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Feasibility of Plasma-Methylated *SFRP2* for Early Detection of Gastric Cancer

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Abstract

Gastric cancer (GC) is fifth most frequently diagnosed cancer and second leading cause of cancer in China. More than 80% of GC are diagnosed at an advanced stage due to low uptake rate of invasive screening method. The performance of methylated *SFRP2* test was evaluated in 236 plasma samples, including 92 patients with GC, 16 intestinal metaplasia patients, 26 gastric fundic gland polyp patients, 13 small adenoma patients, 39 hyperplastic polyp patients, and 50 control patients. The sensitivity of plasma methylated *SFRP2* was compared to serum CEA, CA72-4, CA19-9, and CA242 results in 79 patients with GC. The sensitivities for detecting GC and gastric intestinal metaplasia by methylated *SFRP2* test were 60.9% and 56.3% with a specificity of 86.0%. Methylated *SFRP2* test had significantly higher positive detection rate for patients with GC than gastric fundic gland polyp, small adenoma, and hyperplastic polyp patients. In 79 patients with GC, the sensitivities of CEA, CA72-4, CA19-9, and CA242 for detecting GC were 22.8%, 16.5%, 12.7%, and 11.4%. In comparison, the sensitivity of methylated *SFRP2* test for detecting GC was 58.2%. Plasma methylated *SFRP2* test may become a valuable tool for the noninvasive detection of GC and precursor lesions and showed higher sensitivity than serum tumor markers.

Keywords

Gastric cancer, gastric intestinal metaplasia, methylated *SFRP2*, sensitivity, specificity

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Introduction

Gastric cancer (GC) is responsible for over 1 000 000 new cases in 2018 and an estimated 783 000 deaths, making it the fifth most frequently diagnosed cancer and the third leading cause of cancer deaths worldwide.[1] The incidence rates of GC are markedly elevated in Eastern Asia, including China, Mongolia, Japan, and the Republic of Korea.[2] In China, GC has become the second leading cause of cancer deaths in men and women with 679 100 estimated new cases diagnosed each year, and its 5-year survival rate is low because more than 80% of patients are diagnosed at an advanced stage.[3]

Long-standing screening program and effective early detection method are the most effective strategies to reduce the incidence and mortality of GC. Only 2 countries globally, Japan and Korea, are conducting population-based GC screening.[4] In Japan, X-ray photofluorography is the regular screening method, but high cost and fear for radiation exposure have

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Table 1. Characteristics of Individuals Enrolled in this Study.

	Number (N)	Gender		Age	
		Male, n (%)	Female, n (%)	Min-Max	Average
Total GC	92	67 (72.8%)	25 (27.2%)	31-83	60.7
I	24	18 (75.0%)	6 (25.0%)	36-75	56.3
II	18	16 (88.9%)	2 (11.1%)	44-78	61.3
III	22	12 (54.5%)	10 (45.5%)	42-78	60.7
IV	8	4 (50.0%)	4 (50.0%)	31-74	59.1
Unknown	20	17 (85.0%)	3 (15.0%)	47-83	66.2
IM	16	8 (50.0%)	8 (50.0%)	47-77	61.3
GFGP	26	8 (30.8%)	18 (69.2%)	20-75	51.3
AP	13	9 (69.2%)	4 (30.8%)	51-77	63.9
HP	39	10 (25.6%)	29 (74.4%)	32-88	56.0
Control	50	31 (62.0%)	19 (38.0%)	22-76	35.1

Abbreviations: AP, adenoma patients; GC, gastric cancer; GFGP, gastric fundic gland polyp; HP, hyperplastic polyp; IM, intestinal metaplasia.

led to a low uptake rate.[2] Since endoscopy can detect the early stages of GC, its introduction into communities for GC screening had been highly anticipated, but in reality, its invasiveness has resulted in low acceptance rate.[4] Serum tumor marker tests are simple and noninvasive approaches for screening tumors.[5] However, the low sensitivity of these markers made the tests for them hardly a primary strategy for early GC detection.[6]

