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Research Article

Development of a New Combination Platform for the Early Screening of Gastric Cancer Using Serum Biomarkers

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ABSTRACT

Objective: The combination of pepsinogens (PG I/II) and gastrin-17 (G17) has been used to screen GC in many countries, without satisfactory levels of sensitivity or specificity. The aim of this study was to find a better marker and a new modality in screening early GC.

Methods: We measured the serum levels of PG I/II, G17, and prealbumin (PA) from the serum of 481 healthy individuals, 407 benign gastric diseases (BGD), and 416 GC patients using a latex particle-enhanced turbidimetric immunoassay and Sandwich ELISA. Logistic regression analysis was used to obtain the sensitivity and specificity of the combined detection model.

Results: When PA was combined with the other biomarkers, the sensitivity and specificity were significantly improved in the ROC curve. The combination of PA+G17+PGI+PGR was the best diagnostic combination for both early and late GC. The AUC, sensitivity, and specificity of the combination for discriminating between early GC and healthy individuals were 0.796, 72.1% and 74.2% respectively. For distinguishing patients with early GC from BGD, the AUC, sensitivity and specificity of the combination were 0.696, 66.7% and 65.4%, respectively. The combination of PA+G17+PGI+PGR improved both the sensitivity and the specificity of GC diagnosis compared with those of the traditional combination of G17+PGI+ PGII +PGR.

Conclusion: PA is a valuable indicator for GC and interacts synergistically with PG and G17 in screening for early GC. The new combination platform PA+G17+PGI+PGR may be a potential way for the early screening of GC.

Introduction

Gastric cancer (GC) is an important global cancer, with more than 1 million new cases annually and an estimated 783,000 deaths in 2018, making it the fifth most frequently diagnosed cancer and the third leading cause of cancer death [1, 2]. In some countries, survival rates for GC patients are high, partly due to the early diagnosis. Currently, China also has screening programmes for people at high risk of GC. However, 80% of patients are still in advanced stage, and the 5-year relative survival rate is lower than that of patients in Japan and South Korea [3-5]. For most cases present in late stages of the disease, few opportunities are present for treatment. Therefore, the high rate of recurrence and high risk of disease progression impose regular surveillance to stomach cancer patients. All these facts indicate that search for new and

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noninvasive methods to detect and monitor stomach cancer are of maximum interest.

As serum pepsinogen (PG) has important application value in the diagnosis of GC, serum pepsinogen (pepsinogen I and pepsinogen II) testing has been used as part of large-scale GC screening [6]. Currently, serum pepsinogen I (PGI), pepsinogen II (PGII), and gastrin-17(G17) are the new modalities in screening GC [7]. The PG values and PGI/II ratio are significantly associated with an increased risk of GC [8, 9]. However, the sensitivity of PG and gastrin 17 were not satisfactory for GC screening. There is an urgent need for an effective diagnostic strategy to detect the early stages of the disease and to be sensitive enough to significantly reduce mortality. Therefore, many efforts have been focused on the identification of diagnostic biomarkers for the early detection of GC with adequate sensitivity. The focus and interest of many researchers and clinicians have been put upon many novel diagnostic markers that may be present within early-stage GC.

Prealbumin (PA) is a transport protein in the serum and cerebrospinal fluid that carries the thyroid hormone thyroxine (T4) and retinol-binding protein bound to retinol [10]. PA was traditionally seen as a biomarker of nutritional status [11]. Lower level of serum PA level has been observed in GC [12]. Our study confirmed that PA was deceased in serum of GC patients. Shimura *et al.* showed that serum PA level can be used as a novel prognostic biomarker for patients with gastric cancer [13]. Prealbumin was a potential marker for diagnosis of GC [12, 14]. In this study, we combined the PA with G17, PGI and PGII to explore a new screening scheme for GC. To our knowledge, there has been no datum available for the study of PA and other three markers together for screening GC.

