ORIGINAL ARTICLE

Cancer Science WILEY

Anti-FIRΔexon2 autoantibody as a novel indicator for better overall survival in gastric cancer

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Abstract

There is no clinically available biomarker for efficiently indicating the overall survival or therapy response of gastric cancer (GC). The autoantibodies (Abs) in the sera of anti-far-upstream element-binding protein-interacting repressor-lacking exon2 (FIRΔexon2), anti-sorting nexin 15, and anti-spermatogenesis and oogenesis–specific basic helix–loop–helix 1 were markedly higher in GC patients than in healthy donors (HDs). These Abs were identified by large-scale serological identification of antigens by recombinant cDNA expression cloning screenings and their expression levels were evaluated by amplified luminescence proximity homogeneous assay. In particular, compared with age-matched HDs, the level of anti-FIRΔexon2 Abs in GC patients was significantly higher ($P < .001$). The Spearman's rank correlation analysis between anti-FIRΔexon2 Abs and clinically available tumor markers such as carcinoembryonic antigen (CEA) was statistically insignificant, indicating that FIRΔexon2 Abs is an independent biomarker. We performed receiver-operating curve analysis to evaluate the anti-FIRΔexon2 Ab as a candidate biomarker with CEA and carbohydrate antigen 19-9 (CA19-9). The overall survival of GC patients with high anti-FIRΔexon2 Abs titer was significantly favorable ($P = .04$) than that of GC patients who were below detection level of anti-FIRΔexon2 Abs. However, clinical stages were not apparently correlated with the levels of anti-FIRΔexon2 Ab, CEA, and CA19-9. In conclusion, anti-FIRΔexon2 Abs detected in GC patients is a potential biomarker for monitoring a better prognosis. Hence, anti-FIRΔexon2 Abs is a promising biomarker for indicating better overall survival of gastric cancer patients.

KEYWORDS
AlphaLISA, FIRΔexon2 autoantibody, gastric cancer, gastrointestinal cancer, SEREX

1 | INTRODUCTION

Gastric cancer (GC) remains the third leading cause of cancer-related death worldwide, with a high mortality rate in clinically advanced cases.[1] There is no clinically available or valuable biomarker for evaluating overall survival or therapy response in GC patients at diagnosis except Human Epidermal Growth Factor Receptor 2 expression or microsatellite instability (MSI) status of cancer tissues. This study was designated to identify clinically useful biomarkers indicating survival or therapy response in GC.[2,3] The serological identification of antigens by recombinant cDNA expression cloning (SEREX) is an effective screening method to identify serum antibody (Ab)-type tumor markers as multiple specific immune responses of patients with cancer.[4] SEREX determined tumor-related antigens as potential novel diagnostic markers for digestive organ cancer types.[5,6]

In our previous study, we identified five SEREX antigens in the sera of patients with esophageal squamous-cell carcinoma (ESCC) by the expression cloning assay through λZAP II library construction, including far-upstream element-binding protein-interacting repressor-lacking exon2 (FIRΔexon2; accession number: NM_001271099.1),[7,8] lysyl-tRNA synthetase (KARS; accession number: NM_001130089.1),[9,10] sorting nexin 15 (SNX15; accession number: NM_013306.4),[11] spermatogenesis and oogenesis–specific basic helix–loop–helix 1 (SOHLH1; accession number: NM_001101677.1),[12] and cilia and flagella–associated protein 70 (CFAP70; accession number: NM_145170.3).[13] In addition, recombinant proteins were expressed in *Escherichia coli* as glutathione-S-transferase (GST)-fusion proteins and purified by affinity chromatography using glutathione sepharose, as reported previously.[14] Serum Ab markers were detected using purified GST-fusion proteins as antigens. All five SEREX antigen markers identified were significantly higher in patients with ESCC compared to healthy donors (HDs). Similar results were obtained by receiver-operating curve (ROC) analysis. Furthermore, the area under ROC (AUC) values greater than 0.700 were observed for FIRΔexon2 autoantibodies (Abs) in patients with ESCC. FIRΔexon2 Abs has been reported as a common biomarker for ESCCs.[15-17] The combined ROC analysis of candidate markers with clinically available tumor markers such as antitumor protein 53 (TP53) Abs showed increased AUC values in the sera of patients with ESCC. Furthermore, the DeLong test examined the significance of ROCs among single or combined markers.[18,19] Therefore, anti-FIRΔexon2 Abs with anti-TP53 Abs or carcinoembryonic antigen (CEA) improves the specificity and sensitivity for screening ESCCs.

During cancer development, highly malignant tumors can induce necrosis, leading to the exposure of intracellular antigenic proteins to plasma. Therefore, using combinational Ab detection approaches could allow for the precise early detection of tumors. This study aimed to investigate the significance of anti-FIRΔexon2 Abs and whether it increases the specificity and accuracy of GC diagnosis with other clinically available tumor markers, such as anti-TP53 Abs, CEA, and carbohydrate antigen 19-9 (CA19-9), as reported in other cancer types.[14,20,21] Furthermore, this study explored the significance of anti-FIRΔexon2 Abs in the sera of patients with GC as a potential prognostic biomarker by examining the overall survival (OS).

2 | MATERIALS AND METHODS

2.1 | Clinical samples

The study was performed according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). The sera of patients with GC (n = 96) were obtained from the Department of Frontier Surgery (Chiba University Hospital, Chiba, Japan). Blood samples were obtained from consecutive patients from 2012 to 2015. All the patients were histologically confirmed, and those with cancer were pathologically diagnosed as having GC. All blood samples from cancer patients were taken before any treatment. The sera of age-matched HDs (n = 94) were obtained from the Higashi Funabashi Hospital (Funabashi, Japan) as a control. Healthy donor blood samples were obtained from consecutive patients had undergone brain scans between 2013 and 2014. According to the inclusion criteria for healthy controls, individuals with medication history and lifestyle-related diseases were excluded.[22] Written informed consent was obtained from all participants prior to the study. Each serum sample was centrifuged at 2000 g for 10 minutes and the supernatant was stored at −80°C until further use. Repeated thawing and freezing of the samples were avoided. Clinical data extraction was conducted by one reviewer and checked by a second reviewer.[23] This study was approved by the Local Ethical Institutional Review Board of Chiba University, Graduate School of Medicine and Higashi Funabashi Hospital.