WNT proteins are secreted signaling factors with multiple functions in development and tumorigenesis.[7] Secreted frizzled-related proteins (*SFRPs*), a family of 5 secreted glycoproteins, are identified as possible negative modulators of the WNT signal transduction pathway.[8] Encoding one of these proteins, *SFRP2* gene plays an important role in cell growth, apoptosis, and regulation of cell differentiation, and it is often methylated in human cancers.[9] Several reports found *SFRP2* to be hypermethylated in GC tissues and blood samples,[10,11] suggesting that methylated *SFRP2* may serve as a noninvasive biomarker for early detection of GC. In this study, we evaluated the performance of a plasma methylated *SFRP2* test for the feasibility as a noninvasive screening tool for GC.

Materials and Methods

Sample Collection

Fresh frozen GC tissues (n = 9) and paired adjacent paracancerous tissues (n = 9) were collected at the time of surgery at the Affiliated Hospital of Xuzhou Medical University. All tissue samples were stored at −80°C until use. Plasma specimens were collected from 92 patients with GC, 16 patients with gastric intestinal metaplasia (IM), 26 patients with gastric fundic gland polyp (GFGP), 13 small adenoma (AP, adenomas <1 cm and without dysplasia or villous component) patients, and 39 patients with hyperplastic polyp (HP), and the diagnoses of all patients were histologically confirmed by a pathologist. Control plasma specimens were collected from 50 patients with no evidence diseases or with chronic superficial gastritis as verified by gastroscopy at the Affiliated Hospital of Xuzhou Medical University (Table 1), and colonoscopy was performed on control patients and confirmed no colonic lesions. Ten-milliliter blood was drawn from each patient and stored at 4°C within 6 hours. The plasma fractions were then separated and immediately frozen at −80°C until use. The blood leukocytes of 4 CRC patients, 2 AP patients, and 15 control patients were collected post plasma separation and frozen at −80°C until use. This study was approved by the Institutional Review Board of the Affiliated Hospital of Xuzhou Medical University (ethics committee reference number: XYFY2019-KL121), and informed consent was obtained from all participating patients and control patients.

DNA Extraction, Bisulfite Treatment, and Quantitative Real-Time PCR

Tissue genomic DNA was isolated using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Leukocyte genomic DNA was isolated using VersaPrep DNA extraction kit (Suzhou VersaBio Technologies Co Ltd, Kunshan, China). Plasma DNA was extracted using a circulating free DNA extraction kit (Suzhou VersaBio Technologies Co Ltd) from 3.5-mL plasma and eluted in 100-μL elution buffer. Subsequently, 100-μL purified cfDNA and genomic DNA isolated from tissue or leukocytes were used for bisulfite conversion and the converted DNA was purified and then eluted in 100 μL of elution buffer. DNA bisulfite conversion and purification of the converted product was performed with a bisulfite conversion kit (Suzhou VersaBio Technologies Co Ltd). All the kits were used according to the manufacturer's instructions.

Purified DNA obtained from the above steps was tested by a methylated *SFRP2* test (Suzhou VersaBio Technologies Co Ltd). Methylated *SFRP2* and an internal control (*ACTB*) can be detected simultaneously in the same multiplex qPCR reaction. Three qPCR replicates were performed for each plasma sample, and a single qPCR reaction was performed for each tissue sample. The total qPCR volume was 30 μL with 15-μL DNA and 15-μL PCR master mix. Real-time PCR was performed on LC480-II thermal cycler (Roche Diagnostics, Mannheim, Germany) using the following cycling conditions: activation at 95°C for 30 minutes, 50 cycles of 95°C for 10 seconds, 58°C for 30 seconds, 72°C for 10 seconds, and final cooling to 40°C for 30 seconds.