Materials and Methods

I Serum Samples of Patients

The serum samples were collected from the specimen Banks of Jiangxi Provincial People's Hospital Affiliated to Nanchang University and the Second Affiliated Hospital of Nanchang University. Blood samples were obtained from 416 GC patients aged 20-70 years (average: 59 years). Blood samples were also obtained from 407 benign gastric diseases (BGD) and 481 healthy individuals aged from 20-68 years (average: 56 years). GC cases were a little older than those in the BGD and Healthy groups, and more likely to be men. The clinical, pathological, and demographic information of the subjects is shown in (Table 1). Blood samples from the patients with GC were drawn before surgery. After collection of the blood samples, all participants were confirmed by pathologic diagnosis. All serum samples were obtained by centrifugation at 4°C and then stored in multiple aliquots at -70°C. The protocol used in this study was approved by the Ethics Committee of the two hospitals. All the participants signed a written informed consent form.

Table 1: Patient demographic and clinical information.

	Diagnosis			
	Healthy	BGD	Early stage GC	Late stage GC
Number of case	481	407	226	190
Age				
< 50	196	202	98	91
≥ 50	285	205	128	99
Sex				
Male	261	210	120	110
Female	220	197	106	80
Stage				
I			101	
II			125	
III				93
IV				97

BGD: including inflammation, polyp, ulcer and erosion.

II Determination of Serum Pepsinogens and PA Using a Latex Particle-Enhanced Turbidimetric Immunoassay

Serum PGI, PGII and PA were assayed by a latex particle-enhanced turbidimetric immunoassay (LTIA) using latex bead-immobilized monoclonal antibodies according to the manufacturer's instructions (Huitai Biotech, Nanchang, China). The PGI/II ratio was calculated and reported as a dimensionless fraction. The assay was performed on a Hitachi 7700 P automated analyser.

III Sandwich ELISA Test of Serum Gastrin-17

We performed a sandwich ELISA to determine the optimum conditions for coating and detection of antibodies. A flat-bottomed polystyrene microtiter plate (Nunc A/S, Roskilde, Denmark) was coated with variable dilutions of unlabeled anti-G-17 monoclonal antibodies and incubated at 4°C overnight. The plate was washed three times with phosphate-buffered saline (PBS, pH 7.6) containing 0.05% Tween-20 (PBST) and incubated with a blocking solution containing 3% (w/v) bovine serum albumin (Roche Dx, Mannheim, Germany) for 1 hr at 37°C and washed again as described earlier. After incubation with samples for 1 hr at 37ºC, the plate was washed three times with PBST. Variable dilutions of biotin-labeled anti-G-17 monoclonal antibodies

were added to the wells, and the plate was incubated for 1 hr at 37°C. Three washes using PBST were performed, then 100 μl of avidin-biotin complex stain (Thermo Fisher Scientific Inc., Waltham, MA, USA) was added, and the plate was incubated at room temperature for 30 min. Finally, three additional PBST washes were performed, then 100 μl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich Inc., St. Louis, MO, USA) was added to each well, and the plate was incubated at room temperature for 10 min. The reaction was stopped by adding 1M phosphoric acid (Wako Pure Chemicals Industries, Ltd.). The absorbance value of each well was measured at 450 nm using a microplate reader.

IV Statistical Analysis

The statistical analyses were carried out by using SPSS version 19 software. The levels of serum PA, pepsinogens and gastrin-17 were expressed as the mean ± SD. The t-tests were performed between two groups. The receiving operating characteristics (ROC) curve was generated for the diagnostic value. The area under each ROC curve was used to measure the discriminating ability of the model. Logistic regression analysis was used to obtain the sensitivity and specificity of the combined detection model. Statistical significance was set at a two-sided P value < 0.05.

Results

I Serum Levels of PA, Pepsinogens and G17 in GC, BGD and Healthy Groups

Concentration of serum PA, pepsinogens and G17 are shown in (Figure 1). The serum levels of PA and PGR were significantly lower in GC than those in healthy individuals and BGD, while the levels of G17 and PGII were much higher in GC. With tumor stage, the serum levels of PA gradually decreased, while G17 tended to increase. The serum levels of PGI in BGD were the highest in all experimental groups. Moreover, the detection values of PA, PGI and PGR were the lowest in serum of patients with advanced GC (stage III-IV).

