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SFRP2 and RPRM as methylation based serum biomarkers for the detection of gastric cancer

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Abstract

Background Gastric cancer (GC) has a high mortality rate due to the diagnosis in advanced stages. Aberrant DNA methylation is the earliest event in carcinogenesis and can be noninvasively detected in cell-free DNA (cfDNA) from gastric cancer patients.

Methods A total of 143 serum samples were analyzed, including 33 GC patients, 30 chronic gastritis (ChG) patients, and 80 healthy individuals. Additionally, tissue samples were collected from 30 GC patients (stages I–IV) and 38 ChG patients. Methylation patterns of ten genes were examined in GC cells, as well as in serum and tissue samples from GC, ChG, and control groups using methylation-specific qPCR. Statistical evaluations were conducted on various parameters including Ct differences, categorical variables, sensitivity, and specificity.

Results APC, CDH1, RASSF1A, hMLH1, RUNX3, p16, SFRP2, RNF180, PCDH10, and RPRM were all significantly hypermethylated in the tissues of GC patients compared to those with ChG ($P < 0.001$). SFRP2, RPRM, APC, PCDH10, and RNF180 genes were analyzed in sera of 3 groups. Among them, SFRP2 methylation was detected in 71.87% of GC, 16.6% of ChG and 8.8% of the control group. The methylation frequencies of RPRM were 66.6% in GC, 13.3% in ChG, and 7.5% in the control group. In a dual-gene panel assay combining SFRP2 and RPRM, the sensitivity and specificity for detecting gastric cancer in serum samples were 57.58% and 96.25%, respectively, when comparing the cancer and control groups. The sensitivity was 78.79%, the specificity was 90.00% and AUC was 0.931 for GC and control groups ($P < 0.0001$). The sensitivity was 78.79%, the specificity was 83.33% and AUC was 0.879 for the discrimination of GC and ChG ($P < 0.0001$).

Conclusions Methylation of 10 genes were studied and a prototype early diagnosis tool for GC utilizing SFRP2 and RPRM with high sensitivity and specificity was developed.

Keywords Gastric cancer, Methylation specific qPCR, SFRP2, RPRM, Liquid biopsy

1 Introduction

Gastric cancer (GC) is a complex heterogeneous disease, the currently ranked as the fifth most common malignancy [1] and the third most frequent cause of cancer-related deaths worldwide [2]. The high mortality rate is due to the fact that 80% of patients with

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GC are first diagnosed in advanced stages [3] Currently, endoscopy combined with pathological diagnosis is the gold standard for GC diagnosis, but its use is limited by its dependance on examiner expertise, and patients' reluctance to undergo invasive procedures [4]. Although existing serum tumor markers such as CEA, CA19-9, and CA72-4 are widely used, their specificity and sensitivity are insufficient in the diagnosis of GC, especially in the early stages. For instance, these markers exhibit a sensitivity as low as 20% for early-stage gastric cancer [4]. Consistent with this, sensitivities reported in studies for gastric cancer range from 19 to 47% [5]. CA724 has shown limited diagnostic utility for gastrointestinal tumors, as evidenced by a recent study reporting an area under the curve (AUC) of 0.551 and a sensitivity of approximately 40% [6]. Gastric carcinogenesis is caused by different factors including epigenetic, genetic, and environmental factors [7]. *Helicobacter pylori* infection is one of the best-known environmental risk factors for GC. However, changes at the genetic and epigenetic levels have been the major cause of the increased risk of GC [8] One of the most well-known epigenetic events is the methylation of DNA, and when DNA is methylated in the gene promoter region, genes are inactivated and silenced [9]. Promoter hypermethylation of the CpG islands of tumor suppressor genes (TSGs) is a major event in the initiation of many cancers and affect genes in many pathways such as cell cycle and DNA repair [10]. In cancer patients, aberrant DNA methylation can be noninvasively detected in cell-free DNA (cfDNA) obtained through liquid biopsy, as the levels of tumor-derived cfDNA are typically elevated in the bloodstream [11, 12]. Recent studies have highlighted the clinical potential of cfDNA methylation profiling in the early detection of gastrointestinal cancers, including colorectal, gastric, and esophageal cancer. In a prospective cohort study, a cfDNA-based assay (GutSeer) demonstrated strong diagnostic performance, achieving a sensitivity of 81.5% and a specificity of 94.4% for non-invasive detection of these cancers. These findings support the utility of cfDNA methylation as a promising biomarker for early gastrointestinal cancer screening [13].

Qi et al. reported cfDNA methylation-based detection of gastric cancer using a panel of 21 differentially methylated regions, achieving a sensitivity of 93.90% and specificity of 95.15% in the discovery set, and 88.38% sensitivity and 94.23% specificity in the validation set [14]. cfDNA is a valuable biological material used in the development of biomarkers with high sensitivity and specificity in cancer diagnosis [9, 14]. To date, aberrant methylation of many TSGs, including RUNX3, P16, RASSF1A, RPRM, CDH1, SFRP2, hMLH1 and APC have been used as potential biomarkers for early detection of GC from serum, plasma and tissue samples [3, 11, 15–18]. These genes have been associated with the progression and poor prognosis of GC [19]. This study aimed to investigate the potential of 10 TSGs as diagnostic biomarkers in tissue and serum samples from GC patients and healthy controls and to elucidate their utilization as an early diagnosis tool for GC.

2 Material and methods

2.1 Sample collection

A total of 143 serum samples were analyzed, including 33 GC patients, 30 chronic gastritis (ChG) patients, and 80 healthy individuals. Additionally, tissue samples were collected from 30 GC patients (stages I–IV) and 38 ChG patients.

Tissue samples were collected from 30 patients diagnosed with gastric cancer (GC), covering stages I to IV, and from 38 patients with chronic gastritis (ChG). Serum samples were obtained from 33 GC patients (stages I–IV), 30 ChG patients, and 80 healthy individuals. All samples were obtained from Biruni University Medical School Hospital and Gazi University Medical School Hospital between September 2023 and February 2025.