Serum Tumor Marker Detection

Serum CEA, CA72-4, and CA19-9 levels were measured by using Roche Cobas 8000 electrochemiluminescence instrument and CA242 was test by using Snibe Diagnostic MAGLUMI 4000 instrument at Department of Laboratory Medicine of the Affiliated Hospital of Xuzhou Medical University. The normal reference values were as follows: CEA $\leq$ 5 ng/mL, CA72-4 $\leq$ 6.9 U/mL, CA19-9 $\leq$ 35 U/mL, CA242 $\leq$ 20 IU/mL.

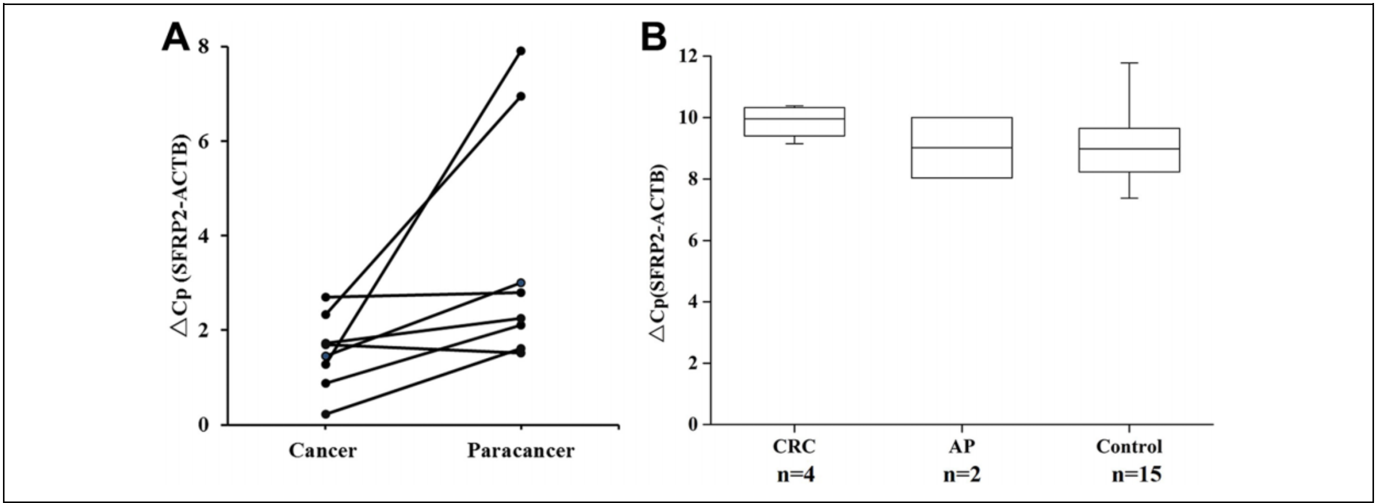

Figure 1. Methylated *SFRP2* level in GC tissues, paired adjacent paracancerous tissues (A) and leukocytes (B). GC, gastric cancer.

Data Analysis

ΔCp was used to determine the methylation level of *SFRP2* in GC tissues, paired adjacent paracancerous tissues, and leukocytes. ΔCp was defined as the difference between the Cp values for the target (*SFRP2*) and the internal control gene (*ACTB*). The results for plasma specimens were considered "invalid" if *ACTB* Cp (output data from Roche LC480II real-time PCR machine defines threshold cycle number as Cp) was greater than 35.0, and methylated *SFRP2* was considered "detected" if its Cp value was less than 39.0. Methylated *SFRP2* was analyzed with a 2/3 rule in which a plasma sample was scored positive if at least 2 of 3 PCR replicates had valid amplification curves (2/3 algorithm). Data were subjected to statistical analysis by IBM SPSS software for Windows version 22.0, and *t* test was used for comparison of 2 samples at the significant level of $P < .05$. Sensitivity and specificity data were used to plot the receiver operating characteristic (ROC) curve. The mean Cp values of IM, GFGP, AP, HP, GC, and control patients for methylated *SFRP2* were used to represent methylation level. Because most control patients were not detected in qPCR reaction, their Cp values were set to 50 (the maximal number of PCR cycles) to plot ROC curve and the chart for methylation levels.[12] The Youden index was used to evaluate the effectiveness of plasma methylated *SFRP2* in detecting IM and patients with GC,[13] it was thus calculated by the following equation:

$$\text{Youden index} = \text{Sensitivity} + \text{specificity} - 100\%.$$

Results

Measured by a methylated *SFRP2* test, *SFRP2* methylation levels were higher in 88.9% (8 of 9) of GC tissues than in their paired adjacent paracancerous tissues ($P < .05$, Figure 1A). In addition, *SFRP2* methylation levels in the leukocytes of patients with GC, AP patients, and normal patients seemed no significant difference ($P > .05$, Figure 1B), thus making plasma methylated *SFRP2* test a candidate for distinguishing GC and normal patients. To evaluate the feasibility of methylated *SFRP2* test for early GC detection, 236 plasma samples were collected from patients of the Affiliated Hospital of Xuzhou Medical University, of which 92 were from patients with GC, 16 from IM patients, 26 from GFGP patients, 13 from AP patients, 39 from HP patients, and 50 from control patients. The patients with GC ranged from 31 to 83 years old with a mean age of 60.7, and 72.8% were male patients. The control patients ranged from 22 to 76 years old with a mean age of 35.1, and 62.0% were males (Table 1).

Of 92 GC plasma samples whose stages were determined based on the surgically resected specimens, methylated *SFRP2* was detected in 50.0% of stage I (12 of 24), 55.6% of stage II (10 of 18), 59.1% of stage III (13 of 22), 87.5% of stage IV (7 of 8), and 70.0% of unknown stage (14 of 20) samples (Figure 2A). The sensitivity for detecting all stage GC by methylated *SFRP2* test was 60.9% (95% confidence interval [CI]: 50.1%-70.7%) with a specificity of 86.0% (95% CI: 72.6%-93.7%). In addition, methylated *SFRP2* test also demonstrated significantly higher positive detection rates for GC and IM patients than control patients ($P < .05$), whereas the positive detection rates for GC and IM patients showed no significant difference ($P = .786$; Figure 2B). Furthermore, the positive detection rate for patients with GC was also significantly higher than benign polyps (GFGP, AP, and HP, $P < .05$). As Cp values of a methylated biomarker reflect its methylation levels, where higher Cp values represent lower methylation levels, the mean Cp values of methylated *SFRP2* for different patient groups were consistent with their disease status (Figure 2C). Particularly, the mean Cp values of methylated *SFRP2* showed a decreasing trend across stage I to IV, and the mean Cp values for GC and IM patients were significantly lower than those for GFGP, AP, HP, and control patients, showing a pattern consist with that of positive detection rates shown in Figure 2A and B.

The ROC curve for plasma methylated *SFRP2* test detecting GC is shown in Figure 3. The area under the curve for GC detection was 0.784 (95% CI: 0.709-0.859). The Youden index

Figure 2. Positive detection rates and methylation levels of methylated *SFRP2* in detecting intestinal metaplasia, GFGP, AP, HP, GC, control, and GC across stages I-IV. A, Sensitivity and specificity of methylated *SFRP2* test in detecting GC. B, The difference of positive detection rates between IM, GFGP, AP, HP, GC, and control patients detected by methylated *SFRP2* test. C, Methylation levels of methylated *SFRP2* in IM, GFGP, AP, HP, GC, and control patients. AP indicates adenoma patients; GC, gastric cancer; GFGP, gastric fundic gland polyp; HP, hyperplastic polyp; IM, intestinal metaplasia.

