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ORIGINAL ARTICLE

Combined Methylation Analysis of SDC2 and TFPI2 in Plasma: A Noninvasive Liquid Biopsy Approach for Early Detection of Colorectal Polyp

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ABSTRACT

Early detection is crucial for improving survival rates in colorectal cancer (CRC). This study evaluates the non-invasive diagnosis of polyps by assessing the methylation status of the TFPI2 and SDC2 genes in plasma. This study enrolled 27 individuals with low-risk polyps (LRP), 27 with high-risk polyps (HRP), and 27 healthy controls. The quantitative methylation levels of TFPI2 and SDC2 genes were analyzed in plasma cell-free DNA (cfDNA) using the methylation-quantification endonuclease-resistant DNA (MethyQESD) method. Increased methylation percentages of both TFPI2 (TFPI2_1 and TFPI2_2) and SDC2 (SDC2_2) were observed in individuals with LRP and HRP. The combination of SDC2 and TFPI2 yielded an Area Under the Curve (AUC) of 0.732 (95% CI 0.78 to 0.96, p=0.001) with a sensitivity of 66% (95% CI 46% - 82%) and specificity of 77% (95 CI 56% - 91%) for LRP. For HRP, the AUC was 0.890 (95% CI 0.596 to 0.843, p<0.001) with a sensitivity of 70% (95% CI 51% - 84%) and specificity of 92% (95 CI 75% - 99%). The combined assessment of SDC2 and TFPI2 methylation presents a potential approach for the early non-invasive detection of CRC and its associated precancerous lesions

Keywords: Colorectal cancer, Methylation, plasma, polyp, SDC2, TFPI2

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Introduction

Despite the progress made in the detection and treatment of colorectal cancer (CRC), it continues to be a significant contributor to cancer-related fatalities worldwide. It is the second leading cause of cancer-related deaths and the third most prevalent malignant tumor among both men and women (1). Surgical interventions become impractical in advanced stages, limiting treatment options and impacting patient prognosis negatively (2). Early detection of CRC is critical for effective treatment, and proactive screening initiatives are vital in reducing its prevalence. Although colonoscopy is the gold standard for CRC diagnosis, its invasiveness, cost, and discomfort present limitations and may not always detect early-stage lesions (3). Molecular biomarkers for CRC detection are currently limited, with stool-based tests such as fecal occult blood testing (FOBT) and fecal immunochemical tests (FIT), as well as blood-based protein markers like CEA and CA19, demonstrating reduced sensitivity and specificity. It is worth noting that most patients prefer blood-based tests over stool-based options (4).

Syndecan-2 (SDC2), a member of the syndecan family of heparan sulfate proteoglycans, has been identified as a crucial actor in cancer advancement through its regulation of cell adhesion, proliferation, and migration in various research studies (5-8). Functioning as a cell surface receptor for extracellular matrix components (9), the SDC2 protein has shown contradictory roles in different types of cancer. It has been characterized as an oncogene in breast cancer (10) and CRC (11) but as a tumor suppressor in osteosarcoma (12). Tissue factor pathway inhibitor 2 (TFPI2), is a serine protease inhibitor (13) and plays a critical role in modulating extracellular matrix digestion and remodeling (13, 14). As a tumor suppressor, TFPI2 can impede cell proliferation, angiogenesis, tumor growth, and metastasis (14) by suppressing matrix metalloproteinases (15). Recent research has highlighted the downregulation of TFPI2 in various cancers, including pancreatic ductal adenocarcinoma (16), prostate cancer (17), non-small-cell lung cancer (18), gastric cancer (19), influencing the progression of malignant tumors and impacting patient survival outcomes (20). Both SDC2 and TFPI2 are frequently methylated in CRC tissues (21, 22), with SDC2 implicated in promoting tumorigenesis in colon cancer cells while TFPI2 is recognized as a tumor suppressor gene in various malignancies (23-26). Studies have consistently shown higher methylation levels in the gene promoters of both SDC2 and TFPI2 in colon cancer cells compared to normal tissue cells (27, 28). The epigenetic silencing of TFPI2 is a prevalent mechanism that plays a role in tumor growth and invasion in cancers like pancreatic ductal adenocarcinoma (16) and hepatocellular carcinoma (29). The role of methylated SDC2 may involve complex functions (30), however methylation in the SDC2 gene has been detected in various samples from CRC patients, including tissue, blood, and stool, indicating its potential role in CRC development and progression (27, 31).Studies have demonstrated the effectiveness of SDC2 and TFPI2 methylation in identifying early CRC in fecal samples (21, 22), as well as their detection in the blood of CRC patients (32, 33). A combined PCR assay targeting SDC2 and TFPI2 in fecal specimens has shown promising results in distinguishing CRC and adenomas from controls (4, 34). In this research, we investigated whether patients with CRC polyps (low risk and high risk) could be differentiated from controls using DNA methylation analysis of SDC2 and TFPI2 in plasma samples.