Serum samples were collected before surgery, chemotherapy, and/or radiotherapy. All subjects included in the present study were informed and gave written informed consent. The study was approved by the ethics committee of Biruni University Medical School (2015-KAEK-79–23-22). All tissue samples were stored at − 80 °C until use. 8 ml of peripheral blood was collected and the blood samples were centrifuged at 2000 × g for 15 min to collect the serum in the tube. The collected serum samples were then stored at − 80 °C for DNA extraction. Inclusion and exclusion criteria were applied to ensure sample consistency and reliability. Control participants were selected among individuals with no history of inflammatory gastric diseases or malignancies. Patients diagnosed with gastric cancer (GC), ranging from stage I to IV based on histopathological evaluation, were included in the study. Chronic gastritis (ChG) patients were enrolled upon endoscopic and histological confirmation of diagnosis. Exclusion criteria for the control group included any prior diagnosis of gastric inflammation or malignancy. For the GC group, individuals with a concurrent history of other malignancies or who had received chemotherapy or radiotherapy prior to sample collection were excluded from the study. These criteria were applied to ensure homogeneity within groups and to minimize potential confounding factors affecting DNA methylation profiles.

2.2 DNA extraction from tissue and serum samples

2.2.1 DNA extraction from tissues

A total of 38 samples of gastric mucosa tissue from patients with ChG and 30 GC tissue samples were obtained using forceps biopsy during esophagogastroduodenal endoscopic examination. Written informed consent was obtained from all patients and healthy volunteers. In addition, the clinical characteristics of GC patients such as differentiation, tumor location, tumor invasion and lymph node status were investigated. All samples were frozen immediately after resection and stored at − 80 °C until use. Patients had never been submitted to chemotherapy or radiotherapy before surgery or had any other diagnosed cancer. Histopathological information for each case was obtained according to approved institutional guidelines. Tissue genomic DNA was isolated using Wizard Genomic DNA Purification Kit (Cat No: A1125) (Promega, Madison, WI, USA) according to the kit protocol and stored at − 20 °C.

2.2.2 Isolation of cell free DNA (cfDNA) from serum samples

Blood samples were taken from 33 GC patients and 30 patients with ChG. Additionally, 80 blood samples were taken from healthy donors as a control group. Serum collection tubes were used. Then, the whole blood was centrifuged at 2000 ×g for 15 min to obtain the serum and serum was stored at − 80 °C.

For cfDNA extraction, serum samples stored in − 80 °C were taken out at room temperature to thaw and, then 4 ml of serum was used to isolate cfDNA using QIAamp

MinElute ccfDNA Mini Kit (Qiagen, Cat No: 55204) according to the manufacturer's protocol and stored at – 20 °C.

2.3 Sodium bisulfite conversion

Extracted DNA was subjected to bisulfite modification for methylation analysis. Bisulfite modification of extracted DNA (maximum of 2 µg) from serum and tissue was carried out using the EZ DNA methylation-Lightning Kit (Zymo Research, Cat No: D5031) following manufacturer's protocol. Bisulfite-modified DNA (BisDNA) was obtained after chemical processing, binding, washing and elution and then immediately stored at – 80 °C until use.

2.4 Methylation specific qPCR

Methylation-specific, fluorescence-based real-time PCR assay (qMSP), MethyLight, was used in the detection of methylated DNA in the sera and tissue samples [20, 21]. A total of 10 carcinogenesis related genes including APC, CDH1, RASSF1A, hMLH1, RUNX3, p16, SFRP2, RNF180, PCDH10, and RPRM were examined. These genes were previously reported to be methylated in various human cancers [22, 23]. Methylation-specific primers and probes specific for the CpG islands close to the promoter of these target genes were designed to amplify the bisulfite-modified DNA. DNA amplification was carried out in a 0.2 mL reaction plate format in an QuantStudio 5 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific Cat No: A34322). Each qMSP reaction was performed in a total volume of 20 µL. Briefly, 2 µL of sodium bisulfite converted DNA was added into a reaction mixture containing 10 µL of 2 × master mix (iTaq Universal Probes Supermix, Cat No:1725134, BIORAD), forward and reverse primers (10 µM) Sentebiolab Biotechnology, Ankara, Turkey, TaqMan probe (5 µM) Sentebiolab Biotechnology, Ankara, Turkey and, finally, dH_2O was added to a final volume. Thermal cycling was initiated with a denaturation step of 5 min at 95 °C. The subsequent thermal profile for qMSP was 95 °C for 15 s, and 60 °C for 30 s. Data obtained after 50 amplification cycles were analyzed for tissue and serum samples, respectively. DNAs which were extracted from bisulfite-treated AGS and MKN45 cell lines were used as positive controls in all amplifications. Genomic DNA extracted from untreated cells were used as negative controls. Standardized quality control criteria were applied to ensure the integrity of DNA samples. Only those that fulfilled the defined standards for concentration and purity both before and after bisulfite conversion were included in the analysis. Due to the limited volume of cfDNA obtained from clinical serum samples, each qMSP assay was performed as a single technical replicate. Specifically, 8 mL of peripheral blood was collected from each patient, yielding approximately 4 mL of serum. After cfDNA extraction and bisulfite conversion, we obtained approximately 20 µL of bisulfite-modified DNA per sample. Since five gene targets were analyzed per patient, this limited DNA volume allowed for only one qMSP reaction per gene. As a result, each gene was tested once per patient, and technical replicates were not feasible. Therefore, the error bars shown in Figs. 1 and 2 represent the standard deviation among biological replicates (i.e., individual patients), not technical variation. To ensure analytical consistency, all experimental protocols—including DNA quantification, bisulfite conversion, and qPCR amplification—were rigorously standardized across all samples.