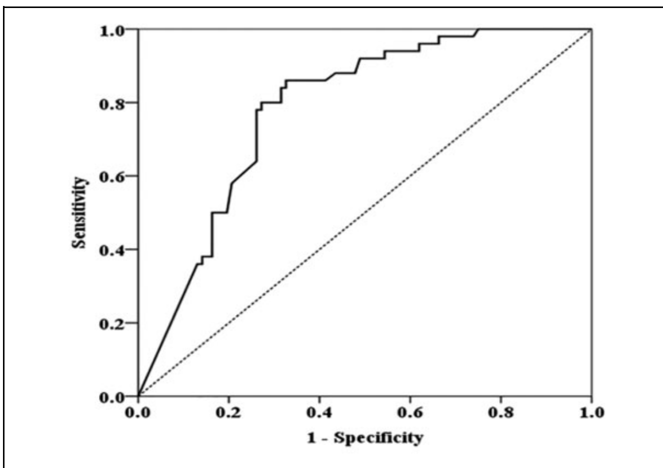

Figure 3. Receiver operating characteristic curve for methylated *SFRP2* test in detecting gastric cancer. AUC = 0.784 (95% CI: 0.709-0.859). AUC indicates area under the curve; CI, confidence interval.

of plasma methylated *SFRP2* in detecting IM and patients with GC were 42.3% and 46.9%, respectively. Furthermore, there was no significant difference among the positive detection rates of methylated *SFRP2* test in detecting GC between different ages, genders, tumor locations, tumor sizes, or tumor differentiation statuses ($P > .05$, Table 2). Overall, the above results demonstrated higher sensitivity of methylated *SFRP2* in detecting patients with GC and IM than benign polyp patients and normal patients.

Among the 92 patients with GC, serum CEA, CA72-4, CA19-9, and CA242 levels were measured in 79 patients. For these patients, the positive detection rates for GC detection by methylated *SFRP2* test and 4 serum tumor markers were showed in Figure 4. While methylated *SFRP2* test detected 55.0% stage I and 50.0% stage II GC, CEA, and CA72-4 had lower positive detection rates in stage I-II GC, and CA19-9 and CA242 missed all of stage I-II GC completely. The positive detection rates for serum CEA, CA72-4, CA19-9, and CA242

Table 2. Results of Methylated *SFRP2* Test in Detecting GC Between Different Ages, Genders, Tumor Locations, Tumor Sizes, and Tumor Differentiation Statuses.

	Methylated *SFRP2* (%)	*P* Value
Age		
≤60 (n = 44)	50.0%	.055
>60 (n = 48)	70.8%	
Gender		
Male (n = 67)	59.7%	.812
Female (n = 25)	64.0%	
Location		.315[a]
Cardia (n = 20)	60.0%	.431[b]
Gastric body (n = 15)	40.0%	1.000[c]
Gastric angle (n = 9)	77.8%	.105[d]
Gastric antrum (n = 20)	60.0%	.315[e]
NA (n = 28)	67.9%	.431[f]
Size		
<3 cm (n = 20)	50.0%	1.000[g]
3-6 cm (n = 24)	50.0%	.176[h]
>6 cm (n = 16)	75.0%	.188[i]
NA (n = 32)	68.8%	
Differentiation status		
Poorly (n = 29)	62.1%	.795[j]
Moderately differentiated (n = 32)	56.3%	.552[k]
Between well and moderately differentiated (n = 3)	33.3%	.582[l]
NA (n = 28)	67.9%	

Abbreviations: GC, gastric cancer; NA, not applicable.
[a]*P* value between cardia and gastric body.
[b]*P* value between cardia and gastric angle.
[c]*P* value between cardia and gastric antrum.
[d]*P* value between gastric body and gastric angle.
[e]*P* value between gastric body and gastric antrum.
[f]*P* value between gastric angle and gastric antrum.
[g]*P* value between <3 cm and 3-6 cm.
[h]*P* value between 3-6 cm and >6 cm.
[i]*P* value between <3 cm and >6 cm.
[j]*P* value between poorly differentiated and moderately differentiated.
[k]*P* value between moderately differentiated and well differentiated.
[l]*P* value between poorly differentiated and well differentiated.

detecting all stage GC were 22.8%, 16.5%, 12.7%, and 11.4%, respectively. In contrast, methylated *SFRP2* test had a 58.2% positive detection rate in detecting all stage GC. The positive detection rate for serum tumor markers was improved with the combined use of all 4 (35.4%) markers, which, however, was still significantly lower than that of methylated *SFRP2* test. And combination of methylated *SFRP2* and 4 serum tumor markers achieved a 68.4% positive detection rate, significantly higher than that of the combination of 4 serum tumor markers only.