Methods

Samples and study population

This research was approved by the Medical Ethics Committee of Baqiyatallah University of Medical Sciences, and all participants provided informed consent for the collection and analysis of blood samples. A total of 27 patients with Low-risk polyp (LRP), 27 with High-risk polyp (HRP), and 27 healthy controls were recruited from Baghiyatollah Hospital. LRP was characterized as adenomas of at least ≤10 mm in size and/or the presence of 2 polyps. HRP was defined as adenomas of ≥10nm in size and/or the presence of more than 3 polyps (35). Blood samples were collected prior to any treatment or surgery. The diagnoses of polyp were confirmed through colonoscopy and pathology. Healthy individuals had negative colonoscopy reports. All participants had no familial history of cancer.

Sample Processing and plasma isolation

A 10-mL portion of peripheral blood samples was obtained through phlebotomy using collection tubes containing EDTA as an anticoagulant. Upon arrival at

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the medical laboratory, the samples were assessed for quality. Samples with low plasma volume, signs of hemolysis, elevated bilirubin levels, or visible particles were excluded from testing, and a repeat blood collection was requested. Within 2 hours of collection, plasma was separated by an initial centrifugation at 1,500 g for 10 minutes at 4 °C, followed by a second centrifugation at 15,000 g for 10 minutes at 4 °C. The isolated plasma was subsequently stored at -80 °C for further analysis.

cfDNA Isolation

cfDNA was extracted from 2–4 mL of plasma using the DNJia CF Kit from ROJETechnologies (Iran) and the QIAvac 24 Plus vacuum system from Qiagen, following the manufacturer's guidelines. The DNA samples were preserved at -80 °C until additional analysis to avoid degradation from multiple freeze-thaw cycles.

Quantitative DNA methylation assessment

For quantitative methylation analysis, the MethyQESD method was employed (36). This method consisted of two separate batches: one for methylation-specific quantification digestion (MQD) with Hin6I and the other for methylation-sensitive endonuclease calibrator digestion (CalD) using methylation-independent endonucleases such as XBaI and DraI. The enzyme digestion and QPCR protocol specifics were provided by Duppel et al (36).To quantitatively assess the methylation levels of SDC2 and TFPI2, real-time PCR was carried out in a final volume of 20 µl. This included 2 µl of digested DNA, 0.5 µl of each primer set listed in Table 1, and 10 µl of RealQ Plus 2x Master Mix Green high ROX™ from Amplicon (Denmark). The cycling program initiated with an initial denaturation at 95 °C for 5 minutes, followed by 45 amplification cycles at 95 °C for 15 seconds, 64°C for 20 seconds, and 72 °C for 30 seconds. The percentage of methylation was determined using the formula: Methylation (%) = $E^{\Delta Ct} \times 100$, where ΔCt = Ct of Calibrator – Ct of methylation quantification (E: PCR efficiency).

Statistical analysis

The research employed the nonparametric Kruskal-Wallis test followed by Dunn's multiple comparisons test to evaluate the differences among the normal, LRP, and HRP groups. The diagnostic accuracy of biomarkers was evaluated through receiver operating characteristic (ROC) curves and calculation of the area under the curve (AUC). The optimal cutoff value for biomarkers was determined using the Youden index. Statistical significance was considered for P-values < 0.05. Statistical analyses were conducted using GraphPad Prism 8.0 and MDCalc 22 software.