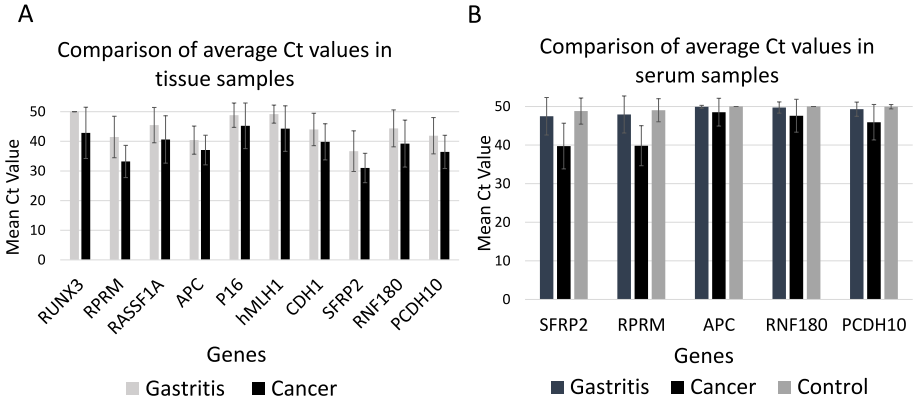


Fig. 1 **a** Average Ct values of 10 genes in GC and ChG tissue samples, **b** Average Ct values of 5 genes in GC, ChG and control group serum samples. Standard deviation was shown by error bars. Error bars indicate standard deviation among biological replicates (individual patients), not technical replicates

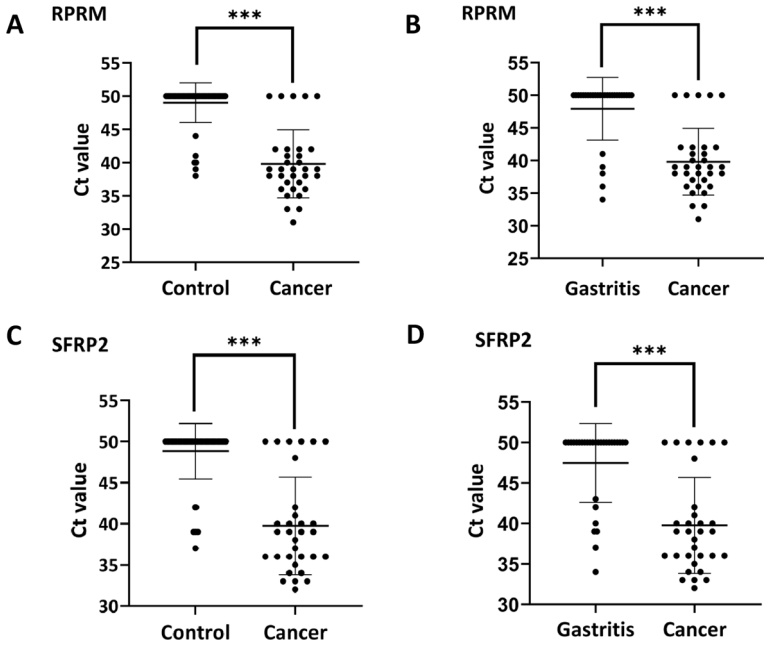


Fig. 2 The distribution of Ct values of qPCR signals in GC, ChG and control groups. **a** RPRM Ct values in control and GC sera, **b** RPRM Ct values in ChG and GC sera, **c** SFRP2 Ct values in control and GC sera, **d** SFRP2 Ct values in ChG and GC sera. Mean and standard deviation were denoted by bars, ****P* < *0.0001*. Error bars indicate standard deviation among biological replicates (individual patients), not technical replicates

The results for serum and tissue samples were considered "negative" if the Ct value (output data from the Quant Studio 5 real-time PCR instrument, which defines the threshold cycle number as Ct) was greater than 40. Methylation was considered "positive" when the Ct value was equal or less than 40 and the amplification curve was valid. In our study, the Ct cut-off value of 40 was applied as an analytical threshold across all target genes, including SFRP2, RPRM, APC, PCDH10, and RNF180, for both tissue and serum samples.

To ensure consistency in downstream analyses, including ROC curve generation and quantitative comparisons, samples with no detectable amplification signal within 50 PCR cycles were assigned a Ct value of 50, corresponding to the total number of amplification

cycles. This approach allows the inclusion of non-amplified cases in numerical analyses and is commonly used in high-sensitivity methylation detection studies. All reactions were performed in a 50-cycle qMSP format, and amplification signals were evaluated according to predefined quality control criteria, including proper curve shape, stable baseline, and the absence of signal in non-template controls (NTCs).

2.4.1 Limit of detection (LOD) determination

To address analytical sensitivity and to further justify the selection of the Ct = 40 threshold, we performed a dilution-based experimental determination of the limit of detection (LOD) using the AGS gastric cancer cell line, which is known to harbor methylated SFRP2 and RPRM promoter regions. Bisulfite-converted DNA from AGS cells (initial concentration: 32 ng/μL) was serially diluted in molecular-grade water to simulate low-concentration clinical samples at 1/100, 1/1000, 1/2000, and 1/3000 dilution levels. qMSP assays targeting SFRP2 and RPRM were then performed using our standard protocol.

At the 1/100 dilution, consistent amplification was detected with Ct values of 37 for SFRP2 and 40 for RPRM. At the 1/1000 dilution, both targets remained detectable, with Ct values of 42 (SFRP2) and 40 (RPRM). Based on these results, and to minimize the risk of non-specific late-cycle amplification, we conservatively selected Ct = 40 as the analytical detection threshold.

Using standard curve-based absolute quantification, the methylated DNA copy number at the 1/1000 dilution was estimated to be approximately 9.5 copies/μL, which we defined as the assay's experimental LOD.

These data have now been included to enhance transparency and support the diagnostic performance claims of our qMSP assay.

2.5 Gastric cancer cell lines

Gastric cancer cell lines (AGS, ATCC, Cat# CRL-1739; MKN45 DSMZ, Cat# ACC 409 were cultured in DMEM-F-12 medium (Cat No: DMEM 12-A, CAPRICORN SCIENTIFIC) supplemented with 10% fetal bovine serum (Cat No: 04–001-1A, Biological Industries), Penicillin–Streptomycin (Cat No: 03.031-1B, SARTORIUS) and L-Glutamine (Cat No: 25030–024, Gibco), and incubated in 5% CO_2 at 37 °C. Cells were harvested for DNA isolation as they reached 80% confluency. The extracted DNA was quantified and subsequently subjected to bisulfite conversion (maximum of 2 μg) using the EZ DNA Methylation-Lightning Kit (Zymo Research, Cat No: D5031), following the manufacturer's protocol. Bisulfite-converted DNA was eluted and stored at − 80 °C until use in methylation-specific PCR (qMSP) assay.