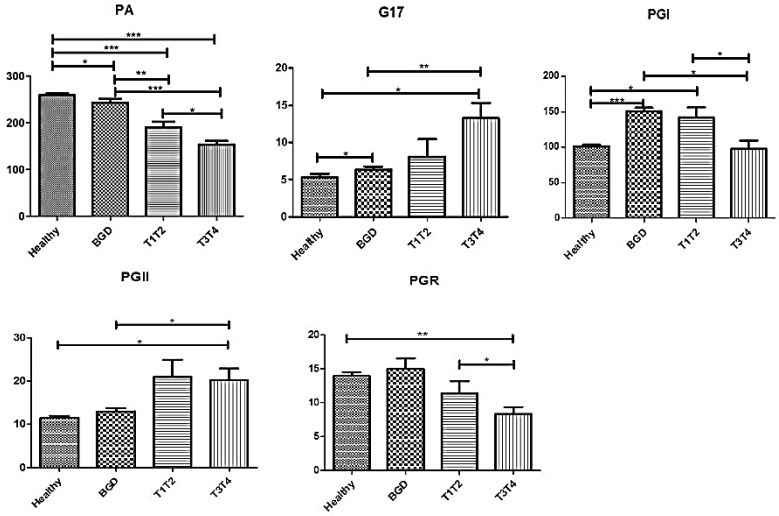

Figure 1: Serum levels of PA, pepsinogens and G17 in GC, BGD and Healthy groups. The serum levels of PA and PGR were significantly lower in GC than those in healthy individuals and BGD. BGD, benign gastric diseases; T1T2, early GC; T3T4, late GC; *, P<0.05; **, P<0.01; ***, P<0.001.

II Evaluation of Diagnostic Value of Serum PA, Pepsinogens and Gastrin-17 Alone for GC

The sensitivity, specificity and AUC are summarized in Table (Supplementary Tables 1-4). The evaluation of diagnostic value of serum PA, pepsinogens and gastrin-17 was analysed using pathological diagnosis as the gold standard. The ROC curves for discriminating between GC and healthy individuals are shown in (Figures 2A & 2B). The AUC areas of PA, PGI, PGII, PGR and G17 for early GC were 0.723, 0.559, 0.594, 0.610 and 0.594 respectively. The AUC areas of PA, PGI, PGII, PGR and G17 for late GC were 0.787, 0.638, 0.658, 0.642 and 0.693 respectively. The sensitivity and specificity of PA were 42.4% and 94.9% for stage I-II, and 88.4% and 66.7% for stage III-IV, respectively (Supplementary Tables 1 & 2). On the other hand, The ROC curves for discriminating GC from BGD are shown in (Figure 2C & 2D). The AUC areas of PA, PGI, PGII, PGR and G17 for early GC were 0.678, 0.598, 0.560, 0.515 and 0.589 respectively. The AUC areas of PA, PGI, PGII, PGR and G17 for late GC were 0.715, 0.546, 0.528, 0.545 and 0.537 respectively. The sensitivity and specificity of PA were 51.5% and 66.8% for stage I-II, and 55.8% and 78.9% for stage III-IV, respectively (Supplementary Tables 3 & 4). In brief, among all of the five markers, the AUC area of PA was the largest in both early and late GC (Figure 2).