2.2 | Screening by expression cloning

We performed recombinant DNA studies with permission from Chiba University Graduate School of Medicine and per the rules of the Japanese government. We used a λZAP II phage cDNA library prepared from the mRNA of T.Tn cells (esophageal cancer cell lines)[24] and a commercially available human fetal testis cDNA library (Uni-ZAP XR Premade Library; Stratagene) to screen for clones immunoreactive against serum IgG from patients with ESCC, as described previously.[25] Then, *E. coli* XL1-Blue MRF' was infected with λZAP II or Uni-ZAP XR phage, and the expression of resident cDNA clones was induced after blotting the infected bacteria onto NitroBind nitrocellulose membranes (Osmonics). Next, we pre-treated the membranes with 10 mmol/L isopropyl-β-D-thiogalactoside (IPTG; Wako Pure Chemicals) for 30 minutes. The membranes with bacterial proteins were rinsed three times with Tris Buffered Saline with Tween 20 (TBST) 20 mmol/L Tris-HCl (pH 7.5), 0.15 mol/L NaCl, and 0.05% Tween-20, and nonspecific binding was blocked by incubating with 1% protease-free bovine serum albumin (Nacalai Tesque, Inc.) in TBST for 1 hour. Then, the

membranes were exposed to 1:2000-diluted sera of ESCC patients for 1 hour.[26] After three washes with TBST, the membranes were incubated for 1 hour with 1:5000-diluted alkaline phosphatase-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories). We developed positive reactions using 100 mmol/L Tris-HCl (pH 9.5) containing 100 mmol/L NaCl, 5 mmol/L MgCl$_2$, 0.15 mg/mL 5-bromo-4-chloro-3-indolylphosphate, and 0.3 mg/mL nitro blue tetrazolium (Wako Pure Chemicals). Furthermore, positive clones were re-cloned twice until monoclonality was obtained, as described previously.[27,28]

We converted monoclonal phage cDNA clones to pBluescript phagemids by in vivo excision using the ExAssist helper phage (Stratagene). Then, plasmid pBluescript-containing cDNA was obtained from the *E. coli* SOLR strain after transformation by the phagemid. Next, we evaluated the sequences of cDNA inserts for homology with identified genes or proteins within the public sequence database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3 | Expression and purification of antigen proteins

We constructed the expression plasmids of GST-fused proteins by recombining the cDNA sequences into pGEX-4T-3 (GE Healthcare Life Sciences). Next, the inserted DNA fragments were ligated into pGEX-4T-3 using Ligation Convenience Kits (Nippon Gene). We used ligation mixtures to transform ECOS™-competent *E. coli* BL21 (DE3; Nippon Gene) and confirmed appropriate recombinants by DNA sequencing and protein expression analyses. Next, the expression of the GST-fusion proteins was induced by treating the transformed *E. coli* with 0.1 mmol/L IPTG for 3 hours. We purified the GST-fused recombinant proteins by glutathione sepharose column chromatography as per the manufacturer's instructions (GE Healthcare Life Sciences) and dialyzed against phosphate-buffered saline, as described previously.[29]

2.4 | Amplified Luminescence Proximity Homogeneous Assay

We performed Amplified Luminescence Proximity Homogeneous Assay (AlphaLISA) using 384-well microtiter plates (white opaque OptiPlate™; PerkinElmer) containing 2.5 μL of 1:100-diluted sera and 2.5 μL of GST or GST-fusion proteins (10 μg/mL) in AlphaLISA buffer (25 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 0.1% casein, 0.5% Triton X-100, 1 mg/mL dextran-500, and 0.05% Proclin-300). The reaction mixture was incubated for 6-8 hours at room temperature. Then, anti-human IgG-conjugated acceptor beads (2.5 μL of 40 μg/mL) and glutathione-conjugated donor beads (2.5 μL of 40 μg/mL) were added and incubated further for 7-21 days at room temperature in the dark. The chemical emission was read on an EnSpire Alpha microplate reader (PerkinElmer), as described previously.[30,31] We calculated selective reactions by subtracting α values of GST control from the values of GST-fusion proteins.

Compared with enzyme-linked immunosorbent assay (ELISA), AlphaLISA is a novel and excellent method to measure Ab levels because of its low variation, stable background, and high specificity. Although AlphaLISA does not involve plate-washing steps, it involves mixing antigens with Abs in the sera followed by the addition of donor and acceptor beads. For instance, Figure 1 shows highly reproducible results, including distributions, *P* values, and positive rates despite using different sets of sera from HDs and patients. The assessment of suitable AlphaLISA conditions in this study revealed that the incubation for 7–21 days is the best to obtain specific antigen–antibody reaction, as well as decrease noise background. Perhaps the precise measurement offered by AlphaLISA could facilitate the establishment of Ab markers, although most of the existing tumor diagnosis methods involve antigen markers, except for the TP53 marker. Compared with the measurement of antigen levels, the measurement of Abs was more sensitive because of the stability of IgG proteins and their amplification by repeated exposure to antigenic proteins.[32,33]

AlphaLISA is a registered trademark of PerkinElmer Inc., as listed on their website (http://www.perkinelmer.com/lab-solutions/resources/docs/GDE_ELISA-to-AlphaLISA.pdf).

2.5 | Statistical analyses

In this study, all statistical analyses were performed using GraphPad Prism 7 (GraphPad Software) and R-3.5.1 statistical software. We used the Mann–Whitney *U*-test to determine the significance of between-group differences. The predictive values of markers for GC were examined by ROC analysis. The cutoff values were set by the values that maximize the sums of the sensitivity and specificity. All tests were two-tailed, and $P < .05$ was considered statistically significant. In addition, we evaluated the Ab group-specific *Z*-scores to facilitate comparison across all Ab groups. Furthermore, *Z*-score analysis was performed after normalization to HD mean values:[34,35]

$$Z - \text{score} = (\text{control mean}) - (\text{individual value}) / (\text{control standard deviation})$$

We performed the combined ROC analysis by adding each *Z*-score. In addition, AUCs were calculated and examined by the Delong or bootstrap tests to compare the significant differences among the single or combined ROCs.[36,37] The Delong test used the following formula:

$$D = \frac{V^r(\theta^r) - V^s(\theta^s)}{\sqrt{S^r + S^s}}$$

3 | RESULTS

3.1 | Higher level of anti-FIRΔexon2 auto-Abs was detected in the sera of patients with GC