2.6 Statistical analysis

Student's t-test was used to compare groups, and differences were regarded as statistically significant at $P < 0.05$. The significance of methylation of target genes and odds ratio (ORs) values were evaluated using the Chi-square or Fisher's exact test in our test groups. To assess the diagnostic potential of methylation of individual genes or their combinations in various groups, the receiver operating characteristic (ROC) curve analysis and Ct values were used to calculate the area under the curve (AUC), OR with 95%

confidence intervals (CIs), sensitivity, and specificity values. $P < 0.05$ was considered to indicate a statistically significant difference.

To assess the diagnostic performance of methylation markers (SFRP2 and RPRM), Receiver Operating Characteristic (ROC) curve analyses were conducted using GraphPad Prism 10.0 software. Sensitivity, specificity, area under the curve (AUC), 95% confidence intervals (CI), and optimal cutoff values were calculated based on the distribution of Ct values obtained from qMSP assays in serum and tissue samples. For all genes Ct values were obtained from amplification plots using the QuantStudio 5 Real-Time PCR System (Applied Biosystems), with a ΔRn threshold set at 10,000. This threshold consistently produced reproducible amplification signals across technical replicates. Ct values exceeding 40 were considered unreliable due to non-specific amplification and increased background noise. Therefore, a Ct value of 40 was established as the analytical cut-off, ensuring high specificity and minimizing the risk of false positives. To further support this threshold, we included dilution-based LOD experiments conducted with AGS gastric cancer cell line DNA, which confirmed that Ct = 40 represents a sensitive and specific detection limit for the qMSP assays.

All analyses were conducted using the GraphPad Prism 10.0 program (GraphPad, Dotmatics).

3 Results

3.1 Patient characteristics

Blood and tissue samples from a total of 37 GC patients, 38 ChG patients and 80 healthy controls were donated within a 18-month time period prior to receiving any treatment. Patients with GC had an average age ± standard deviation of 60.41 ± 11.88 years, those with ChG had an average age of 51.81 ± 16.37 years, and those in the control group had an average age of 44.32 ± 9.49 years. The ratio of male to female patients was 29:8 in the cancer group, 17:21 in the chronic gastritis and 33:7 in the control group. General information of all patients was shown in Table 1. The clinicopathological characteristics of the 37 GC patients were shown in Table 2.

3.2 Methylation status of CpG islands of 10 genes in gastric carcinoma cell lines

AGS and MKN45 (positive control cell lines known as methylated control) cell lines were selected due to their distinct biological and molecular features representative of different gastric cancer subtypes. AGS originates from a primary differentiated gastric tumor,

Table 1 General information of all patients

General Info	Gastric cancer (GC)	Chronic gastritis (ChG)	Control
Gender			
Male	29	17	66
Female	8	21	14
Age			
Average	60.41	51.81	44.32
Range	29–85	21–76	28–62
Gender (Tissue)			
Male	25	16	
Female	5	22	
Gender (Serum)			
Male	25	13	66
Female	8	17	14

Table 2 Clinical and demographic characteristics of gastric cancer patients

Variables	GC patients, n (%)
Gender	
Male	29 (78.4)
Female	8 (21.6)
Age	
< 50 years	5 (13.5)
≥ 50 years	31 (83.8)
NA	1 (2.7)
Tumor size	
< 5 cm	30 (81.08)
≥ 5 cm	5 (13.51)
NA	2 (5.41)
Differentiation	
Well differentiated	10 (27.03)
Moderately differentiated	4 (10.81)
Poorly differentiated	12 (32.43)
NA	11 (29.73)
Tumor location	
Cardia	11 (29.73)
Antrum	18 (48.65)
Korpus	6 (16.22)
NA	2(5.40)
Tumor invasion	
pT1-pT2	13 (35.14)
pT3-pT4	15 (40.54)
NA	9 (24.32)
Lymph node status	
pN0	7 (18.92)
pN1-N2-N3	18 (48.65)
NA	12 (32.43)
H. pylori infection	
Positive	10 (27.03)
Negative	19 (51.35)
NA	8 (21.62)

while MKN45 is derived from a metastatic, poorly differentiated carcinoma. Including both cell lines allowed us to evaluate methylation events across early and advanced GC models.

The hypermethylation status of SFRP2, P16, RASSF1A, RUNX3, PCDH10, RNF180, APC, hMLH1, and CDH1 related CpG islands in gastric cancer cell lines (AGS and MKN45) was detected by qMSP. Both cell lines didn't have any detectable methylation in hMLH1 and CDH1 genes. Methylation of SFRP2, RASSF1A, RPRM, PCDH10, and APC was detected in both cell lines, while methylation of P16, RUNX3, and RNF180 was detected only in the AGS cell line (Supplementary Fig. 1).

3.3 Methylation profile of CpG islands of 10 genes in GC tissues

We investigated the methylation status of CpG islands of ten genes in the DNA obtained from the tissues of GC and ChG patients by using qMSP. Ct values were used to determine the methylation level of genes in GC and ChG tissues and given in Fig. 1a. All genes were significantly hypermethylated in GC tissues compared to ChG.

The RPRM and SFRP2 genes specifically showed lower Ct values when compared with the average Ct values in tissue samples, suggesting a higher level of methylation in cancer tissues. There was a 9 Ct difference between cancer and gastritis patients for RPRM gene ($P < 0.0001$), while this value was 6 Ct for SFRP2 gene ($P = 0.0003$).

Table 3 showed the hypermethylation frequencies of certain genes in the tissues of cancer and chronic gastritis. In GC, the frequency of the methylation of 10 genes ranged from 30% to 96.7%. The genes that were most frequently methylated were SFRP2, RPRM, RNF180, APC, and PCDH10. SFRP2 had the highest frequency of methylation (96.7%, 29/30) in GC tissues.

3.4 Detection of RPRM, APC, RNF180, PCDH10, and SFRP2 CpG island methylation in serum samples

The five most frequently hypermethylated genes in the GC tissues (SFRP2, RPRM, APC, PCDH10 and RNF180) were examined in 33 GC cases from serum samples. In addition, sera from 30 ChG patients and 80 healthy controls were analyzed for these genes.