Results

Characteristics of the study population

A total of 27 individuals with LRP, 27 patients with HRP, and 27 healthy individuals who underwent colonoscopy confirmation were included in the study. The average age of the individuals in the LRP was 35-86 (62.52±11.17) years, 33-86 (59.89±10.84) years in the HRP group, and 35-71 (56.11±8.45) years in the healthy group. The clinical characteristics of the study samples are detailed in Table 2.

Methylation status of the SDC2 in plasma from LRP & HRP

First, we analyzed average percentages data between groups by Kruskal-Wallis test that showed methylation ratio of SDC2_1 was not different between control, LRP and HRP groups (Figure 1A), however analysis revealed a significantly higher methylation level of SDC2_2 in LRP and HRP groups (Figure 1B) in comparison to control group.

This higher methylation level was more in HRP (P value: 0.002) versus LRP (P value: 0.030). Subsequently, receiver operating characteristic curves (ROCs) were generated to assess the reliability of SDC2_2 for polyp diagnosis across LRP and HRP samples. The analysis indicated a notably high accuracy in polyp detection with an area under the AUC of 0.707. (95% CI 0.569 to 0.822, $p = 0.005$) a sensitivity of 86% (95%CI 68 - 96%) and a specificity of 58% (95%CI 37 - 77%), for LRP (Figure 1C), and AUC of 0.751 (95% CI 0.621 to 0.854, $p < 0.001$) a sensitivity of 100% (95%CI 89 - 100%), a specificity of 58% (95%CI 37 - 77%) for HRP (Figure 1D).

Methylation status of the TFPI2 in plasma from LRP & HRP

Similar to SDC2, we firs compared methylation ratio of TFPI2_1 and TFPI2_2 in control, LRP and HRP groups that showed significant differences in HRP vs control (P value: 0.001) in TFPI2_1 (Figure

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2A) and significant differences in LRP vs control (P value: 0.01) and also HRP vs control (P value <0.0001) in TFPI2_2 (Figure 2B). The ROC analysis conducted to assess the reliability of the methylation status of TFPI2_1 for polyp diagnosis across LRP and HRP samples demonstrated a significantly high accuracy in polyp detection, with an AUC of 0.758 (95% CI 0.628 to 0.860, $p < 0.001$) a sensitivity of 100% (95%CI 89 - 100%), a specificity of 42% (95%CI 23 - 63%) for LRP (Figure 2C). TFPI2_2 showed AUC of 0.879 (95% CI 0.768 to 0.950, $p = 0.001$) a sensitivity of 90% (95%CI 73 - 98%), a specificity of 46% (95%CI 27 - 67%) for LRP (Figure 2D), and AUC of 0.729 (95% CI 0.593 to 0.840, $p < 0.001$) a sensitivity of 97% (95%CI 84 - 100%), a specificity of 61% (95%CI 41 - 80%) for HRP (Figure 2E).

Methylation status of the SDC2 and TFPI2 in plasma from LRP & HRP

To evaluate the combination of the SDC2 and TFPI2 genes to detect polyps we used SDC2_2 and TFPI2_2 for LRP versus control and SDC2_2, TFPI2_1 and TFPI2_2 for HRP versus control. As shown in Figure 3A, combination of the SDC2_2 and TFPI2_2 increased the diagnostic power and significance (AUC = 0.732; 95% CI 0.78 to 0.96, p = 0.001) with sensitivity of 66% (95% CI 46% - 82%) and specificity of 77% (95 CI 56% - 91%) for LRP (Figure 3A). Combination of SDC2_2, TFPI2_1 and TFPI2_2 showed AUC of 0.890 (95% CI 0.596 to 0.843, p < 0.001) with sensitivity of 70% (95% CI 51% - 84%) and specificity of 92% (95 CI 75% - 99%) for HRP (Figure 3B).

Table 1. Primer sequence for the NDRG4 and TFPI2 genes (human reference genome: GRCh38/hg38).

