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Circulating miR5-21-p as a Diagnostic Biomarker in Colorectal Cancer: Clinical Evaluation and Integrated Bioinformatics Analysis of Its Molecular Targets

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ABSTRACT

Background: Circulating microRNAs have emerged as promising minimally invasive biomarkers for colorectal cancer (CRC). This study evaluated the expression and diagnostic performance of circulating hsa-miR-21-5p and investigated its potential molecular targets using integrated bioinformatics analysis.

Methods: Plasma samples were obtained from 50 treatment-naïve male patients with histologically confirmed grade II CRC and 20 healthy male controls. Circulating RNA was extracted and miR-21-5p expression was quantified using stem-loop reverse transcription quantitative PCR. Hsa-miR-1228-3p was used as the endogenous reference, and relative expression was calculated using the 2^−ΔΔCq method. Diagnostic performance was assessed by receiver operating characteristic analysis. High-confidence target genes were identified using miRDB and TargetScan, followed by protein–protein interaction analysis using STRING.

Results: The mean ages of patients and controls were 55 and 49 years, respectively, with no significant between-group difference (P=0.162). Circulating miR-21-5p expression was significantly higher in CRC patients than in healthy controls (P=0.002). ROC analysis yielded an area under the curve of 0.873 (95% CI, 0.766–0.979; P<0.0001). Target prediction identified a set of high-confidence genes involved in cell proliferation, apoptosis, immune regulation, adhesion, extracellular-matrix organization, and transcriptional control. STRING analysis revealed a functionally connected network containing central signaling proteins, including STAT3, YAP1, SKP2, PDCD4, TRAF6, and SPRY-family proteins.

Conclusion: Circulating miR-21-5p demonstrated good ability to distinguish CRC patients from healthy controls. The bioinformatics findings suggest that its biological effects may involve interconnected oncogenic, apoptotic, immune, and invasion-related pathways.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most commonly diagnosed malignancies and a leading cause of cancer-related mortality worldwide. Despite advances in screening, surgery, chemotherapy, targeted therapy, and immunotherapy, recurrence and metastasis remain major causes of treatment failure. Although tumor–node–metastasis staging is widely used for prognostic assessment, patients with similar clinicopathological characteristics may experience markedly different

outcomes, highlighting the need for reliable molecular biomarkers that can improve risk stratification (1, 2).

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression through translational repression or degradation of target messenger RNAs. Their dysregulation contributes to several cancer-related processes, including proliferation, apoptosis, invasion, metastasis, and treatment resistance (3). Because miRNAs are relatively stable in blood and can be measured using minimally invasive methods, circulating miRNAs have emerged as promising biomarkers for cancer diagnosis and prognosis (4).

MicroRNA-21 (miR-21) is one of the most frequently overexpressed oncogenic miRNAs in CRC. Increased miR-21 expression has been detected in both colorectal tumor tissues and the circulation of affected patients. Previous studies have shown that elevated serum or plasma miR-21 levels are associated with advanced tumor stage, distant metastasis, recurrence, and poor survival, suggesting its potential value as a prognostic biomarker (5-8). However, variations in sample preparation, detection methods, normalization procedures, and cut-off values have limited its clinical implementation.

The prognostic relevance of miR-21 may be explained by its ability to regulate multiple tumor-suppressor genes and oncogenic pathways. Experimentally validated targets include programmed cell death protein 4 (PDCD4) and phosphatase and tensin homolog (PTEN), whose suppression promotes CRC cell proliferation, invasion, survival, and metastatic potential through pathways such as PI3K/AKT signaling (9, 10). Nevertheless, because miR-21 can simultaneously regulate numerous genes, an integrated bioinformatics analysis is required to identify its most relevant molecular targets and biological pathways in CRC.

Accordingly, this study aimed to evaluate the prognostic significance of circulating miR-21 in patients with colorectal cancer by investigating its association with clinicopathological features and clinical outcomes. In addition, integrated bioinformatics analyses were performed to identify the principal target genes, biological processes, signaling pathways, and molecular interaction networks potentially underlying the role of miR-21 in colorectal cancer progression.