Ct values were used to determine the methylation level of genes in GC, ChG and control sera, and average Ct values were given in Fig. 1b. When we examined the serum samples, the Ct difference between cancer and gastritis patients was 8 Ct for RPRM gene, while this difference was 7 Ct for SFRP2 gene (Fig. 2, $P < 0.0001$ for both). The difference in Ct values between the control and cancer patients was 9 Ct for both genes (Fig. 2, $P < 0.0001$ for both).

In the patient sera, SFRP2 methylation was detected in 71.87% (23/32) of GC patients, while methylation of this gene was observed in 16.6% (5/30) of ChG patients and 8.8% (7/80) of controls (Table 3). The methylation frequencies of RPRM were 66.6% (22/33) in GC patients, 13.3% (4/30) in ChG patients, and 7.5% (6/80) in controls (Table 3).

On the other hand, APC, RNF180, and PCDH10 methylation were detected in only 6.3% (2/32), 12.5% (4/31), and 12.5% (4/31) of GC patients, respectively. No methylation of these genes was observed in ChG or control sera (Table 3).

All qMSP reactions were performed as single technical replicates. Error bars in Figs. 1 and 2 represent biological variability (i.e., standard deviation across individual patient samples), not technical replicates. Individual Ct values for each gene in all samples were provided in Supplementary Table 1).

Table 3 Frequency of promoter hypermethylation for individual genes in tissue and serum samples from GC, ChG and control groups

Genes	Frequency of the methylation				
	GC Tissue, n = 30, Methylation%	ChG Tissue, n = 38, Methylation%	GC Serum, n = 33, Methylation%	ChG Serum, n = 30, Methylation%	Control Serum, n = 80, Methylation%
RUNX3	43.3%	0%	NA	NA	NA
RPRM	90%	55.3%	66.6%	13.3%	7. 5%
RASSF1A	53.3%	26.3%	NA	NA	NA
APC	90%	60.5%	6.25%	0%	0%
P16	30%	7.9%	NA	NA	NA
hMLH1	33.3%	2.6%	NA	NA	NA
CDH1	60%	31.6%	NA	NA	NA
SFRP2	96.7%	81.6%	71.8%	16.6%	8.8%
RNF180	66.6%	34.2%	12.5%	0%	0%
PCDH10	86.6%	44.7%	12.5%	0%	0%

3.5 Performance of RPRM, SFRP2, APC, PCDH10 and RNF180 for the detection of GC

Fisher's exact test was performed to assess the association of DNA methylation for the RPRM, SFRP2, APC, PCDH10 and RNF180 to detect GC. Parameters such as sensitivity, specificity, odds ratio (OR) and P value were given in Table 4. Youden index was calculated for each biomarker as an integrated metric combining sensitivity and specificity to assess the diagnostic performance in Table 4.

The sensitivity of SFRP2 and RPRM were 71.88% and 66.67%, and the specificity of SFRP2 and RPRM were 91.25% and 92.50%, respectively, for cancer and control groups. When the cancer and gastritis groups were analyzed, the sensitivity of SFRP2 and RPRM were 71.88% and 66.67%, and the specificity of SFRP2 and RPRM were 83.33% and 88.67%, respectively. The other three genes had sensitivities ranging from 6.25% to 12.90% for GC (Table 4).

3.6 The combined dual-gene panel performance of RPRM and SFRP2 methylation for the diagnosis of gastric cancer.

We used a dual-gene panel to assess whether the combined detection of SFRP2 and RPRM methylation could improve early gastric cancer (GC) detection. To evaluate diagnostic performance, we conducted receiver operating characteristic (ROC) curve analyses using Ct values obtained from qMSP assays on serum samples from GC, chronic gastritis (ChG), and control groups. Sensitivity, specificity, area under the curve (AUC), and 95% confidence intervals (CI) were calculated for different classification strategies. A Ct cut-off value of 40 was used to define methylation positivity (Fig. 3).

In the dual-gene panel where both SFRP2 and RPRM methylation were required to be positive for GC detection, the sensitivity was 57.58% and the specificity was 96.25% in distinguishing GC from control samples (AUC = 0.966; 95% CI 0.938–0.994, Fig. 3a). When compared with gastritis patients, the sensitivity remained at 57.58%, and the specificity was 90.00% (AUC = 0.903; 95% CI 0.814–0.991, Fig. 3b).

Table 4 Diagnostic performance of individual gene methylation (RPRM, SFRP2, APC, PCDH10, RNF180) and combined SFRP2–RPRM panel for the detection of gastric cancer using serum samples

Gene methylation	Sensitivity, % (95% CI)	Specificity, % (95% CI)	Odds ratio (95% CI)	P value*	Youden index
Cancer control					
SFRP2	71.88 (54.63–84.44)	91.25 (83.02–95.70)	26.65 (8.93–79.55)	< 0.0001	0.631
RPRM	66.67 (49.61–80.25)	92.50 (84.59–96.52)	24.67 (8.23–79.11)	< 0.0001	0.592
APC	6.25 (1.11–20.15)	100 (95.42–100)	–	0.0798	0.063
PCDH10	12.90(5.13–28.85)	100(95.42–100)	–	0.0053	0.129
RNF180	12.90(5.13–28.85)	100(95.42–100)	–	0.0053	0.129
SFRP2 + RPRM	57.58(40.81–72.76)	96.25 (89.55–98.98)	36.71. (8.17–165.6)	< 0.0001	0.538
SFRP2or RPRM	78.79(62.25–89.32)	90.00 (81.49–94.85)	45.48 (13.27–131.1)	< 0.0001	0.688
Cancer gastritis					
SFRP2	71.88 (54.63–84.44)	83.33(66.44–92.66)	12.78(3.72–43.78)	< 0.0001	0.552
RPRM	66.67(49.61–80.25)	88.67(70.32–94.69)	13(3.76–39.44)	< 0.0001	0.553
APC	6.25 (1.11–20.15)	100 (88.65–100)	–	0.4923	0.063
PCDH10	12.90(5.13–28.85)	100(88.65–100)	–	0.1128	0.129
RNF180	12.90(5.13–28.85)	100(88.65–100)	–	0.1128	0.129
SFRP2 + RPRM^{+}	57.58(40.81–72.76)	90.00 (74.38–96.54)	8.47 (2.19–29.63)	< 0.0010	0.476
SFRP2 or RPRM^{+}	78.79(62.25–89.32)	83.33 (66.44–92.66)	29.00 (7.15–92.53)	< 0.0001	0.621