III Determination of New Joint Detection Platforms

In order to screen out a new joint detection platform, different combinations are compared in (Figure 3). Figures 3A & 3B are the ROC curves obtained by healthy individuals as a reference. Logistic Regression analysis showed that PA+G17+PGI+PGR was the best diagnostic combination for the early GC. The AUC, sensitivity and specificity of this combination in the diagnosis of early GC were 0.796, 72.1% and 74.2%, respectively (Table 2). Supplementary Table 5 demonstrated that the AUC, sensitivity and specificity of the combination in the diagnosis of late GC were respectively 0.824, 79.4% and 74.1% (Supplementary Table 5). The following Logistic Regression

analysis was about the discriminating between BGD and GC. The ROC curves showed that the combination of PA+G17+PGI+PGR was one of the best diagnostic curves in different combinations (Figures 3C & 3D). The AUC, sensitivity and specificity of the combination in the diagnosis of early GC were 0.696, 66.7% and 65.4%, respectively (Table 3), while an AUC of 0.759, a sensitivity of 65.5%, and a specificity of 73.8% were for the late GC (Supplementary Table 6). Comprehensively considering logistic regression analysis of GC vs healthy (Figures 3A & 3B) and GC vs BGD (Figures 3C & 3D), the PA+G17+PGI+PGR combination was the best platform for screening GC.

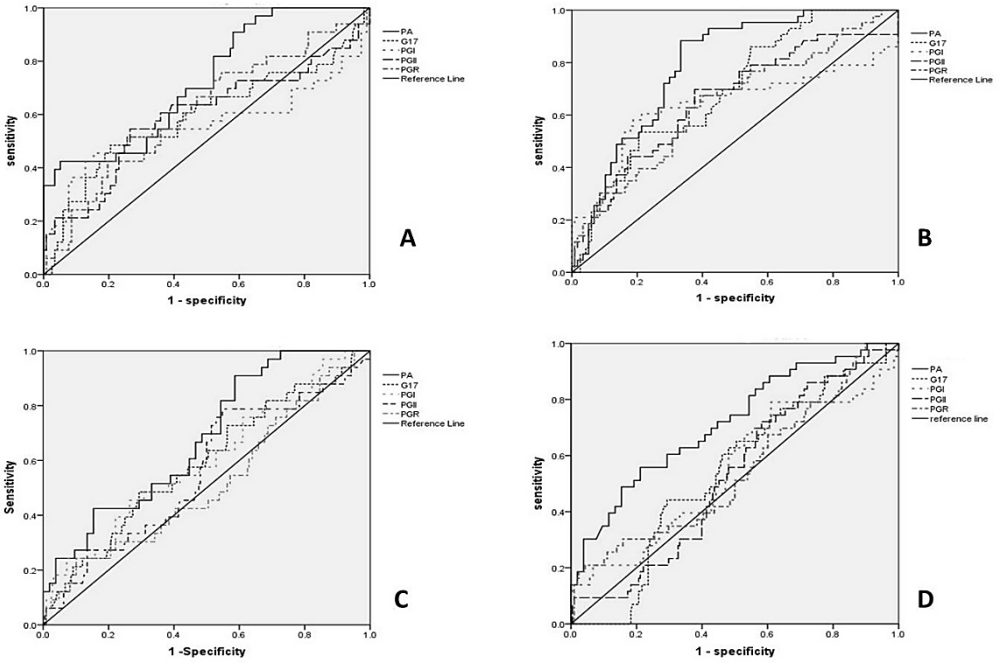

Figure 2: ROC curves for PA, PGI, PGII, PGR and G17 alone. The AUC area of PA was the largest in both early and late GC. **A)** The ROC curves for discriminating between early GC and healthy individuals. **B)** The ROC curves for discriminating between late GC and healthy individuals. **C)** The ROC curves for discriminating between early GC and BGD. **D)** The ROC curves for discriminating between late GC and BGD.

