qu, Y., Zhang, J., Liu, B., and Zhu, Z. (2008) IPO-38 is identified as a novel serum biomarker of gastric cancer based on clinical proteomics technology. *J. Proteome Res.* **7**, 3668–3677
 23. Jørgensen, J. T., and Hersom, M. (2012) HER2 as a prognostic marker in gastric cancer—a systematic analysis of data from the literature. *J. Cancer* **3**, 137–144
 24. Koskensalo, S., Mrena, J., Wiksten, J. P., Nordling, S., Kakkola, A., Hagström, J., and Haglund, C. (2010) MMP-7 overexpression is an independent prognostic marker in gastric cancer. *Tumour Biol.* **31**, 149–155
 25. Mrena, J., Wiksten, J. P., Kakkola, A., Nordling, S., Ristimäki, A., and Haglund, C. (2010) COX-2 is associated with proliferation and apoptosis markers and serves as an independent prognostic factor in gastric cancer. *Tumour Biol.* **31**, 1–7
 26. Tseng, C. W., Yang, J. C., Chen, C. N., Huang, H. C., Chuang, K. N., Lin, C. C., Lai, H. S., Lee, P. H., Chang, K. J., and Juan, H. F. (2011) Identification of 14-3-3 β in human gastric cancer cells and its potency as a diagnostic and prognostic biomarker. *Proteomics* **11**, 2423–2439
 27. Lee, J. W., Choi, H. S., Gyuris, J., Brent, R., and Moore, D. D. (1995) Two classes of proteins dependent on either the presence or absence of thyroid hormone for interaction with the thyroid hormone receptor. *Mol. Endocrinol.* **9**, 243–254
 28. Altincicek, B., Tenbaum, S. P., Dressel, U., Thormeyer, D., Renkawitz, R., and Baniahmad, A. (2000) Interaction of the corepressor Alien with DAX-1 is abrogated by mutations of DAX-1 involved in adrenal hypoplasia congenita. *J. Biol. Chem.* **275**, 7662–7667
 29. Lykke-Andersen, K., Schaefer, L., Menon, S., Deng, X. W., Miller, J. B., and Wei, N. (2003) Disruption of the COP9 signalosome Csn2 subunit in mice causes deficient cell proliferation, accumulation of p53 and cyclin E, and early embryonic death. *Mol. Cell. Biol.* **23**, 6790–6797
 30. Vazquez-Ortiz, G., Pina-Sanchez, P., Vazquez, K., Duenas, A., Taja, L., Mendoza, P., Garcia, J. A., and Salcedo, M. (2005) Overexpression of cathepsin F, matrix metalloproteinases 11 and 12 in cervical cancer. *BMC Cancer* **5**, 68
 31. Wex, T., Levy, B., Wex, H., and Brömme, D. (2000) Human cathepsins W and F form a new subgroup of cathepsins that is evolutionary separated from the cathepsin B- and L-like cysteine proteases. *Adv. Exp. Med. Biol.* **477**, 271–280
 32. Santamaría, I., Velasco, G., Pendás, A. M., Paz, A., and López-Otín, C. (1999) Molecular cloning and structural and functional characterization of human cathepsin F, a new cysteine proteinase of the papain family with a long propeptide domain. *J. Biol. Chem.* **274**, 13800–13809
 33. Colgan, S. P., Eltzschig, H. K., Eckle, T., and Thompson, L. F. (2006) Physiological roles for ecto-5'-nucleotidase (CD73). *Purinergic Signal.* **2**, 351–360
 34. Resta, R., Yamashita, Y., and Thompson, L. F. (1998) Ecto-enzyme and signaling functions of lymphocyte CD73. *Immunol. Rev.* **161**, 95–109
 35. Yamashita, Y., Hooker, S. W., Jiang, H., Laurent, A. B., Resta, R., Khare, K., Coe, A., Kincade, P. W., and Thompson, L. F. (1998) CD73 expression and fyn-dependent signaling on murine lymphocytes. *Eur. J. Immunol.* **28**, 2981–2990
 36. Strohmeier, G. R., Lencer, W. I., Patapoff, T. W., Thompson, L. F., Carlson, S. L., Moe, S. J., Carnes, D. K., Mrsny, R. J., and Madara, J. L. (1997) Surface expression, polarization, and functional significance of CD73 in human intestinal epithelia. *J. Clin. Invest.* **99**, 2588–2601
 37. Borsellino, G., Kleiweietfeld, M., Di Mitri, D., Sternjak, A., Diamantini, A., Giometto, R., Höpner, S., Centonze, D., Bernardi, G., Dell'Acqua, M. L., Rossini, P. M., Battistini, L., Röttschke, O., and Falk, K. (2007) Expression of ectonucleotidase CD39 by Foxp3⁺ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood* **110**, 1225–1232
 38. Dwyer, K. M., Deaglio, S., Gao, W., Friedman, D., Strom, T. B., and Robson, S. C. (2007) CD39 and control of cellular immune responses. *Purinergic Signal.* **3**, 171–180
 39. Deaglio, S., Dwyer, K. M., Gao, W., Friedman, D., Usheva, A., Erat, A., Chen, J. F., Enjyoji, K., Linden, J., Oukka, M., Kuchroo, V. K., Strom, T. B., and Robson, S. C. (2007) Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J. Exp. Med.* **204**, 1257–1265