SFRP2, secreted frizzled-related protein 2; Reprimo, TP53 Dependent G2 Arrest Mediator Homolog; APC Regulator Of WNT Signaling Pathway; PCDH10, Protocadherin 10; RNF180, ring finger protein 180; CI, confidence interval

*Fisher's exact test, + ROC curve analysis

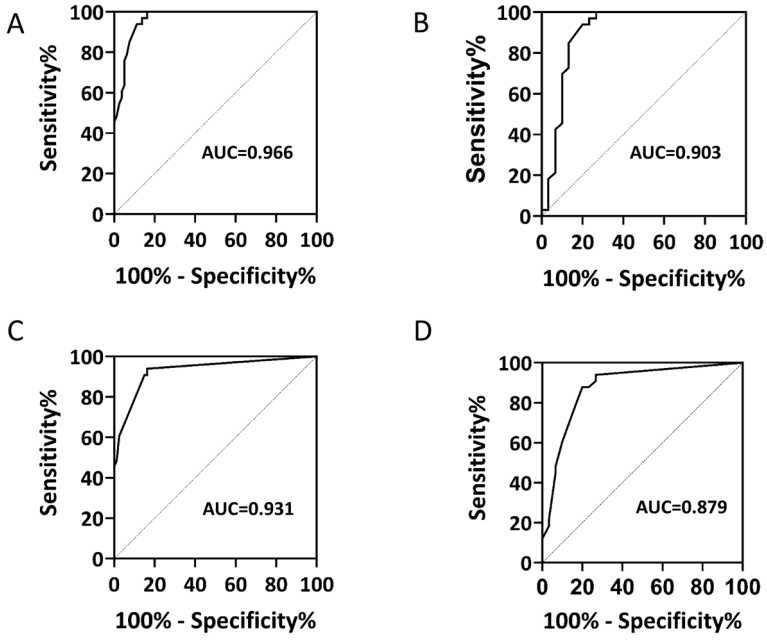


Fig. 3 The ROC curve for methylated SFRP2 and RPRM in combination, for the detection of GC in the control, gastritis, and cancer groups. AUC indicates area under the curve; CI, confidence interval. **a** ROC curve for the methylated SFRP2 and RPRM test in detecting GC between cancer and control groups AUC = 0.966; 95% CI 0.938–0.994; p < 0.0001. **b** ROC curve for the methylated SFRP2 and RPRM test in detecting GC between cancer and gastritis groups AUC = 0.903; 95% CI 0.814–0.991;p < 0.0001 **c:** ROC curve for the methylated SFRP2 or RPRM test in detecting GC between cancer and control groups AUC = 0.931; 95% CI 0.874–0.988;p < 0.0001 **d** ROC curve for the methylated SFRP2 or RPRM test in detecting GC between cancer and gastritis groups AUC = 0.879; 95% CI 0.789–0.970;p < 0.0001

In contrast, when either SFRP2 or RPRM methylation was considered sufficient to classify a sample as GC, the sensitivity increased to 78.79%, with a specificity of 90.00% in the GC vs. control group (AUC = 0.931; 95% CI 0.874–0.988, Fig. 3c). For the GC vs. ChG comparison under the same condition, the sensitivity was 78.79% and specificity was 83.33% (AUC = 0.879; 95% CI 0.789–0.970, Fig. 3d).

To statistically evaluate serum methylation differences between early-stage (pT1–pT2) and advanced-stage (pT3–pT4) gastric tumors, Ct values from qMSP assays were compared using the non-parametric Mann–Whitney U test due to the relatively small sample size and potential non-normality. The analysis was performed using GraphPad Prism, and no statistically significant difference was observed ($P = 0.3303$). This result supports the observation that methylation levels detected by qMSP are comparable across early and late-stage tumors, reinforcing the potential utility of this assay for early gastric cancer detection.

4 Discussion

GC carcinogenesis process is a multistep process that includes numerous genetic and epigenetic alterations. Aberrant promoter methylation of tumor suppressor genes is an important epigenetic mechanism that leads to gene silencing and occurs in the early stages of gastric carcinogenesis [21]. A number of genes, including CDH-1, hMLH1, p16, RUNX3, DAPK1, SFRP2, and RNF180, were discovered to be methylated in the tissue and blood samples of patients who suffer GC [11, 24].

Although there are a number of clinical studies, there is still a need for reliable serum biomarkers for GC. In our study, we have selected CpG regions of 10 genes known to be hypermethylated in GC and studied their methylation profiles in tumor biopsy samples from GC and gastritis patients (Table 3).

Given the crucial role of DNA methylation in gastric cancer, the selection of target genes for methylation analysis was based on both biological significance and prior experimental evidence. Accordingly, the gene panel included in this study was selected from genes whose methylation profiles have been documented in the literature and whose roles in cancer development have been well established.

The panel of ten tumor suppressor genes analyzed (APC, CDH1, RASSF1A, hMLH1, RUNX3, p16, SFRP2, RNF180, PCDH10, and RPRM) was selected based on previous studies highlighting frequent promoter hypermethylation across various human malignancies, particularly gastric cancer. Several of these genes, such as CDH1, hMLH1, and RUNX3, have been shown in multiple studies to play important roles in gastric carcinogenesis through epigenetic silencing by DNA methylation [11, 15–18].

Additionally, genes like RASSF1A and PCDH10 have been widely investigated for their diagnostic and prognostic value in gastrointestinal tumors [9] SFRP2 and RPRM, in particular, have emerged as promising methylation based biomarkers in several gastric cancer studies, demonstrating high sensitivity and specificity [11, 25–29].

The selection of these genes was also supported by our preliminary findings confirming their methylation in the gastric cancer cell lines AGS and MKN45 used in this study. Therefore, the final gene panel was established based on both biological rationale and prior empirical evidence supporting their involvement in gastric cancer-related methylation events. Among these genes, RPRM, APC, SFRP2, PCDH10 and RNF180 were found to be frequently methylated in tumor tissues ranging from 66.6% to 96.7%.