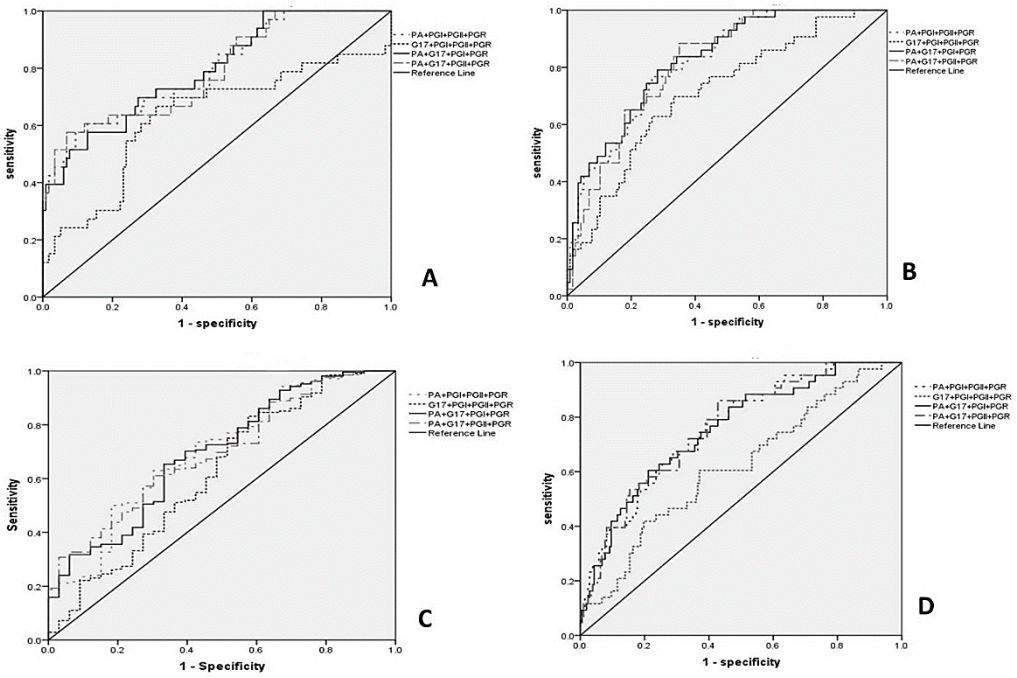

Figure 3: ROC curves for discriminating GC from healthy individuals or BGD using combination platforms. Logistic Regression Analysis showed that the combination of PA+G17+PGI+PGR was the best platform for screening GC. **A)** The ROC curves for discriminating early GC from healthy individuals. **B)** The ROC curves for discriminating between late GC and healthy individuals. **C)** The ROC curves for the differential diagnosis of early GC and BGD. **D)** The ROC curves for discriminating between late GC and BGD.

Table 2: Diagnostic values of different combinations for detecting early GC from healthy.

	AUC	95% CI	Sensitivity	Specificity
PA+G17+PGI+PGR	0.796	0.708-0.885	0.721	0.742
G17+PGI+PGII+PGR	0.629	0.506-0.752	0.647	0.655
PA+PGI+PGII+PGR	0.795	0.706-0.884	0.589	0.906
PA+G17+PGII+PGR	0.789	0.698-0.881	0.576	0.912

Table 3: Diagnostic values of different combinations for detecting early GC from BGD.

	AUC	95% CI	Sensitivity	Specificity
PA+G17+PGI+PGR	0.696	0.604-0.792	0.667	0.654
G17+PGI+PGII+PGR	0.619	0.507-0.732	0.484	0.682
PA+PGI+PGII+PGR	0. 696	0.605-0.791	0.687	0.630
PA+G17+PGII+PGR	0.695	0.603-0.785	0.697	0.611

Discussion

Despite of the technique advances, gastric cancer (GC) remains a severe health problem. An effective diagnostic strategy is urgently required for detection of early stage of GC with adequate sensitivity that could significantly reduce mortality rates. At present, PGI, PGII and G17 are used for screening GC in many countries [7]. However, the effect of this screening scheme is unsatisfactory in both specificity and sensitivity. Our study demonstrated that PA is a valuable GC screening indicator. In this research we combined the PA with G17, PGI and PGII to explore a new screening scheme for GC.