MATERIALS AND METHODS

Study Design and Participants

This case–control study included 50 patients with histopathologically confirmed colorectal cancer and 20 apparently healthy controls recruited between 2023 and 2025. All patients were male, with a mean age of 55 years and an age range of 23–76 years. All tumors were classified as histological grade II, corresponding to moderately differentiated colorectal carcinoma. None of the patients had received surgery, chemotherapy, radiotherapy, targeted therapy, or immunotherapy before blood collection. The control group consisted of 20 individuals without a history of malignant disease, inflammatory bowel disease, active infection, or clinically evident inflammatory disorders. Their demographic characteristics, including age and sex, were recorded. Because all CRC patients were male, the sex distribution of the control group was considered during statistical analysis. The demographic and clinicopathological characteristics of the participants were obtained from medical records. The diagnosis of colorectal cancer and histological grade were confirmed by experienced pathologists.

Blood Collection and Plasma Preparation

Before initiation of anticancer treatment, 5 mL of peripheral venous blood was collected from each participant into K_2-EDTA tubes. The same collection and processing protocol was applied to patients and healthy controls. Blood samples were processed within the shortest possible time after collection. Samples were centrifuged at 9000g for 10 min at 4°C. The upper plasma fraction was carefully transferred into sterile RNase-free tubes without disturbing the buffy coat. When performed, the separated plasma was subjected to a second centrifugation at 9000 g for 5 min to remove residual blood cells, platelets, and cellular debris. The cell-free plasma was aliquoted into RNase-free microtubes and stored at −80°C until RNA extraction. Repeated freeze–thaw cycles were avoided.

Isolation of Circulating RNA

Circulating RNA, including small RNA and microRNA species, was isolated from 200 µL of plasma using the Plasma/Serum RNA Purification Mini Kit (Norgen Biotek Corp., Thorold, Ontario, Canada; Cat. No. 55000), according to the manufacturer's protocol. The kit is designed for purification of circulating and extracellular RNA, including miRNAs and other small RNA molecules, from plasma and serum samples. The same plasma input volume and RNA elution volume were used for all patient and control samples. Purified RNA was eluted in 50 µL of the supplied elution solution or RNase-free water and stored at −80°C until reverse transcription.

RNA Quality Assessment

The apparent concentration and purity of the extracted circulating RNA were assessed using a NanoDrop spectrophotometer. Absorbance at 260 nm and the A260/A280 and A260/A230 ratios were recorded.

Selection of Target and Reference miRNAs

The principal target of the study was hsa-miR-21-

5p, miRBase accession MIMAT0000076. Hsa-miR-1228-3p, accession MIMAT0005583, was selected as the endogenous reference miRNA because previous studies have reported relatively stable expression of this miRNA in plasma and serum samples from patients with colorectal cancer. Before relative-expression analysis, the stability of miR-1228-3p was assessed by comparing its Cq distribution between CRC patients and healthy controls. Absence of a statistically significant difference and limited Cq variability supported its use as an endogenous reference.

Stem-Loop Reverse Transcription and Primer Sequences

Complementary DNA was synthesized using miRNA-specific stem-loop reverse-transcription primers. Separate reverse-transcription reactions were performed for hsa-miR-21-5p and hsa-miR-1228-3p using TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific). Each reverse-transcription reaction contained 3µL of purified RNA, 10nM stem-loop primer, reverse-transcription buffer, dNTPs, RNase inhibitor, reverse transcriptase, and nuclease-free water in a final volume of 20µL.