We selected five genes that were most commonly methylated in tumor tissues after completing the analysis using tumor samples, and we used these five genes to do methylation analysis in serum samples. In our study, RPRM was methylated in 66.6% of sera from GC patients and only in 7.5% of sera from controls. However, Bernal et. al. reported that RPRM was methylated in 95.3% of plasma from GC patients from Chile, South America [25] This difference might be due to the use of a different population in our study or the use of qPCR methodology and a different primer–probe set in our study instead of conventional PCR in their study. In another study, RPRM methylation ratio was 66.7% and 6.7% in plasma of GC patients and controls, respectively [11]. Guo et. al. also reported that RPRM methylation ratio was 60.7% and 1.8% in plasma of GC patients and controls, respectively [26]. Wen et. al. reported the methylation of RPRM with a sensitivity of 82.0% and specificity of 89.0% in blood samples in GC [27]. RPRM methylation in the sera of GC patients range from 60 to 95% according to these studies. Our results for RPRM are in the range of these findings. RPRM methylation was also increased in intestinal metaplasia as a precursor lesion for GC, and might be an early biomarker in GC carcinogenesis [30].

We observed only 6.25% APC methylation in sera from GC patients and no methylation in control samples. There are studies indicating higher or similar degrees of APC methylation in GC. Leung et. al. found 17% of APC methylation in plasma of GC patients in Chinese Population [20]. In contrast, Balgkouranidou et. al. found 83.6% of APC methylation in plasma of GC patients in Greece [31]. APC methylation ratios varied in

different studies and the range was wide between 17% and 83.6%. We have also found the ratio of 6.25% which was at the lower end of the range. This wide difference might be due to the population difference, and APC methylation might be different between eastern and western populations.

We found SFRP2 methylation in 71.8% of sera in GC patients and 8.8% of sera in the control group. Miao et. al. reported SFRP2 methylation in GC with the sensitivity of 60.9% and the specificity of 86%. The sensitivity was 56.3% for gastric intestinal metaplasia in the same study [28]. In another study, methylation of SFRP2 and RNF180 was investigated in GC circulating DNA and methylation of both genes reached a sensitivity of 87.8% when random forest modelling method was used [29]. Interestingly, SFRP2 methylation was found in 61.5% of GC patients when stool DNA was analysed [32]. The percentages of SFRP2 methylation in sera and stool samples of GC patients range from 60 to 70%, and there is a consistency among different studies in general. According to Pimson et al., the methylations of PCDH10 and RASSF1A in the blood samples of patients with gastric cancer were 94.1% and 83.2%, respectively, whereas these levels were 2.97% and 5.45% in control samples [33]. In our study, PCDH10 methylation was found in 12.5% of sera in GC patients and none in the control group. However, PCDH10 methylation in GC tissue samples was 86.6%. This discrepancy may be caused by low methylation levels of PCDH10 in GC tissues, because average Ct value in tissue samples was 36.4 for PCDH10 methylation, however, it was 31 for SFRP2 methylation. We obtained the same results for APC methylation in which, the percentage of APC methylation was 90% for GC tissues as opposed to be 6.25% for the GC sera samples. Similarly, average Ct value in tissue samples was only 37.1 for APC methylation and the transition of the low amount of APC methylation in GC tissues to sera of the patients will be very low and would not be detected. In our study, RNF180 methylation was found in 12.5% of sera in GC patients and none in the control group. In contrast, RNF180 methylation in GC tissue samples was 66.6%. This discrepancy between tissue and serum results may be caused by low methylation levels of RNF180 in GC tissues. The average Ct value of RNF180 in GC tissue samples was only 39. In a study with 198 GC patients, RNF180 methylation percentage was 76% in GC tissues and 56% in GC plasma [34]. Methylation of RNF180 and SEPT9 was analysed together in plasma of 74 GC patients. RNF180 had a sensitivity of 32.4% and a specifity of 89.7%. The sensitivity was 40.5% and the specifity was 85.3% when RNF180 and SEPT9 were evaluated together [35]. Methylation detection rate for APC, PCDH10 and RNF180 was lower or at the low end of the range in our study compared to similar studies in plasma of GC patients. This might be due to the selection of different parts of CpG regions related to these genes or the population difference.

When we analyzed the association of the methylation of target genes and clinicopathological characteristics of GC patients such as tumor location, tumor invasion, *H. Pylori* infection, lymph node status, we have not found any significant association between the methylation and clinical parameters. This might be due to relatively low number of our patients enrolled in the study. Moreover, we have not observed a difference in serum methylation between early stage (pT1 and pT2) and late stage (pT3 and pT4) tumors, suggesting that it's possible to detect early stage tumors by qMSP.

In our study, methylation detection rate for RPRM and SFRP2 was high in sera of GC patients. Therefore, we focused on SFRP2 and RPRM methylation in further analysis of

sensitivity and specifity in GC diagnosis. The combination of SFRP2 and RPRM methylation was analyzed in cancer and control groups in our study. When SFRP2 or RPRM methylation was positive, the sensitivity was 78.79%, and the specificity was 90.00%. As discussed above, there are studies focusing on the methylation of SFRP2 or RPRM separately in GC. We have also analyzed the use of SFRP2 and RPRM methylation in cancer and gastritis groups, and the sensitivity was 78.79%, however the specificity was 83.33% which was moderately lower compared to the analysis of cancer and control groups. Nevertheless, it was still possible to discriminate between cancer and gastritis patients with 83.33% specificity.

Notably, RPRM and SFRP2 exhibited 9- and 6-cycle lower Ct values, respectively, in GC samples compared to ChG, both in tissue and serum. Since lower Ct values correspond to higher methylation levels in qMSP assays, these differences reflect significantly increased methylation in GC patients. Such pronounced Ct differences translated into robust diagnostic accuracy, further supporting the potential of these methylation biomarkers in the non-invasive and early detection of gastric cancer.