Gene	Primer sequence (forward/reverse)	Position	Product size
SDC2-1	TCGGGAGTGCAGAAACCAAC GCTCAGGCTCGGGGACT	chr8:96,494,022-96,494,150	129
SDC2-1	GTACTCTGCTCCGGATTCGT CCAAGGTGAGCAGGATCCAC	chr8:96,494,164-96,494,302	139
TFPI2-1	CATGAATCAGCCACCCCTCAG GGCAAGGCGTCCGAGAAAG	chr7:93,890,705-93,890,842	138
TFPI2-2	CTTGCGACGATGCTTGCTG TCCTGTAGAAAGCGAGACGTG	chr7:93,890,043-93,890,166	124

Table 2. Baseline characteristics of CRC patients and controls in the present study.

	Count	Age range min-max (mean± SD)	P-value	Sex (male/female)	P-value
Control	27	35-71 (56.11±8.45)	0.075	12/15	0.95
LRP	27	35-86 (62.52±11.17)		12/15	
HRP	27	33-86 (59.89±10.84)		16/11	

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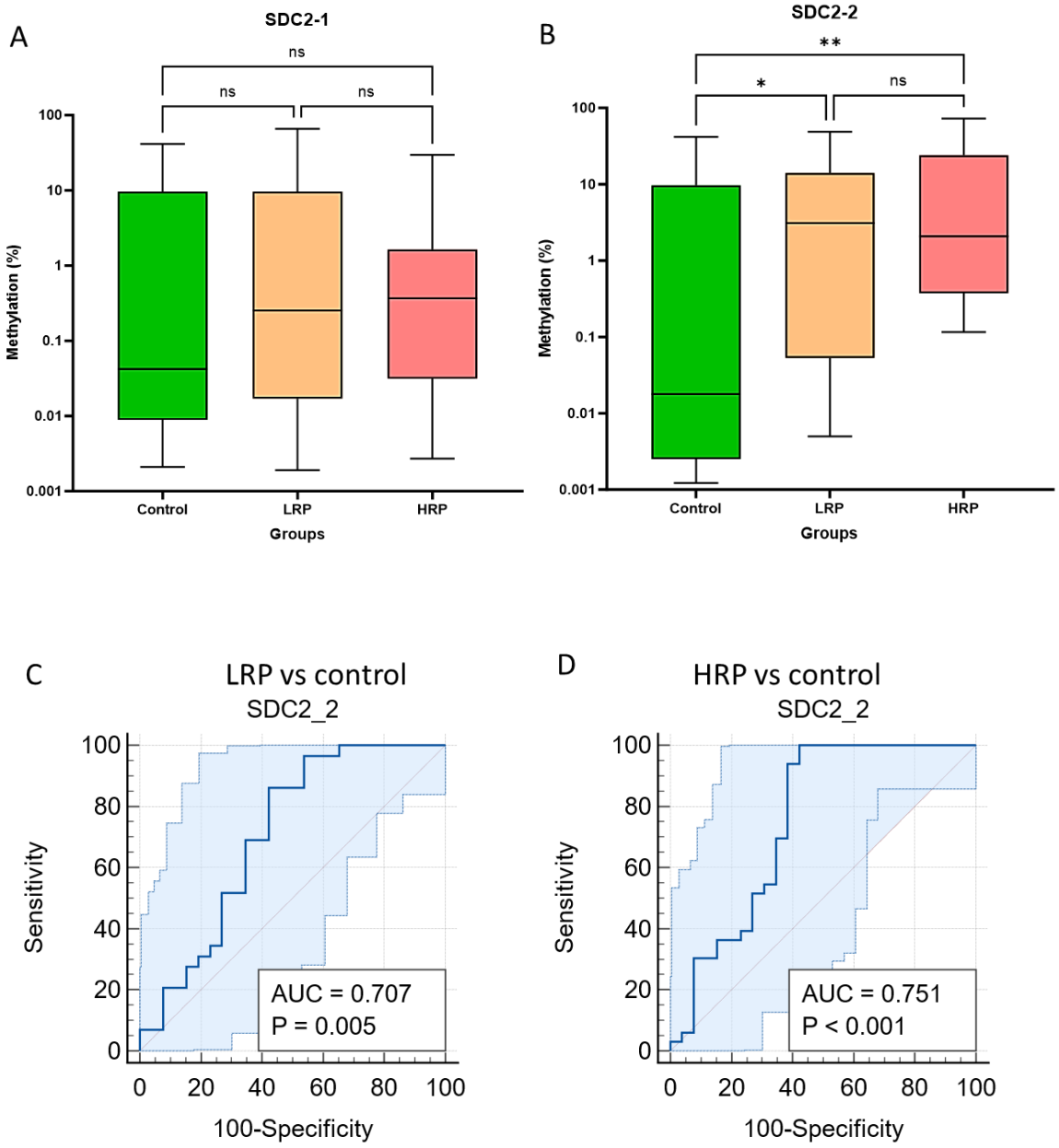