We analyzed the levels of serum auto-Abs, against FIRΔexon2, CFAP70, KARS, SNX15, or SOHLH1, by AlphaLISA in the sera of HDs (Table S1) and patients with GC. The levels of FIRΔexon2, SNX15, and
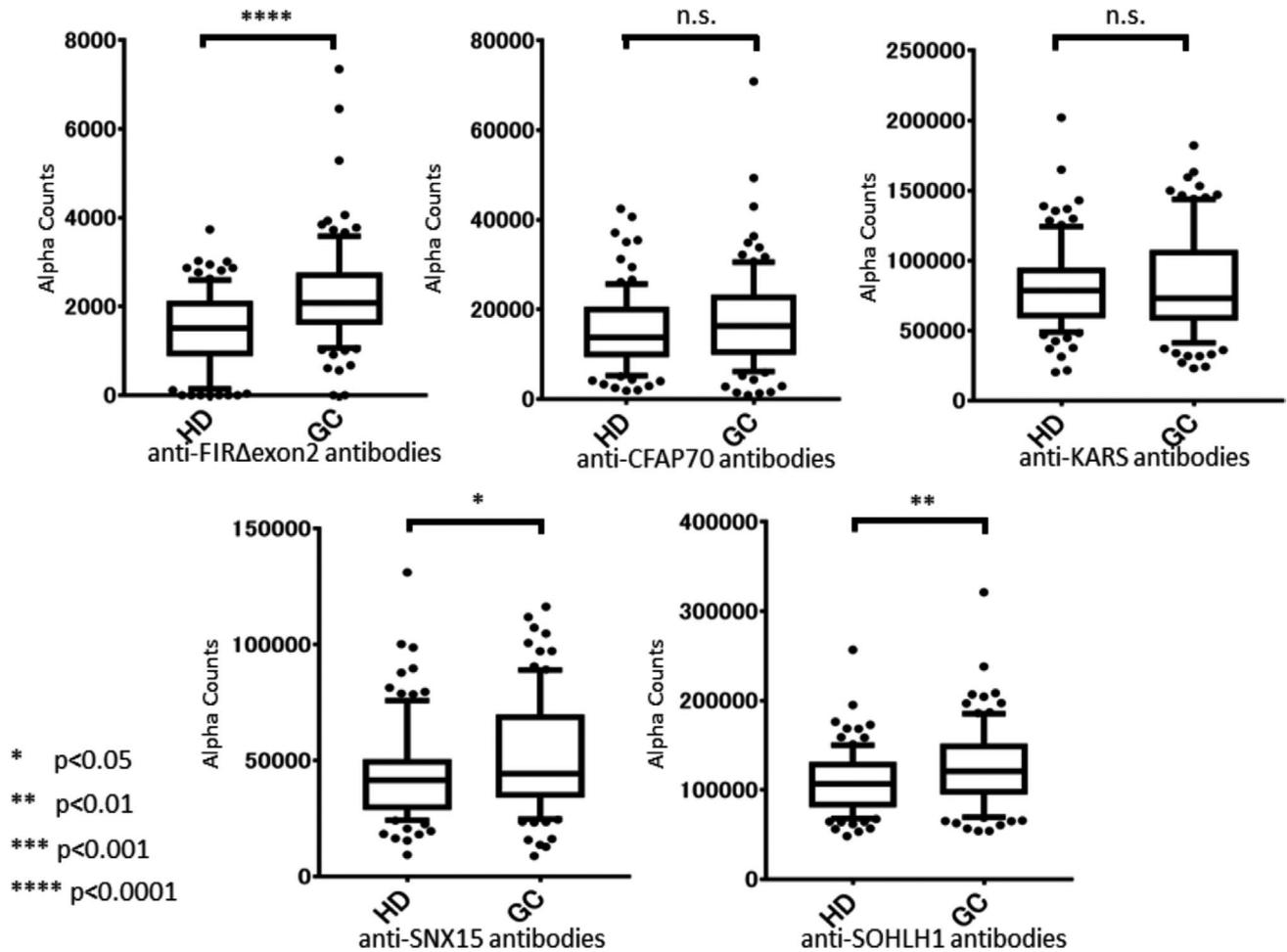
* p<0.05
** p<0.01
*** p<0.001
**** p<0.0001

FIGURE 1 Comparison of levels of antibodies against serological identification of antigens by recombinant cDNA expression cloning (SEREX) and FIRΔexon2 antigens in patients with gastric cancer (GC). The levels of antibodies (Abs) against FIRΔexon2, KARS, SNX15, SOHLH1, and CFAP70 Abs in healthy donors (HD) and patients with GC measured by amplified luminescence proximity homogeneous assay (AlphaLISA). The serum Ab levels measured by AlphaLISA are shown using a box-whisker plot. The box plots display the 10th, 20th, 50th, 80th, and 90th percentiles. In addition, *P* values (calculated using the Mann–Whitney *U* test) compared with HD specimens are shown

TABLE 1 The percentage of antibody-positive cases on AlphaLISA assay

	FIRΔexon2-Abs (%)	*P* value	CFAP70-Abs (%)	*P* value	KARS-Abs (%)	*P* value	SNX15-Abs (%)	*P* value	SOHLH1-Abs (%)	*P*-value
Healthy subjects, n = 94	1 (1)		5 (5)		3 (3)		5 (5)		2 (2)	
Gastric cancer, n = 96	13 (14)	<.001	6 (6)	.126	10 (10)	.760	11 (12)	.034	13 (14)	.003

Note: P values were calculated by Mann–Whitney *U* test.

SOHLH1 Abs were markedly higher in patients with GC compared with HDs (Figure 1). We determined the cutoff value as the average plus two standard deviations (SD) of HDs (95% confidence interval). The percentages of Ab-positive cases were as follows: FIRΔexon2 13/96, 14%; CFAP70 6/96, 6%; KARS 10/96, 10%; SNX15 11/96, 12%; and SOHLH1 Abs 13/96, 14%, as shown in Table 1. Table 2 is a list of the clinical features of GC patients. Anti-FIRΔexon2, SNX15, and SOHLH1 Abs levels were not significantly associated with gender, age, clinical stages, CEA, and CA19-9 level. Table 3 is the diagnostic accuracy of Ab-positive cases significant difference with HD.

3.2 | Anti-FIRΔexon2 auto-Ab is the novel tumor maker for patients with GC

Using Spearman's rank correlation analysis, we explored the existence of a correlation between anti-FIRΔexon2, anti-CFAP70, anti-KARS, anti-SNX15, or anti-SOHLH1 Abs and clinically used tumor markers. The correlation coefficient between FIRΔexon2 Abs and clinically used tumor markers was not significant (Figure 2). Anti-CFAP70 Abs positively correlated with anti-KARS, anti-SNX15, and anti-SOHLH1 Abs, but not with anti-FIRΔexon2 Abs (Figure 2).

TABLE 2 List of clinical features of gastric cancer patients

Gastric cancer	FIRΔexon2-Abs (positive rate %)	P value	SNX15-Abs (positive rate %)	P value	SOHLH1-Abs (positive rate %)	P value
Gender						
Male (65)	9 (14)		6 (9)		7 (11)	
Female (31)	4 (13)	.899	5 (16)	.321	6 (19)	.250
Age						
=71 y (50)	4 (8)		6 (12)		7 (14)	
>71 y (46)	9 (20)	.098	5 (11)	.862	6 (13)	.891
Stage						
0, I, II (69)	11 (16)		7 (10)		9 (13)	
III, IV (25)	2 (8)	.324	3 (12)	.797	3 (12)	.893
ND (2)	0 (0)		1 (50)		1 (50)	
CEA						
Positive (16)	2 (13)		1 (6)		2 (13)	
Negative (80)	11 (14)	.894	10 (13)	.474	11 (14)	.894
CA19-9						
Positive (14)	1 (5)		3 (21)		4 (29)	
Negative (82)	12 (15)	.449	9 (11)	.274	9 (11)	.075
ND (1)	0 (0)		0 (0)		0 (0)	

Note: Pearson's chi-square test.