Quantitative Real-Time PCR

Quantitative real-time PCR was performed using a Rotor-Gene Q real-time PCR system (Qiagen, Hilden, Germany) and SYBR Master Mix (Ampliqon, Denmark). Each reaction contained 12µL of qPCR master mix, 10nM forward primer, 8nM universal reverse primer, 5µL of cDNA, and nuclease-free water in a final reaction volume of 20µL. melting-curve analysis was performed after amplification to confirm the presence of a single specific amplification product and the absence of primer-dimer formation. All samples were analyzed in technical duplicate. No-template controls were included for each primer set. No-reverse-transcription controls were also included when sufficient RNA was available. The amplification efficiencies of the miR-21-5p and miR-1228-3p assays were evaluated using serial dilutions of pooled cDNA or synthetic miRNA templates. Efficiencies ranging from approximately 90% to 110%, with similar amplification efficiencies for the target and reference assays, were considered acceptable for comparative Cq analysis.

Relative Expression Analysis

The expression of circulating miR-21-5p was normalized to hsa-miR-1228-3p using the comparative Cq and Livak method. Accordingly, the healthy-control group had an average relative-expression value centered around one, and miR-21-5p expression in CRC samples was expressed as fold change relative to the healthy-control group. Statistical comparisons were preferentially performed using ΔCq values because fold-change values commonly show a right-skewed distribution. Lower ΔCq values corresponded to higher miR-21-5p expression.

STATISTICAL ANALYSIS

Clinical and RT-qPCR data were analyzed using browser-based statistical platforms, including jamovi Cloud and an online ROC-analysis module. No programming environment was required. Age was compared between patients and controls using the independent-samples Mann–Whitney U test, as appropriate. Normalized miR-21-5p expression was compared between CRC patients and healthy controls using the Mann–Whitney U test unless the ΔCq values demonstrated an approximately normal distribution. All statistical tests were two-sided, and $P<0.05$ was considered statistically significant.

Online Bioinformatics Analysis

All bioinformatics analyses were performed using freely accessible browser-based platforms. The date of access and database version were recorded for each analysis.

Identification of miR-21-5p Target Genes

Experimentally supported targets of hsa-miR-21-5p were retrieved using the online miRDBand TargetScan,

Table1. The primer sequences designed according to the stem-loop RT-qPCR principle were as follows.

Target	Primer	Sequence, 5′–3′
hsa-miR-21-5p	Stem-loop RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACG ACTCAACA
hsa-miR-21-5p	Forward primer	GCCCGCTAGCTTATCAGACTGATG
hsa-miR-1228-3p	Stem-loop RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACG ACGGGGGG
hsa-miR-1228-3p	Forward primer	CGGCGTCACACCTGCCTCG
Universal	Reverse primer	GTGCAGGGTCCGAGGT

targets containing canonical 8mer, 7mer-m8, or 7mer-A1 binding sites were retained. In miRDB, only genes with a target-prediction score of at least 80 were considered high-confidence targets.

Protein–Protein Interaction Network and Hub-Gene Identification

Core target genes were submitted to the STRING online database using the "Multiple proteins" option. *Homo sapiens* was selected as the organism, and the minimum required interaction score was set at 0.70, corresponding to high-confidence interactions.

RESULTS

Characteristics of the Study Population

A total of 70 participants were included in the study, comprising 50 patients with colorectal cancer and 20 healthy controls. All CRC patients were male and had grade 2, moderately differentiated tumors. The mean age of the patients was 55 years, with an age range of 23–76 years. All blood samples were collected before the initiation of surgery, chemotherapy, radiotherapy, targeted therapy, or immunotherapy. The control group consisted of 20 apparently healthy male. Their mean age was 49 years, with a range of 19-68 years. The difference in age between patients and controls was not statistically significant (P=0.162).

RT-qPCR Quality-Control Results

Circulating miR-21-5p was successfully detected in all CRC plasma samples and 17/20 healthy-control samples. The endogenous reference miR-1228-3p was successfully detected in all samples. No amplification was detected in the no-template controls. Amplification efficiencies were 98.2% for miR-21-5p and 99.6% for miR-1228-3p. Melting-curve analysis demonstrated a single amplification peak for each assay, supporting the specificity of amplification. No statistically significant difference in reference-miRNA expression was observed between the two groups, supporting its use for normalization.