Consistent with these findings, our study revealed that methylation-positive serum samples exhibited Ct values greater than 30. This observation is consistent with the biological nature of circulating cell-free DNA (cfDNA), where methylated target sequences are typically present at low abundance, especially in early-stage cancers. Ct values exceeding 30 are not uncommon in plasma/serum qMSP studies. Similar findings have been reported in previous studies [28] documented Ct values above 30 as analytically valid and biologically expected in the context of cfDNA methylation detection.

To further substantiate this diagnostic accuracy, we included dilution-based LOD experiments using AGS gastric cancer cell line DNA, which experimentally validated Ct = 40 as a sensitive and specific analytical threshold for our qMSP assay.

Our study introduces an original dual-gene methylation panel combining these two markers, specifically evaluated for its efficacy in the non-invasive detection of gastric cancer using serum samples. This panel demonstrated high diagnostic accuracy, with a sensitivity of 78.79%, specificity of 90.00%, and an AUC of 0.931 in distinguishing gastric cancer patients from healthy controls.

These findings highlight the dual-gene panel's strong discriminatory performance, even under clinically challenging conditions, supporting its potential as a non-invasive and reliable diagnostic tool. Importantly, we plan to validate this panel in larger, multicenter cohorts to facilitate the development of a clinically applicable, methylation-based early detection kit for gastric cancer. This study represents a critical first step toward that goal and contributes an innovative perspective to the field of gastric cancer biomarker development.

Although this study presents promising results, we acknowledge several limitations that warrant consideration. One of the primary limitations is the relatively small sample size, particularly in subgroup analyses. This may limit the statistical power to detect associations with clinicopathological features and may affect the generalizability of the findings. Despite this, the observed methylation differences for SFRP2 and RPRM across gastric cancer tissue, serum, and cell lines were statistically significant and reproducible, strengthening the credibility of our data.

Another limitation is the absence of an external validation cohort. This study represents an initial, exploratory phase, and further multicenter studies with larger, more

diverse populations are essential to confirm and generalize the diagnostic potential of the identified methylation biomarkers.

Moreover, due to limitations in cfDNA yield, technical replicates were not performed in the qMSP assays. From each patient, 8 mL of peripheral blood was collected, yielding approximately 4 mL of serum. After cfDNA extraction and bisulfite modification, we obtained about 20 µL of bisulfite-converted DNA per sample. Since five target genes were analyzed per patient, this limited volume permitted only a single qMSP reaction per gene, per individual. Therefore, each gene was analyzed once per patient, and the error bars shown in Figs. 1 and 2 represent biological variability (inter-patient differences) rather than technical variation.

In addition, although qMSP is a sensitive and specific technique, potential technical variability such as inconsistencies in DNA input quantity, bisulfite conversion efficiency, and PCR amplification conditions may introduce some degree of variability. To address this, all experimental protocols were rigorously standardized, and technical replicates demonstrated high reproducibility, minimizing these effects.

Moreover, although clonal bisulfite sequencing was not performed in the current study, we agree that such high-resolution profiling would provide deeper insights into site-specific methylation heterogeneity and would help confirm the qMSP results at single-molecule resolution. We plan to incorporate clonal bisulfite sequencing in future validation studies to strengthen our findings and further investigate methylation density and distribution within the SFRP2 and RPRM promoter regions.

Similarly, we acknowledge the absence of a reference control gene (such as ACTB (β-actin) or GAPDH) for DNA input normalization. However, due to the qualitative design of our qMSP assay and consistent input volume across all samples, interpretative reliability was maintained. Analytical rigor was ensured through standardization of PCR conditions, use of positive controls (AGS, MKN45), and NTC monitoring. Future studies will incorporate reference gene normalization to enable quantitative assessment of methylation burden.

Finally, fully methylated commercial control DNA was not used for absolute quantification or standard curve generation. Instead, bisulfite-treated DNA from methylated GC cell lines (AGS and MKN45) served as positive controls. Inclusion of commercial fully methylated controls will be considered in future studies to further enhance analytical calibration and validation.

Collectively, these limitations do not undermine the study's main findings but rather highlight areas for refinement in future research aimed at developing a clinically applicable, non-invasive early detection assay for gastric cancer.

5 Conclusion

These findings demonstrate the potential use of RPRM and SFRP2 methylation for GC detection. In conclusion, we had an analysis tool utilizing two genes for the early diagnosis of GC by liquid biopsy with high sensitivity and specificity.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1007/s12672-025-03472-5.

Additional file 1. Figure S1. qPCR results for methylation status of SFRP2, P16, RASSF1A, RUNX3, PCDH10, RNF180, APC, hMLH1, and CDH1 in GC cell lines AGS and MKN45. Standard deviation was shown by error bars.

Additional file 2.

Acknowledgements
The author "Esra Aydemir" declares she is an Editorial Board Member of <Discover Oncology>and confirms that she was not involved in the handling or decision-making of her own submission.

Author contributions
Serap Dede, Esra Aydemir, Çağrı Şakalar, and Furkan Ayaz conceptualized the study. Serap Dede, Barış Yılmaz, Gamze Çubukcı, Muradiye Acar, Aydın Yavuz, Gizem Aksu, Saygın Altıner and Osman Yüksel, collected the clinical samples, conducted the experiments. Serap Dede, Esra Aydemir, Çağrı Şakalar, and Furkan Ayaz analyzed the data, wrote the manuscript, read and approved the final version of the manuscript.

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Data availability
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request. The qMSP Ct values generated and analysed during the current study are available from the corresponding author on reasonable request. Primer and probe sequences designed specifically for this study are considered proprietary, as they are planned to be used in future for diagnostic kit development, and therefore are not publicly available. These sequences were, however, shared confidentially with the reviewers during peer review.

Declarations

Ethics approval and consent to participate
The study was approved by the ethics committee of Biruni University Medical School (2015-KAEK-79–23-22). All subjects included in the present study were informed and gave written informed consent. All methods were carried out in accordance with relevant guidelines and regulations. The required consents were taken from the individuals.

Consent to publication
Not applicable.

Competing ınterests
The authors have no relevant financial or non-financial interests to disclose. The author "Esra Aydemir" declares she is an Editorial Board Member of < Discover Oncology > and confirms that she was not involved in the handling or decision-making of her own submission.

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