Abbreviation: ND, Not Detected.

TABLE 3 Summary estimates of the diagnostic accuracy to predict gastric cancer patients

	Sn (95% CI)%	Sp (95% CI)%	PPV (95% CI)%	NPV (95% CI)%	LR+ %	DOR (95% CI)
anti-FIRΔexon2 Abs	13.5 (8.1-21.8)	98.9 (94.2-100)	92.9 (68.5-99.6)	52.8 (45.5-60.1)	12.7	14.6 (2.2-156.9)
anti-SNX15 Abs	11.5 (6.5-19.4)	94.7 (88.2-97.7)	68.8 (44.4-85.8)	51.2 (43.8-58.5)	2.2	2.3 (0.8-6.1)
anti-SOHLH1 Abs	13.5 (8.1-21.8)	97.9 (92.6-99.6)	86.7 (62.1-97.6)	52.6 (45.2-59.8)	6.4	7.2 (1.8-32.6)

Abbreviations: DOR, diagnostic odds ratio; LR−, likelihood ratio of a negative test result; LR+, likelihood ratio of a positive test result; NPV, negative predictive value; PPV, positive predictive value; Sn, sensitivity; Sp, specificity.

Sn, Sp ets, Wilson–Brown test; DOR, Baptista-Pike test.

Using Venn diagram analysis, we explored the existence of duplicate cases between anti-FIRΔexon2, anti-SNX15, or anti-SOHLH1 Abs and clinically used tumor markers. A Venn diagram analysis revealed that Abs-positive cases such as anti-FIRΔexon2, anti-SNX15, and anti-SOHLH1 Abs were not elevated CEA, CA19-9 and without anti-*Helicobacter pylori* (*H. pylori*) detection. Groups of anti-FIRΔexon2 Abs: FIRΔexon2 (2 positive cases), *H. pylori* (44 positive cases), FIRΔexon2 + *H. pylori* (8 positive cases), CA19-9 (6 positive cases), *H. pylori* + CA19-9 (4 positive cases), CEA (11 positive cases), CA19-9 + CEA (2 positive cases), CEA + FIRΔexon2 (2 positive cases), FIRΔexon2 + *H. pylori* + CA19-9 (1 positive case), *H. pylori* + CA19-9 + CEA (1 positive case). Groups of anti-SNX15 Abs: SNX15 (3 positive cases), *H. pylori* (47 positive cases), SNX15 + *H. pylori* (5 positive cases), CA19-9 (6 positive cases), *H. pylori* + CA19-9 (3 positive cases), CEA (12 positive cases), CA19-9 + CEA (2 positive cases), CEA + SNX15 (1 positive case), SNX15 + *H. pylori* + CA19-9 (2 positive cases), *H. pylori* + CA19-9 + CEA (1 positive case). Groups of anti-SOHLH1 Abs: SOHLH1 (5 positive cases), *H. pylori* (48 positive cases), SOHLH1 + *H. pylori* (4 positive cases),

CA19-9 (6 positive cases), *H. pylori* + CA19-9 (3 positive cases), CEA (11 positive cases), CA19-9 + CEA (2 positive cases), CEA + SOHLH1 (2 positive cases), SOHLH1 + *H. pylori* + CA19-9 (2 positive cases), *H. pylori* + CA19-9 + CEA (1 positive case). Therefore, anti-FIRΔexon2, anti-SNX15, and anti-SOHLH1 Abs were relatively independent from CEA, CA19-9, and anti-*H. pylori* Abs (Figure 3). Specifically, the combination of clinically available tumor markers, CEA and CA19-9, with FIRΔexon2 Abs as a novel biomarker potentially improves the diagnostic efficiency and support the early detection of GC.

3.3 | Anti-FIRΔexon2 Abs increased the AUC of CEA or CA19-9 in the ROC analysis

We performed ROC analysis to evaluate the ability of candidate tumor markers—anti-FIRΔexon2, anti-SNX15, anti-SOHLH1 Abs—for detecting patients with GC. The AUC of anti-FIRΔexon2 Abs (0.702) was the highest among anti-SNX15 Abs (0.618) or anti-SOHLH1 Abs (0.581) in
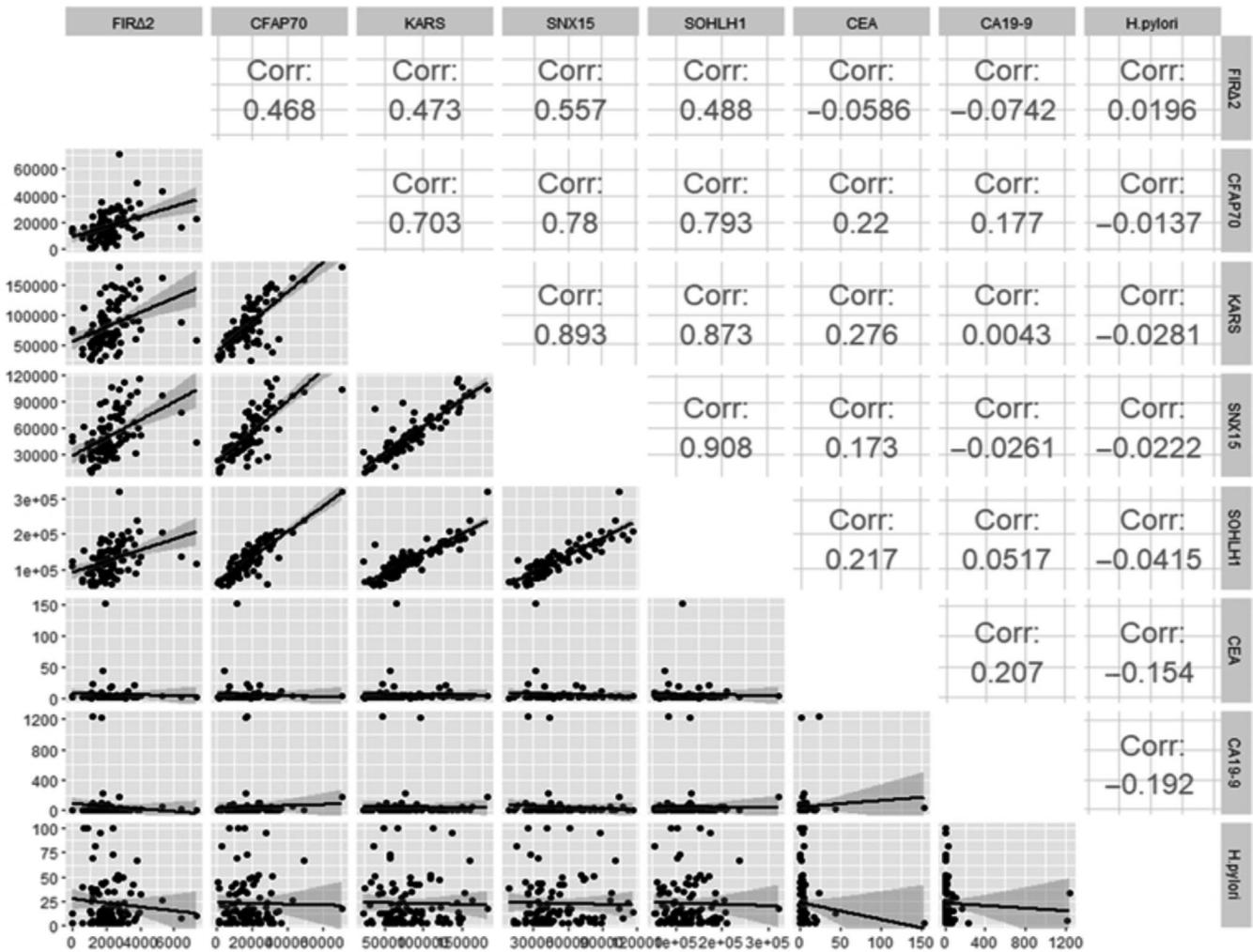
FIGURE 2 The correlation coefficient between candidate markers and clinically used tumor markers. The between-group correlation was assessed using the Spearman's rank correlation coefficient. Lower triangular matrix, the pairwise scatter plots between variables; upper triangular matrix, the Spearman's rank correlation coefficients among each paired measurement