Pepsinogen (PG) is a precursor of an aspartic acid protease secreted by the gastric mucosa. As serum pepsinogen has important application value in the diagnosis of GC, currently serum PG is one of the standard modalities in screening GC [6, 8, 15, 16]. The PGI/II ratio is significantly associated with an increased risk of GC [9, 17-19]. But the sensitivity of the detection of PG is not high for the early diagnosis of GC. Gastrin-17 (G17) is another biomarker for the diagnosis of GC [20]. Serum G17 levels are abnormal in GC and precancerous lesions such as atrophic gastritis, intraepithelial neoplasia [21, 22]. However, the sensitivity and specificity of G17 for GC are not high either. In order to improve the accuracy of early detection of GC, many countries now combine G17 with PG to form a joint detection platform for early detection of GC [7]. Although this combination improves the specificity of GC screening, its sensitivity is unsatisfactory [20]. Therefore, it is necessary to add new elements to explore new combinations to improve the accuracy of GC screening.

Prealbumin (PA, TTR) is a normal serum protein synthesized primarily in the liver, the choroid plexus and the retina. PA binds and transports the thyroid hormones and the retinol-binding protein-retinal complex [23-25]. The expression level of PA is to a certain degree correlated with the clinical stage, lymph node metastasis and differentiation of patients in gastric cancer [12]. Serum PA is useful for predicting the prognosis of patients with gastric cancer [13]. The serum level of PA was significantly lower in GC than those in healthy individuals and BGD (Figure 1). Our results showed the AUC area of PA was the largest among all of the markers in both early and late GC (Figure 2). In comparison to PG and G17, PA had a higher sensitivity in detecting stage I GC. On this basis, we further explored whether the combination of PA and other markers could improve the accuracy of GC detection. Wang *et al.* suggested the benefit of the combination of PA with ApoC-I and ApoC-III for the diagnosis of GC, particularly test specificity [12]. Traditionally, the most used combinations are G17+ PGI + PGII +PGR and PGI + PGII +PGR [20, 26-28]. The study by Kikuchi *et al.* showed the sensitivity of G17+ PGI + PGII +PGR in the diagnosis of GC was only 12%. Even though the specificity was 98%, the results were not as good as expected [20]. Mizuno *et al.* found that the sensitivity of PGI + PGII +PGR to diagnose GC was 36.8%, but the specificity was lower than that of Kikuchi [26].

Our study suggests that the combination of PA with PG and G17 can improve the detection of early-stage GC. According to different permutation and combination, our joint detection platform was divided into four groups for comparison, namely PA+G17+PGI+PGR, PA+G17+PGII+PGR, PA+ PGI+ PGII+PGR, and G17+PGI+ PGII+PGR. After logistic regression analysis and comparison, the optimal combination was selected for the early screening of GC. The results showed that PA+G17+PGI+PGR were the most qualified combination for both early and late GC (Figure 3). The AUC, sensitivity and specificity of this combination in the diagnosis of early GC from healthy individuals were 0.796, 72.1% and 74.2%, respectively (Table 2). The AUC, sensitivity and specificity for discriminating between early GC and BGD were respectively 0.696, 66.7% and 65.4% (Table 3). This diagnostic accuracy is significantly better than the traditional combination of G17+ PGI + PGII +PGR. The AUC, sensitivity and specificity of the traditional combination in the diagnosis of early GC from healthy individuals were 0.629, 64.7% and 65.5%, and 0.619, 48.4% and 68.2% between early GC and BGD, respectively (Tables 2 & 3). Although the traditional combination will produce a large number of false positives and false negatives in GC screening, due to the lack of a better combined screening platform, most countries still use this platform to screen GC. Now we have found a better platform than the traditional combination, which is our new combination platform PA+G17+PGI+PGR. The new PA combination has higher detection sensitivity for early GC than the traditional combination (72.1% versus 64.7%), and also higher specificity (74.2% versus 65.5%), respectively (Table 2). At the same time, the sensitivity and specificity of our new platform in the detection of advanced GC were significantly higher than those of the traditional combination (79.4% versus 68.2%) and 74.1% versus 66.3%, respectively (Supplementary Table 5). In addition, our PA+G17+PGI+PGR combination was also more accurate than the traditional G17+PGI+ PGII +PGR in the differential diagnosis of GC and BGD (Table 3 & Supplementary Table 6). All of these results strongly suggest that PA is a valuable indicator for GC and, most

importantly, that it interacts synergistically with PG and G17 in screening for early GC.