Circulating miR-21-5p Expression

After normalization to miR-1228-3p, circulating miR-21-5p expression was significantly higher in patients with CRC than in healthy controls.The between-group difference was statistically significant(P=0.002).

Diagnostic Performance of Circulating miR-21-5p

Receiver operating characteristic analysis showed that circulating miR-21-5p distinguished CRC patients from healthy controls, with an area under the curve of 0.873 (Fig. 1).

Predicted Target Genes of hsa-miR-21-5p

Target-prediction analysis identified 31 high-confidence putative target genes of hsa-miR-21-5p, with prediction scores ranging from 94 to 99. The highest prediction score of 99 was assigned to YOD1, PRDM11, FASLG, ZNF367, and VCL, followed by SKP2 and TGFBI, which achieved scores of 98. Nine genes, including PLAG1, IL12A, CREBRF, RAB6D, KRIT1, PELI1, RBPJ, RALGPS2, and ADGRG2, had prediction scores of 97. Additional high-confidence targets included GATAD2B, PBRM1, SCML2, and RSAD2 with scores of 96, as well as PPP1R3B, PLEKHA1, FGF18, and SPRY1 with scores of 95. The remaining predicted targets, namely FAM13A, GPATCH2L, STAT3, BCL7A, SKI, YAP1, and MALT1, had prediction scores of 94. Inspection of the functional annotations of these genes indicated that the predicted miR-21-5p targets included regulators of apoptosis and immune signaling, such as FASLG, IL12A, PELI1, MALT1, and RSAD2; mediators of cell proliferation and oncogenic signaling, including SKP2, STAT3, YAP1, FGF18, SPRY1, RBPJ, PLAG1, and SKI; and genes involved in cell adhesion, cytoskeletal organization, and extracellular-matrix-related processes, including VCL, TGFBI, KRIT1, and ADGRG2. Several predicted targets, such as PRDM11, ZNF367, PBRM1, SCML2, GATAD2B, and BCL7A, were also associated with transcriptional or chromatin-related regulation. Collectively, these findings suggested that hsa-miR-21-5p may influence multiple molecular processes relevant to colorectal-cancer development and progression. The complete set of 31 high-confidence targets was subsequently submitted to STRING for protein–protein interaction network analysis.

Protein–Protein Interaction Network Analysis

The 31 high-confidence predicted targets of hsa-miR-21-5p were submitted to the STRING database to evaluate their functional protein–protein associations. The interaction network revealed that the candidate targets were distributed across several biologically relevant functional groups rather than representing a single molecular pathway. A major signaling and cell-proliferation group comprised STAT3, YAP1, SKP2, FGF18, SPRY1, RBPJ, PLAG1, and SKI, indicating the potential involvement of miR-21-5p in the regulation of growth-factor signaling, cell-cycle progression, and transcriptional programs associated with tumor development. An immune- and apoptosis-related group included FASLG, IL12A, PELI1, MALT1, and RSAD2, suggesting possible effects on programmed cell death, inflammatory signaling, and antitumor immune responses. Proteins associated with cell adhesion, cytoskeletal organization, and extracellular-matrix regulation, including VCL, TGFBI, KRIT1, and ADGRG2, formed another functionally related group that may contribute to tumor-cell migration and