Figure 1. Methylation of SDC2 locations in LRP and HRP plasma. A: Methylation assessment of SDC2_1 revealed no notable variances among the groups. B: Methylation analysis of SDC2_2 showed significant differences in LRP (P value: 0.030) and HRP (P value: 0.002) groups in comparison to control group. C,D: ROC curve analysis assessing the methylation of SDC2_2 for polyp detection in LRP and HRP plasma samples. High-risk polyp (HRP), Low-risk polyp (LRP), p≤0.05 (*), p≤0.01 (**), area under the ROC curve (AUC).

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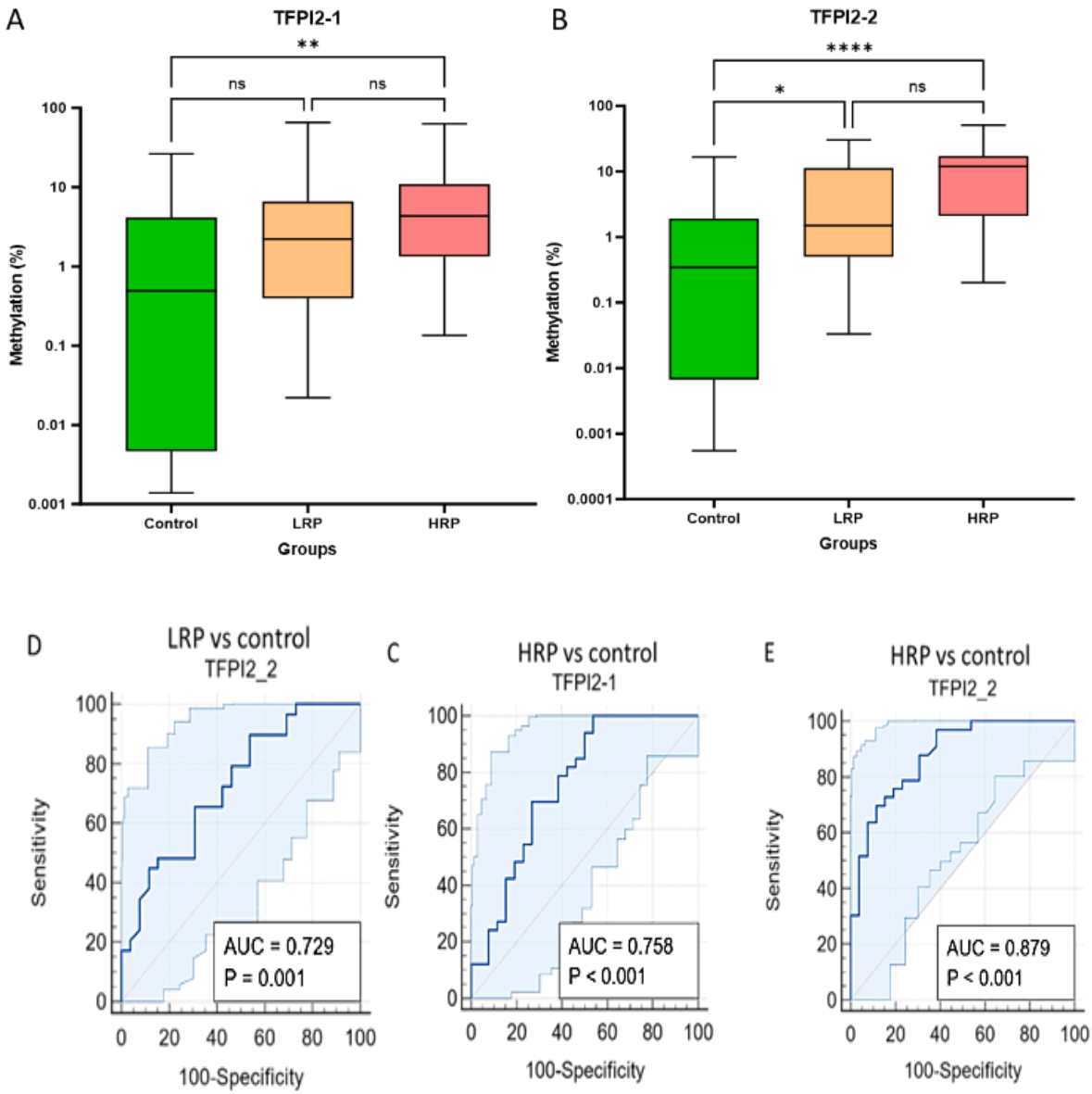