patients with GC (Figure 4A). Combined with CEA, the AUC of anti-FIRΔexon2 Abs (0.719) was higher than that of anti-SNX15 Abs (0.640) or anti-SOHLH1 Abs (0.624) in patients with GC (Figure 4B). Similarly, the combined AUC of anti-FIRΔexon2 (0.706) was larger than those of anti-SNX15(0.624) or anti-SOHLH1 (0.620) Abs with CA19-9 in patients with GC was indicated (Figure 4C). Furthermore, combined with both CEA and CA19-9, anti-FIRΔexon2 Abs exhibited the highest AUC (0.699) in the sera of patients with GC (Figure 4D). We examined the AUC according to clinical stage, early (0, I, and II) or advanced (III and IV), in patients with GC (Figure 5). The highest AUCs were obtained by anti-FIRΔexon2 Abs in both early (0.721) and advanced clinical stages (0.660), compared with those of anti-SOHLH1 Abs, CEA, or CA19-9 in patients with GC. The AUCs of early clinical stage cancer types were as follows: FIRΔexon2 (0.721), SOHLH1 (0.608), CA19-9 (0.598), CEA (0.594), SNX15 (0.577), CFAP70 (0.554), and KARS (0.504; Figure 5A). For advanced clinical stage cancer types, the AUCs were as follows: FIRΔexon2 (0.660), SOHLH1 (0.647), CA19-9 (0.576), CEA (0.577), SNX15 (0.592), CFAP70 (0.560), and KARS (0.506; Figure 5B). In addition, the AUCs of anti-FIRΔexon2 Abs were higher in early-stage

cancer (0.721) than in advanced-stage cancer (0.660). In early-stage GC, the AUC of FIRΔexon2 Abs with CEA (0.731) was higher than those of CEA + CA19-9 (0.701) or CA19-9 (0.698; Figure 5C). In advanced-stage GC, the AUC of anti-FIRΔexon2 Abs with CEA and CA19-9 Abs (0.755) was the highest (Figure 5D). Moreover, the Ab-specific Z-scores were calculated to assess the significance of anti-FIRΔexon2 Abs to CEA and CA19-9 (Table S2) and in clinical stages (Table S3). Overall, anti-FIRΔexon2 Abs exhibited statistically higher AUC than CEA or CA19-9 (Figure 4) and valuable marker independent on clinical stages of patients with GC (Figure 5).

3.4 | Anti-FIRΔexon2 Abs is a novel biomarker for better OS of patients with GC

Univariate analysis revealed that the FIRΔexon2 Ab-positive group (n = 13, 11 early stage and 2 advanced stage) had significantly longer OS than the FIRΔexon2 Ab-negative group (n = 82, 58 early stage and 25 advanced stage; P = .0405; Figure 6A).[38]