 40. Kobie, J. J., Shah, P. R., Yang, L., Rebhahn, J. A., Fowell, D. J., and Mosmann, T. R. (2006) T regulatory and primed uncommitted CD4 T cells express CD73, which suppresses effector CD4 T cells by converting 5'-adenosine monophosphate to adenosine. *J. Immunol.* **177**, 6780–6786
 41. Canbolat, O., Durak, I., Cetin, R., Kavutcu, M., Demirci, S., and Oztürk, S. (1996) Activities of adenosine deaminase, 5'-nucleotidase, guanase, and cytidine deaminase enzymes in cancerous and non-cancerous human breast tissues. *Breast Cancer Res. Treat.* **37**, 189–193
 42. Eroglu, A., Canbolat, O., Demirci, S., Kocaoglu, H., Eryavuz, Y., and Akgül, H. (2000) Activities of adenosine deaminase and 5'-nucleotidase in cancerous and noncancerous human colorectal tissues. *Med. Oncol.* **17**, 319–324
 43. Kondo, T., Nakazawa, T., Murata, S. I., and Katoh, R. (2006) Expression of CD73 and its ecto-5'-nucleotidase activity are elevated in papillary thyroid carcinomas. *Histopathology* **48**, 612–614
 44. Sadej, R., Spychala, J., and Skladanowski, A. C. (2006) Ecto-5'-nucleotidase (eN, CD73) is coexpressed with metastasis promoting antigens in human melanoma cells. *Nucleosides Nucleotides Nucleic Acids* **25**, 1119–1123
 45. Saitoh, M., Nagai, K., Nakagawa, K., Yamamura, T., Yamamoto, S., and Nishizaki, T. (2004) Adenosine induces apoptosis in the human gastric cancer cells via an intrinsic pathway relevant to activation of AMP-activated protein kinase. *Biochem Pharmacol.* **67**, 2005–2011
 46. Vannoni, D., Bernini, A., Carlucci, F., Civitelli, S., Di Pietro, M. C., Leoncini, R., Rosi, F., Tabucchi, A., Tanzini, G., and Marinello, E. (2004) Enzyme activities controlling adenosine levels in normal and neoplastic tissues. *Med Oncol.* **21**, 187–195
 47. Sadej, R., Spychala, J., and Skladanowski, A. C. (2006) Expression of ecto-5'-nucleotidase (eN, CD73) in cell lines from various stages of human melanoma. *Melanoma Res.* **16**, 213–222
 48. Wang, L., Zhou, X., Zhou, T., Ma, D., Chen, S., Zhi, X., Yin, L., Shao, Z., Ou, Z., and Zhou, P. (2008) Ecto-5'-nucleotidase promotes invasion, migration and adhesion of human breast cancer cells. *J. Cancer Res. Clin. Oncol.* **134**, 365–372
 49. Spychala, J., Lazarowski, E., Ostapowicz, A., Ayscue, L. H., Jin, A., and Mitchell, B. S. (2004) Role of estrogen receptor in the regulation of ecto-5'-nucleotidase and adenosine in breast cancer. *Clin. Cancer Res.* **10**, 708–717
 50. Salas, T. R., Petrusseva, I., Lavrik, O., Bourdoncle, A., Mergny, J. L., Favre, A., and Saintomé, C. (2006) Human replication protein A unfolds telomeric G-quadruplexes. *Nucleic Acids Res.* **34**, 4857–4865
 51. de Lange, T. (2005) Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev.* **19**, 2100–2110
 52. d'Adda di Fagagna, F., Reaper, P. M., Clay-Farrace, L., Fiegler, H., Carr, P., Von Zglinicki, T., Saretzki, G., Carter, N. P., and Jackson, S. P. (2003) A DNA damage checkpoint response in telomere-initiated senescence. *Nature* **426**, 194–198
 53. Bodnar, A. G., Ouellette, M., Forkis, M., Holt, S. E., Chiu, C. P., Morin, G. B., Harley, C. B., Shay, J. W., Lichtsteiner, S., and Wright, W. E. (1998) Extension of life-span by introduction of telomerase into normal human cells. *Science* **279**, 349–352
 54. Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., Coviello, G. M., Wright, W. E., Weinrich, S. L., and Shay, J. W. (1994) Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**, 2011–2015
 55. Shay, J. W., and Bacchetti, S. (1997) A survey of telomerase activity in human cancer. *Eur. J. Cancer* **33**, 787–791
 56. Allgayer, H., Babic, R., Gruetzner, K. U., Tarabichi, A., Schildberg, F. W., and Heiss, M. M. (2000) c-erbB-2 is of independent prognostic relevance in gastric cancer and is associated with the expression of tumor-associated protease systems. *J. Clin. Oncol.* **18**, 2201–2209
 57. Chan, A. O., Chu, K. M., Lam, S. K., Wong, B. C., Kwok, K. F., Law, S., Ko, S., Hui, W., Yueng, Y. H., and Wong, J. (2003) Soluble E-cadherin is an independent pretherapeutic factor for long-term survival in gastric cancer. *J. Clin. Oncol.* **21**, 2288–2293
 58. Jüttner, S., Wissmann, C., Jöns, T., Vieth, M., Hertel, J., Gretschel, S., Schlag, P. M., Kemmner, W., and Höcker, M. (2006) Vascular endothelial growth factor-D and its receptor VEGFR-3: two novel independent prognostic markers in gastric adenocarcinoma. *J. Clin. Oncol.* **24**, 228–240