Figure 2. Methylation of TFPI2 locations in LRP and HRP plasma. A: Methylation analysis of TFPI2_1 showed significant differences between in HRP (P value: 0.001). B: Methylation analysis of TFPI2_2 showed significant differences in LRP (P value: 0.01) and HRP (P value <0.0001) groups in comparison to control group. C. ROC curve analysis assessing the methylation of TFPI2_1 for the detection of polyp in HRP plasma samples and TFPI2_2 for the detection of polyp in LRP (D) and HRP (E) plasma samples. High-risk polyp (HRP), Low-risk polyp (LRP), p≤0.05 (*), p≤0.01 (**), area under the ROC curve (AUC).

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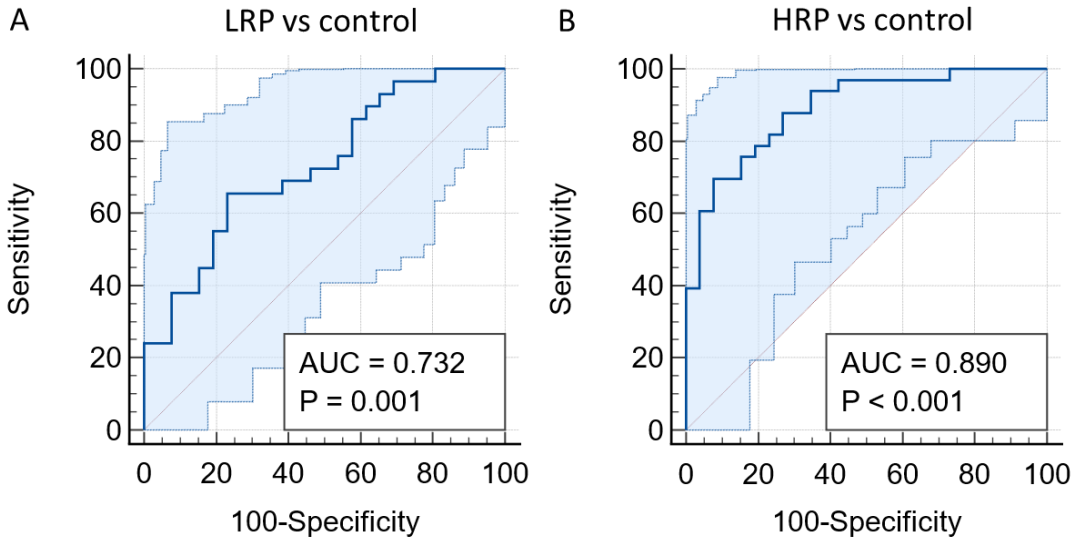

Figure 3. Methylation of SDC2 and TFPI2 locations in LRP and HRP plasma. A, combination of the SDC2_2 and TFPI2_2. B, Combination of SDC2_2, TFPI2_1 and TFPI2_2. Low-risk polyp (LRP), High-risk polyp (HRP), area under the ROC curve (AUC).