FIGURE 3 Venn diagram analysis among candidate markers and clinically used tumor markers. Results of Venn diagram analysis for differentially detected markers identified in gastric cancer (GC) patients. Venn diagrams were used to display the proportions of antibody-positive cases shared among the tumor markers. Each candidate marker indicates the presence or absence of overlapping cases with clinically available tumor markers. Shown are antibody-positive cases. pts, patients
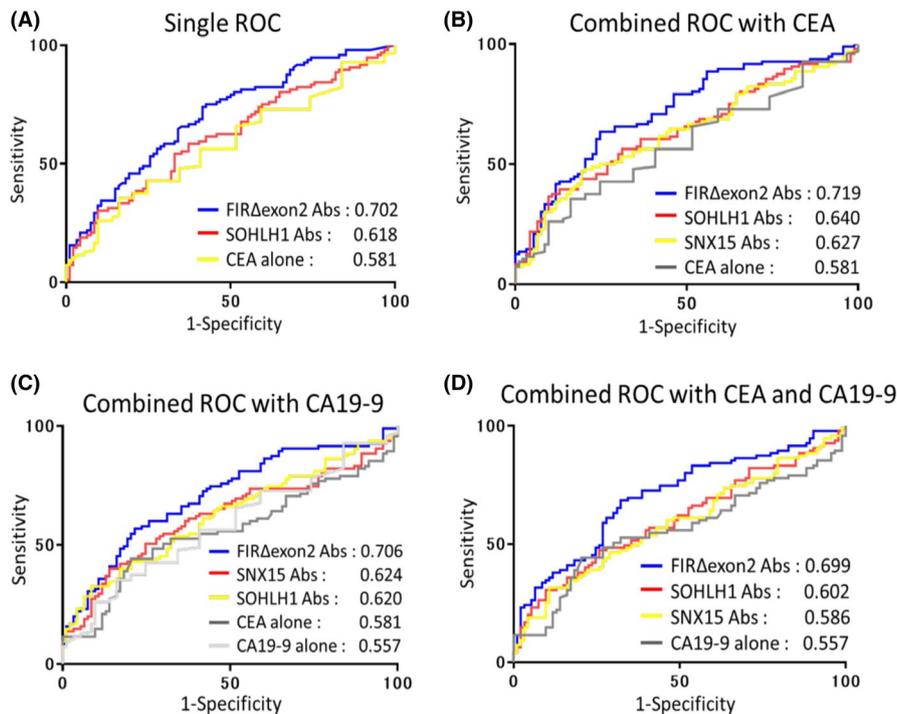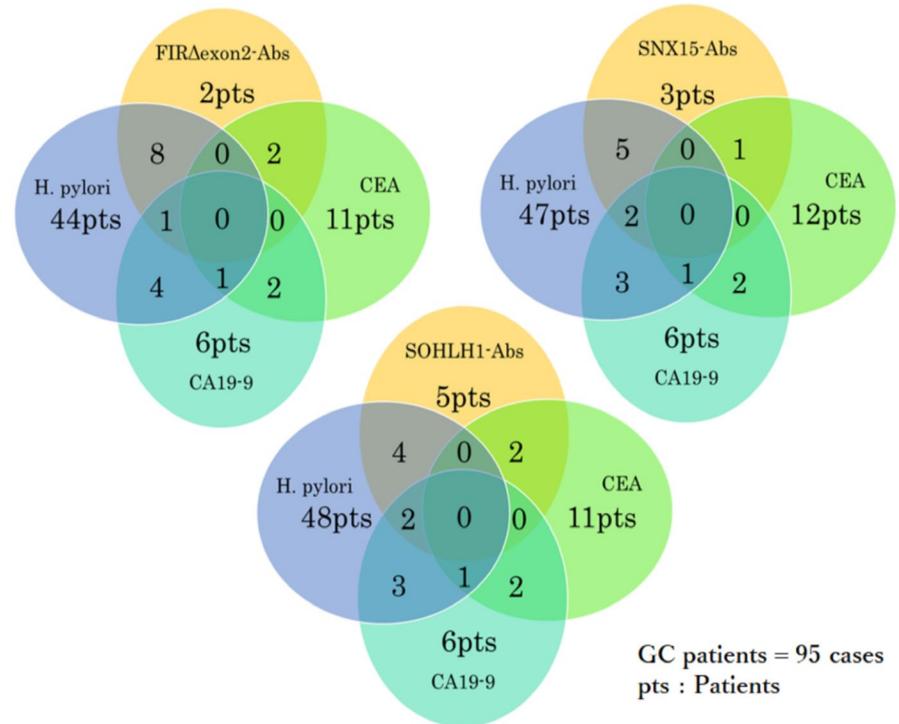

FIGURE 4 The comparison of area under the curve (AUC) values in patients with gastric cancer (GC). The overall diagnostic efficiency of seven antibodies (Abs) was assessed by comparing the receiver-operating curves (ROCs). The ROC curves were generated and AUC values were calculated using GraphPad Prism 7. A, The ROC analysis for individual candidate marker and CEA/CA19-9 markers. The values are shown in the descending order of AUC. Shown are the top Ab lists. B–D, The ROC analysis for the combination of candidate markers and CEA/CA19-9 markers. The ROC analysis of the detected candidate markers and two clinically used tumor markers (CEA and CA19-9) were created according to the Z-score data normalized to the standard deviation (SD) of the quantified α count data of 96 patients with GC and 94 healthy donors. The values are shown in descending order of AUC. Shown are the top Ab lists