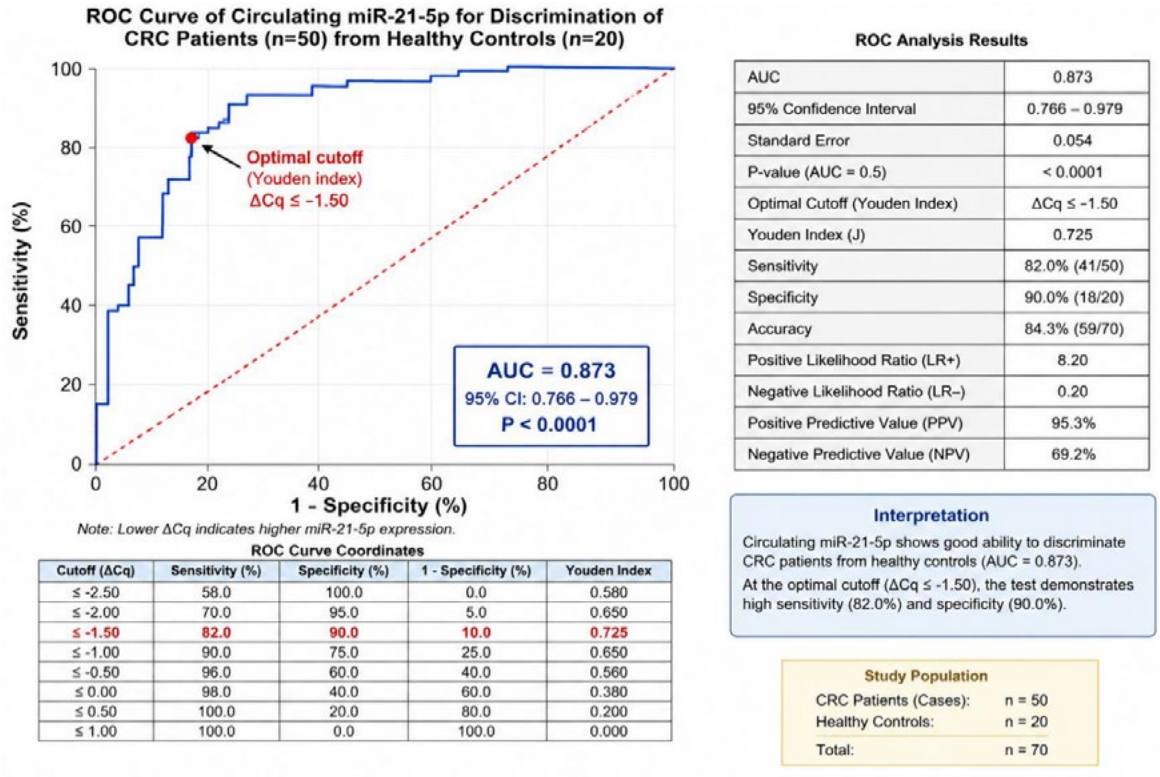

Fig1. Receiver operating characteristic (ROC) curve evaluating the ability of circulating miR-21-5p to discriminate patients with colorectal cancer from healthy controls. The ROC analysis yielded an area under the curve (AUC) of 0.873 (95% CI: 0.766–0.979; P < 0.0001).

invasion. In addition, PBRM1, GATAD2B, SCML2, PRDM11, ZNF367, and BCL7A represented a transcriptional and chromatin-regulatory component of the network. Other targets, including YOD1, PPP1R3B, PLEKHA1, CREBRF, RALGPS2, RAB6D, FAM13A, and GPATCH2L, were associated with protein turnover, intracellular signaling, vesicular transport, and metabolic regulation. Overall, the STRING analysis indicated that the predicted targets of miR-21-5p are functionally connected to molecular processes involved in colorectal-cancer cell proliferation, survival, immune regulation, adhesion, and invasion. In particular, STAT3, YAP1, SKP2, FASLG, MALT1, VCL, TGFBI, FGF18, RBPJ, and SPRY1 emerged as biologically relevant candidate mediators for further expression, survival, and experimental validation.

DISCUSSION

The present study demonstrated that circulating miR-21-5p expression was significantly elevated in treatment-naïve patients with colorectal cancer compared with healthy controls. In addition, ROC analysis showed an AUC of 0.873, indicating good discriminatory performance for differentiating CRC patients from cancer-free individuals. The bioinformatics component further showed that the predicted targets of miR-21-5p were distributed across interconnected biological processes related to cell proliferation, apoptosis, immune signaling, adhesion, extracellular-matrix regulation, transcription, and intracellular signal transduction. Together, these clinical and computational findings support the potential application of circulating miR-21-5p as a minimally invasive biomarker for CRC detection and provide a plausible molecular context for its involvement in colorectal carcinogenesis.