Discussion

Gastric cancer is one of the most common malignancies worldwide, and several new methods for early GC detection have been published in recent years.[14,15] DNA methylation plays a significant role in gastric carcinogenesis.[16] Therefore, using DNA methylation in serum or plasma as a molecular biomarker may be an alternative strategy for GC early detection and screening.

Aberrant hypermethylation of WNT antagonist genes is associated with the development of GC,[17] and *SFRP2*, one of these genes, has been published in several studies. Nojima et al observed methylated *SFRP2* in 95.6% (44 of 46) GC tissues.[17] Zhang et al found a significantly higher frequency of *SFRP2* methylation in the plasma DNA of patients with GC than that of controls (71.9% vs 42.9%).[11] Cheng et al showed that *SFRP2* was significantly downregulated in GC as compared to adjacent paracancerous tissues ($P < .01$), and methylated *SFRP2* was detected in 73.3% (22 of 30) GC tissues, 20% (6 of 30) adjacent paracancerous tissues, and 66.7% (12 of 18) serum samples from patients with GC but 0.0% (0 of 18) in controls.[10]

In this study, we demonstrated that plasma methylated *SFRP2* test had 60.9% sensitivity and 86.0% specificity for GC detection, similar to the results from earlier studies.[10,17] Compared to those studies, more patients with GC were enrolled, and methylated *SFRP2* was analyzed for different age and gender groups, different tumor locations, sizes, stages, and differentiation statuses. Therefore, our study presented a more comprehensive evaluation of methylated *SFRP2* levels in the blood samples of patients with GC. Meanwhile, the results of this study showed that methylated *SFRP2* test detected 56.3% gastric IM, a significantly higher positive detection rate than that for control patients (Figure 2B, $P = .001$), but the positive detection rates of plasma methylated *SFRP2* for GC and IM patients showed no significant difference (Figure 2B). These results might be due to the smaller sample size of IM samples than that of patients with GC (16 vs 92). Moreover, most patients with GC with clear TNM stages were early stage GC (58.3%), which could also account for similar positive detection rates between IM and GC groups. Furthermore, gastric IM is a relatively frequent precancerous lesion[18] so that screening and intervention for gastric IM has been considered as a primary strategy for prevention and early screening of GC.[19] Taken together, our results suggested that methylated *SFRP2* may also be a viable biomarker for detecting early stage GC and precursor lesions.

Serum tumor marker test has become a common method for screening GC. He et al reported the results of combining serum AFP, CEA, CA125, and CA19-9 to improve the sensitivity for GC diagnosis. Whereas the sensitivities of AFP, CEA, CA125, and CA19-9 individually for the detection of GC ranged from 4.7% to 20.8%, the combined test showed a sensitivity of 40.3%.[20] Reported in another study, the sensitivities of CA72-4, CEA, CA125, and CA19-9 for early GC detection were 33.0%, 25.5%, 31.1%, and 38.7%, respectively, but when used in combination, the 4 markers showed an increased sensitivity of 66.0%.[21] In this study, we compared the sensitivities of serum CEA, CA72-4, CA19-9, and CA242 to that of plasma methylated *SFRP2* for GC detection. While the sensitivities of CEA, CA72-4, CA19-9, and CA242 were 22.8%, 16.5%, 12.7%, and 11.4%, the combination of all 4 markers improved