The CEA-negative group (n = 80, 60 early stage and 20 advanced stage) exhibited longer OS than the CEA-positive group (n = 16, 9 early stage and 7 advanced stage; P = .0873; Figure 6B). No clinically significant difference was indicated. The CA19-9 Ab-negative group (n = 82, 61 early stage and 21 advanced stage) exhibited longer OS than the CA19-9 Ab-positive group (n = 14,
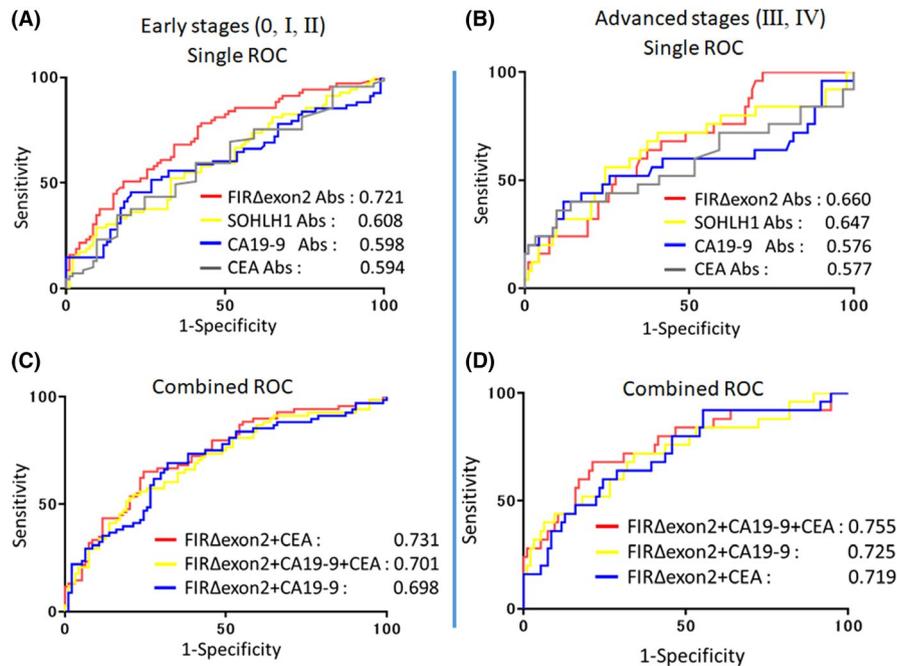
FIGURE 5 Receiver-operating curve (ROC) analysis depicting the diagnostic efficiency of FIRΔexon2 Abs in combination with CEA and CA19-9 markers for early- or advanced-stage gastric cancer (GC). The ROC analysis of detected candidate markers and two clinically used tumor markers (CEA and CA19-9) was created according to the Z-score data normalized to the standard deviation (SD) of the quantified α count data of patients with GC and healthy donors. A, The AUC values of candidate markers for early-stage cancer. B, The AUC values of candidate markers for advanced-stage cancer. C, The ROC analysis depicting the diagnostic efficiency of FIRΔexon2 Abs in combination with CEA/CA19-9 markers for early-stage cancer. D, The ROC analysis depicting the diagnostic efficiency of FIRΔexon2 Abs in combination with CEA/CA19-9 markers for advanced-stage cancer
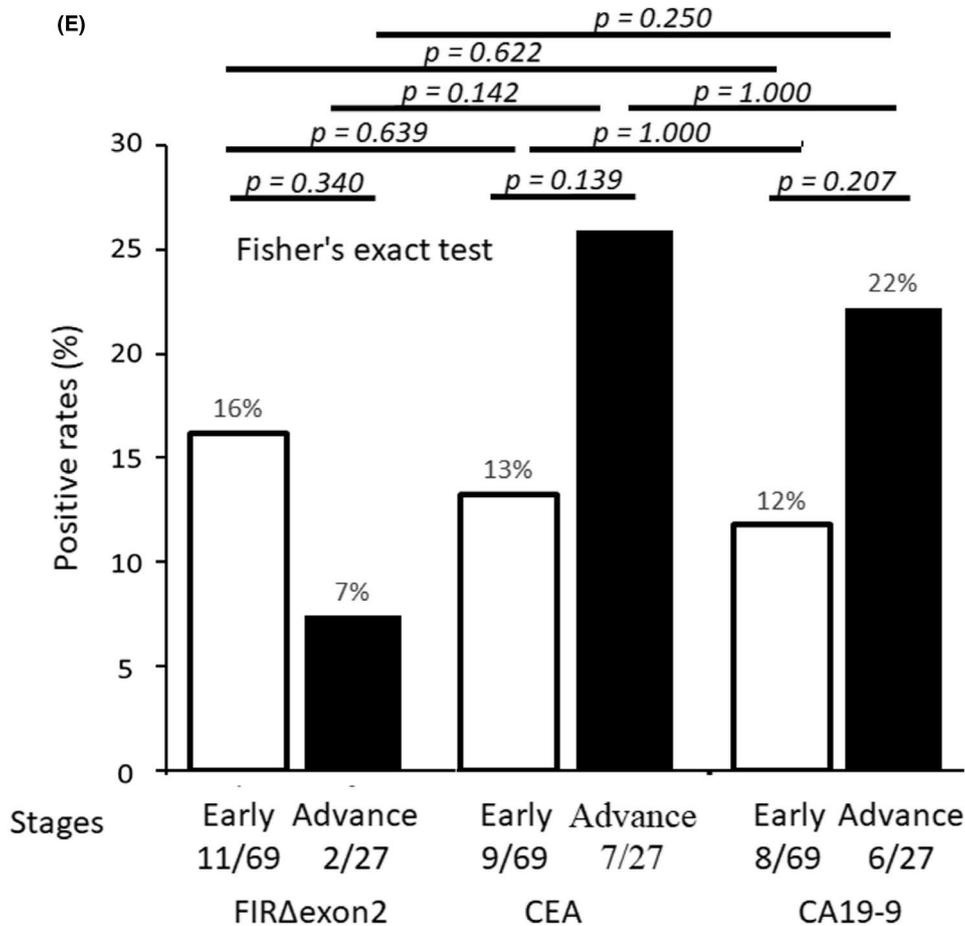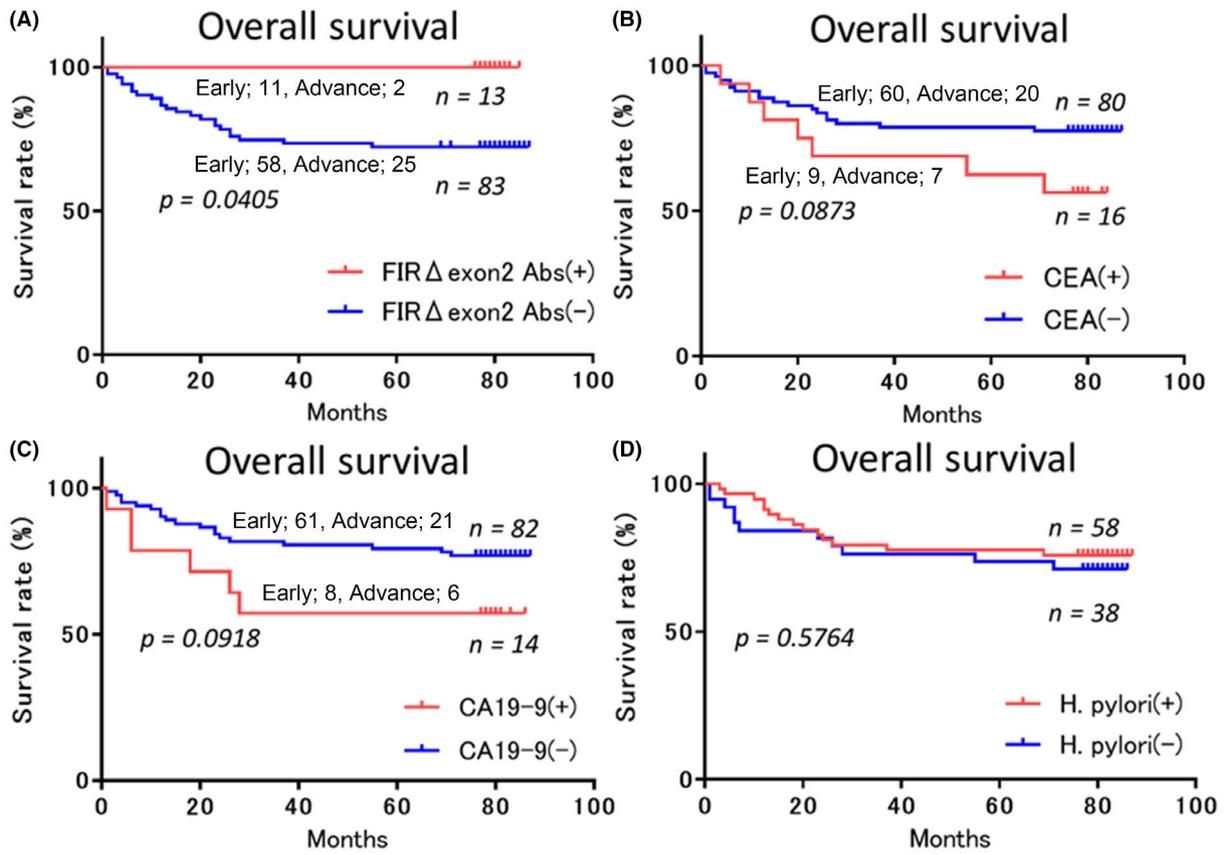
8 early stage and 6 advanced stage; $P = .0918$; Figure 6C). No clinically significant difference was indicated. In addition, *H. pylori* did not affect the OS ($P = .5764$; Figure 6D). Overall, anti-FIRΔexon2 Ab is an independent marker of clinical stages, as we observed no statistically significant difference between patients with early- and advanced-stage GC (Figure 6E).