Discussion

Multiple studies have confirmed the efficacy of methylated SDC2 and TFPI2 in CRC detection in fecal samples (21, 22) and the blood of CRC patients (32, 33). Additionally, a combined PCR test that targets SDC2 and TFPI2 in fecal samples has demonstrated promising results in distinguishing CRC and adenomas from control groups (34). In this investigation, we examined the methylation status of two specific sites in SDC2 and TFPI2 in 27 Low-risk polyp (LRP) patients, 27 High-risk polyp (HRP) patients, and 27 healthy individuals. Our research revealed increased methylation of SDC2_2 and TFPI2_2 in both LRP and HRP cases.

In a study by Hu et al. in 2017, TFPI2 hypermethylation was detected using qMSP analysis, yielding a sensitivity of 61% and a specificity of 84% in 80 CRC tissue samples across all stages (I-IV) with an AUC of 0.759 (95% CI: 0.685–0.834) (37). TFPI2 methylation was also identified in fecal DNA from stage I to III CRC patients, showing sensitivities ranging from 76% to 89% and specificities from 79% to 93% (22). Studies also suggested that postoperative assessment of TFPI2 methylation could potentially serve as a marker for surgical outcomes (28). In our study, TFPI2_2 showed increased methylation ratio on both LRP and HRP (Figure 1B). TFPI2_2 showed sensitivity of 90% (95%CI 73 - 98%), a specificity of 46% (95%CI 27 - 67%) for LRP (Figure 2D), and a sensitivity of 97% (95%CI 84 - 100%), a specificity of 61% (95%CI 41 - 80%) for HRP (Figure 2E). However, there was no significant difference in the methylation rate at the SDC2_1 site between the groups.A combined PCR test targeting SDC2 and TFPI2 in stool samples achieved an AUC value of 0.98 for CRC, with a specificity of 96% and sensitivity of 96%. Additionally, it obtained an AUC value of 0.87 for adenomas, with a specificity of 95% and sensitivity of 80% (34).

Consistent with this study, we found that TFPI2 can improve the sensitivity of SDC2 while maintaining high specificity. Also, they showed, in most SDC2 hypomethylated CRC samples, TFPI2 is hypermethylated. This difference in methylation may be linked to the etiological heterogeneity of CRC in different regions of the colon. However, in this study, we were unable to classify patients based on the origin of their polyps. Zhang et al. introduced a two-biomarker panel (SDC2, TFPI2) for CRC detection in stool samples, attaining a sensitivity of 93.4% and specificity of 94.3%, along with an 81.3% sensitivity for adenoma samples (30).

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Ruixue Lei et al. identified SDC2 and TFPI2 as suitable methylation markers in CRC samples, highlighting a positive relationship between SDC2 and TFPI2 methylation levels and microsatellite instability (MSI) scores in CRCs with high microsatellite instability (38). In a recent investigation by Ma L. and co-authors (2022), a panel of four methylation markers (SDC2, TFPI2, WIF1, and NDRG4) for detecting CRC in plasma has demonstrated a sensitivity of 95.56% and specificity of 91.86% (39). In our study, the combination of SDC2 and TFPI2 enhanced the detection of polyps. The combination of SDC2_2 and TFPI2_2 exhibited a sensitivity of 66% (95% CI 46% - 82%) and specificity of 77% (95% CI 56% - 91%) for Low-risk polyps (LRP) compared to controls, and a sensitivity of 70% (95% CI 51% - 84%) and specificity of 92% (95% CI 75% - 99%) for High-risk polyps (HRP) compared to controls (Figure 3A, B). However, our current study is constrained by certain limitations. Firstly, a more comprehensive validation on a larger scale is required to accurately assess the effectiveness. The restricted number of adenoma cases, particularly the lack of pathology details regarding location, villous, and serrated adenomas, leads to inadequate statistical power for precise determination of the test's sensitivity and for conducting further analysis on these precancerous lesions.We evaluated the clinical utility of TFPI2 and SDC2 methylation in plasma cell-free DNA as a non-invasive biomarker for LRP and HRP. TFPI2 can improve the sensitivity of SDC2 methylation-specific detection of precancerous lesions while retaining a high level of specificity. The combined detection of SDC2 and TFPI2 offers a straightforward and precise screening method for polyp detection, demonstrating potential as a biomarker for the early non-invasive identification of CRC and related precancerous lesions.

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