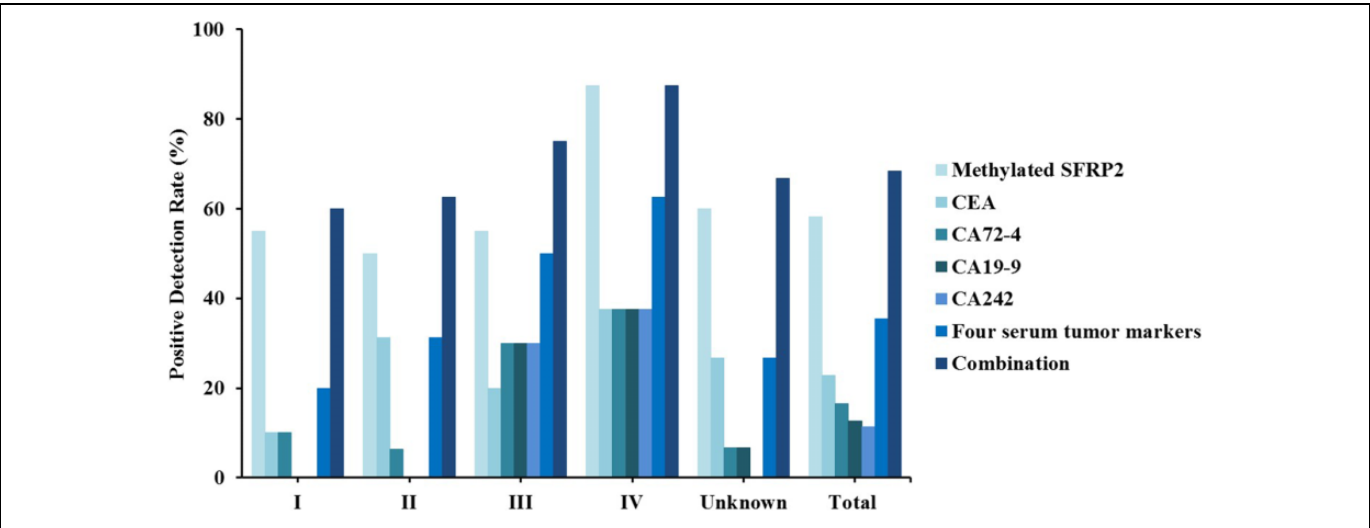

Figure 4. The positive detection rates of serum CEA, CA72-4, CA19-9, CA242, and plasma methylated *SFRP2* test for detecting GC. GC indicates gastric cancer.

the sensitivity to 35.4%. In contrast, methylated *SFRP2* alone demonstrated a significantly higher sensitivity of 58.2%. The data from this study and previous studies indicated that plasma methylated *SFRP2* had significantly higher sensitivity for GC detection than any serum tumor marker alone. Therefore, plasma methylated *SFRP2* could be a more sensitive biomarker for early GC detection. Moreover, the combination of serum tumor markers with plasma methylated *SFRP2* could achieve even higher sensitivity, suggesting the possible application of this combination in clinics for early GC detection as well as the assessment for the therapeutic effects of different treatments and the prognosis of patients with GC.

However, there are several limitations in this study. For example, the lack of the comparison between plasma methylated *SFRP2* and serum tumor markers in normal patients. Meanwhile, the number of patients enrolled in this study was relatively low, especially when patients were further divided into IM, GFGP, AP, HP groups as well as different stages of patients with GC. Further increasing the number of patients of each group will make it possible to distinguish the diagnostic performance of plasma methylated *SFRP2* assay for these diseases. Moreover, we could collect more specimens from multiple clinical centers and combined plasma methylated *SFRP2* with other biomarkers, such as serum PGI concentration and the PGI/II ratio, to evaluate and enhance its sensitivity in detecting early stage GC in the further studies.

Conclusion

We evaluated the feasibility of using methylated *SFRP2* test for the early detection of GC and precursor lesions. Its sensitivities for GC and gastric IM were 60.9% and 56.3% with a specificity of 86.0%. The results thus indicated that plasma methylated *SFRP2* test may be a valuable tool for the noninvasive detection of GC and precursor lesions.

Authors' Note

Jin Miao, Yi Liu, and Guodong Zhao has been contributed equally to this work. The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request. This study was approved by the Institutional Review Board of the Affiliated Hospital of Xuzhou Medical University (Ethics Committee reference number: XYFY2019-KL121), the informed consent was obtained from all participating patients and healthy control patients, and the study was performed according to the Declaration of Helsinki principles.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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