4 | DISCUSSION

This study revealed that the anti-FIRΔexon2 Ab-positive group exhibited markedly better OS than the anti-FIRΔexon2 Ab-negative group in GC patients. This study reported that anti-FIRΔexon2 Ab in the sera is a novel diagnostic marker and predicted better survival for patients with GC identified by SEREX screening and AlphaLISA.[21] Compared with age-matched HDs, anti-FIRΔexon2, anti-SNX15, and anti-SOHLH Abs in the sera of patients with GC were markedly higher (Table 1 and Figure 1). In addition, no correlation was noted between anti-FIRΔexon2 Abs and the seven tumor markers (anti-CFAP70, anti-KARS, anti-SNX15,

anti-SOHLH1 Abs, CEA, CA19-9, and anti-*H. pylori* Abs; Figures 2 and 3), indicating that anti-FIRΔexon2 Abs is an independent marker for patients with GC. The proportion of early gastric cancer was 72% (69/96), therefore Abs were presumably detected due to the immune responses against early-stage gastric cancer; however, the routine tumor markers were less detected in the sera of those patients. In this study, no clinically significant properties were found in patients with high Abs. The ROC analysis revealed similar results. The AUCs of anti-FIRΔexon2 (0.702) and anti-SNX15 Abs (0.618) were higher than that of CEA (0.581; Figure 4A). In addition, anti-FIRΔexon2 Abs with CEA exhibited the highest AUC (0.719) in GC patients (Figure 4B). The AUC of anti-FIRΔexon2 with CA19-9 was higher than that of CA19-9 alone (0.557) in GC patients (Figure 4C). Moreover, anti-FIRΔexon2 Abs with both elevated CEA and CA19-9 exhibited the highest AUC (0.699; Figure 4D). Accordingly, anti-FIRΔexon2 Ab is a potential novel biomarker for GC patients.[17,20,21] The combined ROC analysis of anti-FIRΔexon2 Abs, anti-SNX15, or anti-SOHLH1 Abs with CEA or CA19-9 revealed increased AUC values in the sera of GC patients. In particular, the combination of CEA and anti-FIRΔexon2

FIGURE 6 The overall survival (OS) curves of patients with gastric cancer (GC) according to each tumor marker. Patients with GC were categorized into two groups based on the presence or absence of each tumor marker. A, The Kaplan–Meier curves for the OS of patients in all stages for FIRΔexon2 Abs ($P = .0405$). B, The Kaplan–Meier curves for the OS of patients in all stages for CEA ($P = .0873$). C, The Kaplan–Meier curves for the OS of patients in all stages for CA19-9 ($P = .0918$). D, The Kaplan–Meier curves for the OS of patients in all stages for *Helicobacter pylori* (0.5764). The statistical analyses were performed using the log-rank test. E, Positive rates by early or advanced stage for each tumor marker

(A) Overall survival

Early; 11, Advance; 2   n = 13

Early; 58, Advance; 25   n = 83

p = 0.0405

FIRΔexon2 Abs(+)
FIRΔexon2 Abs(−)

(B) Overall survival

Early; 60, Advance; 20   n = 80

Early; 9, Advance; 7   n = 16

p = 0.0873

CEA(+)
CEA(−)

(C) Overall survival

Early; 61, Advance; 21   n = 82

Early; 8, Advance; 6   n = 14

p = 0.0918

CA19−9(+)
CA19−9(−)

(D) Overall survival

n = 58

n = 38

p = 0.5764

H. pylori(+)
H. pylori(−)

(E)

p = 0.250
p = 0.622
p = 0.142   p = 1.000
p = 0.639   p = 1.000
p = 0.340   p = 0.139   p = 0.207

Fisher's exact test

16%
7%
13%
22%
12%

Positive rates (%)

Stages

	Early	Advance	Early	Advance	Early	Advance
	11/69	2/27	9/69	7/27	8/69	6/27
	FIRΔexon2		CEA		CA19-9	

Abs exhibited higher AUC in the early or advanced stages of GC patients (Figure 5C). Previously, the significance of ROCs among single or combined markers was assessed by comparing the AUC using Delong tests.[18,19] In all stages of GC patients, anti-FIRΔexon2 Abs + CEA or FIRΔexon2 Abs + CA19-9 or FIRΔexon2 Abs + CEA +CA19-9 were markedly higher than that of CEA and CA19-9 alone (Table S2, middle column), as well as in early or advanced stages of GC patients (Table S3). Thus, anti-FIRΔexon2 Abs with elevated CEA or CA19-9 improved the specificity and sensitivity for the follow-up of GC patients. Nevertheless, further prospective multi-institutional studies will be required to determine the sensitivity and specificity of this combinational detection approach.

Alternate splicing activities induce the secretion of soluble programmed death-ligand 1 (PD-L1) in melanoma cells.[39] The local immune responses indicated by highly expressed HLA-DR antigen reflects better prognosis in colorectal cancer.[40,41] In cancers, aberrant RNA splicing generates altered protein expression, and therefore potentially activates local immune responses.[42] In this study, a high level of anti-FIRΔexon2 Abs in the sera of GC patients indicated better overall survival (Figure 6A). FIR is a splicing variant of PUF60, the poly (U)-binding-splicing factor known as a transcriptional repressor of the *c-myc* gene. In colorectal cancer, a dominant-negative form of FIR, lacking exon2 (FIRΔexon2), is overexpressed.[43-47] The elevated expression of alternative RNA splicing forms of FIR family, FIR and FIRΔexon2, has been reported in many cancers.[7,8,14-17] Anti-PUF60 or anti-FIR family autoAbs have been detected in the sera of dermatomyositis patients,[48] Sjogren's syndrome,[49] and early-stage colon cancer[14]; however, little information is available about its detection significance in the sera of cancer patients. Tumor-specific antigens (TSAs) are antigens derived from mutational frameshifts, splice variants, gene fusions, and other processes.[50] A mass spectrometry analysis identified ribosomal proteins, hnRNPs, splicing-related factors, poly (A)-binding proteins, and mRNA-binding proteins as coimmunoprecipitated proteins with FIR and FIRΔexon2,[51] suggesting that FIRΔexon2 engages in multistep posttranscriptional regulation and promotes tumor proliferation and invasion of GC.[51] In addition, a subset of genetic variants detected through screening of patients with some autosomal dominant hereditary cancer types, such as hereditary breast and ovarian cancer or Lynch syndrome, was reported to influence RNA splicing.[52] Notably, Lynch syndrome presents better prognosis with high microsatellite instability that potentially induces tumor-infiltrating lymphocytes. Thus, anti-FIRΔexon2 Ab, which is caused by alternative splicing of FIR, reflects the immunoreactivity of the host. As the FIR family interacts with splicing factors,[53] the abnormal expression of FIRΔexon2 potentially induces pathogenic alternative splicing changes in numerous genes.[51,54]

Alternatively, GC patients with high anti-FIRΔexon2 Abs titer present better OS, possibly as a result of reaction to aberrant splicing variant form FIRΔexon2, which possibly induces tumor-infiltrating lymphocytes. Together, this study identified five SEREX antigen markers and established that anti-FIRΔexon2 Abs is a candidate biomarker for monitoring better OS and prognosis of GC patients.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.