Conclusion

This study is the first attempt to combine PA and other GC markers into a new detection platform for the screening of GC. The screening effect of our new combination of PA+G17+PGI+PGR is much stronger than that of the traditional combination of G17+PGI+ PGII +PGR, especially for the screening of early GC. This proves that our study will explore a new and more effective way for the early screening of GC.

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Author Contributions

Conception and design: Weiqun Rao, Yuefei Yu; Provision of study materials or patients: Limin Ding, Honglang Li, Guoxing Xie; Collection and assembly of data: Honglang Li, Guoxing Xie; Data analysis and interpretation: Jiquan Yu, Yanjiao Du, Wei Tang; Manuscript writing: Yanjiao Du, Wei Tang.

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Availability of Data and Materials

All data generated or analysed in this study are included in this published article.

Ethical Approval

The protocol used in this study was approved by the Ethics Committee of Jiangxi Provincial People's Hospital Affiliated to Nanchang University and the Second Affiliated Hospital of Nanchang University. Specimens were collected within the diagnostic procedure after no clinical use was indicated any longer. All the participants signed a written informed consent form.

Consent for Publication

Not applicable.

Conflicts of Interest

None.

Supplementary Table 1: Diagnostic values of PA, pepsinogens and G17 for detecting early GC from healthy.

	AUC	95% CI	Sensitivity	Specificity
PA	0.723	0.626-0.820	0.424	0.949
G17	0.594	0.468-0.719	0.485	0.795
PGI	0.559	0.420-0.617	0.455	0.846
PGII	0.594	0.470-0.718	0.545	0.735
PGR	0.610	0.497-0.722	0.424	0.803

Supplementary Table 2: Diagnostic values of PA, pepsinogens and G17 for detecting late GC from healthy.

	AUC	95% CI	Sensitivity	Specificity
PA	0.787	0.716-0.858	0.884	0.667
G17	0.693	0.606-0.780	0.535	0.795
PGI	0.638	0.522-0.755	0.721	0.291
PGII	0.658	0.558-0.758	0.698	0.624
PGR	0.642	0.543-0.741	0.674	0.607

Supplementary Table 3: Diagnostic values of PA, pepsinogens and G17 for detecting early GC from BGD.

	AUC	95% CI	Sensitivity	Specificity
PA	0.678	0.587-0.768	0.909	0.413
G17	0.589	0.483-0.696	0.485	0.707
PGI	0.598	0.491-0.706	0.455	0.736
PGII	0.560	0.453-0.667	0.788	0.452
PGR	0.515	0.404-0.625	0.242	0.899

Supplementary Table 4: Diagnostic values of PA, pepsinogens and G17 for detecting late GC from BGD.

	AUC	95% CI	Sensitivity	Specificity
PA	0.715	0.629-0.801	0.558	0.789
G17	0.537	0.451-0.623	0.441	0.707
PGI	0.546	0.445-0.647	0.790	0.390

PGII	0.528	0.441-0.616	0.860	0.279
PGR	0.545	0.446-0.644	0.209	0.957

Supplementary Table 5: Diagnostic values of different combinations for detecting late GC from healthy.

	AUC	95% CI	Sensitivity	Specificity
PA+G17+PGI+PGR	0.824	0.757-0.892	0.794	0.741
G17+PGI+PGII+PGR	0.713	0.624-0.802	0.682	0.663
PA+PGI+PGII+PGR	0.818	0.750-0.886	0.767	0.735
PA+G17+PGII+PGR	0.813	0.746-0.880	0.884	0.650

Supplementary Table 6: Diagnostic values of different combinations for detecting late GC from BGD.

	AUC	95% CI	Sensitivity	Specificity
PA+G17+PGI+PGR	0.759	0.685-0.834	0.655	0.738
G17+PGI+PGII+PGR	0.616	0.524-0.709	0.605	0.630
PA+PGI+PGII+PGR	0.758	0.684-0.833	0.790	0.611
PA+G17+PGII+PGR	0.761	0.687-0.835	0.860	0.572

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