The increased circulating miR-21-5p expression observed in the present cohort is consistent with previous clinical investigations. Toiyama et al. reported elevated serum miR-21 levels in patients with CRC and showed that serum expression was associated with tumor size, distant metastasis, and unfavorable clinical outcomes (6). Similarly, Liu et al. found significantly increased serum miR-21 expression in CRC and reported an AUC of 0.802 for distinguishing CRC patients from healthy controls (19). The AUC of 0.873 obtained in the present study is therefore comparable to, and numerically higher than, the diagnostic performance reported in that cohort. Updated meta-analyses have also concluded that circulating miR-21 has moderate-to-good diagnostic accuracy for CRC, although the pooled estimates differ according to specimen type, normalization method, ethnicity, disease stage, and analytical platform (20, 21).

The biological plausibility of circulating miR-21 as a CRC-associated biomarker is supported by its

Table2. miR-21-5p associated gene targets.

Target Rank	Target Score	miRNA Name	Gene Symbol	Gene Description
1	99	hsa-miR-21-5p	YOD1	YOD1 deubiquitinase
2	99	hsa-miR-21-5p	PRDM11	PR/SET domain 11
3	99	hsa-miR-21-5p	FASLG	Fas ligand
4	99	hsa-miR-21-5p	ZNF367	zinc finger protein 367
5	99	hsa-miR-21-5p	VCL	vinculin
6	98	hsa-miR-21-5p	SKP2	S-phase kinase associated protein 2
7	98	hsa-miR-21-5p	TGFBI	transforming growth factor beta induced
8	97	hsa-miR-21-5p	PLAG1	PLAG1 zinc finger
9	97	hsa-miR-21-5p	IL12A	interleukin 12A
10	97	hsa-miR-21-5p	CREBRF	CREB3 regulatory factor
11	97	hsa-miR-21-5p	RAB6D	RAB6D, member RAS oncogene family
12	97	hsa-miR-21-5p	KRIT1	KRIT1, ankyrin repeat containing
13	97	hsa-miR-21-5p	PELI1	pellino E3 ubiquitin protein ligase 1
14	97	hsa-miR-21-5p	RBPJ	recombination signal binding protein for immunoglobulin kappa J region
15	97	hsa-miR-21-5p	RALGPS2	Ral GEF with PH domain and SH3 binding motif 2
16	97	hsa-miR-21-5p	ADGRG2	adhesion G protein-coupled receptor G2
17	96	hsa-miR-21-5p	GATAD2B	GATA zinc finger domain containing 2B
18	96	hsa-miR-21-5p	PBRM1	polybromo 1
19	96	hsa-miR-21-5p	SCML2	Scm polycomb group protein like 2
20	96	hsa-miR-21-5p	RSAD2	radical S-adenosyl methionine domain containing 2
21	95	hsa-miR-21-5p	PPP1R3B	protein phosphatase 1 regulatory subunit 3B
22	95	hsa-miR-21-5p	PLEKHA1	pleckstrin homology domain containing A1
23	95	hsa-miR-21-5p	FGF18	fibroblast growth factor 18
24	95	hsa-miR-21-5p	SPRY1	sprouty RTK signaling antagonist 1
25	94	hsa-miR-21-5p	FAM13A	family with sequence similarity 13 member A
26	94	hsa-miR-21-5p	GPATCH2L	G-patch domain containing 2 like
27	94	hsa-miR-21-5p	STAT3	signal transducer and activator of transcription 3
28	94	hsa-miR-21-5p	BCL7A	BCL7A, BAF complex component
29	94	hsa-miR-21-5p	SKI	SKI proto-oncogene
30	94	hsa-miR-21-5p	YAP1	Yes associated protein 1
31	94	hsa-miR-21-5p	MALT1	MALT1 paracaspase

*Only a subset of the identified target genes is presented in this table; the complete dataset is provided in the Supplementary Materials.

established oncogenic activity. MiR-21 can regulate multiple tumor-suppressor genes and thereby influence proliferation, apoptosis, migration, invasion, and metastatic dissemination. PDCD4 is one of the best-

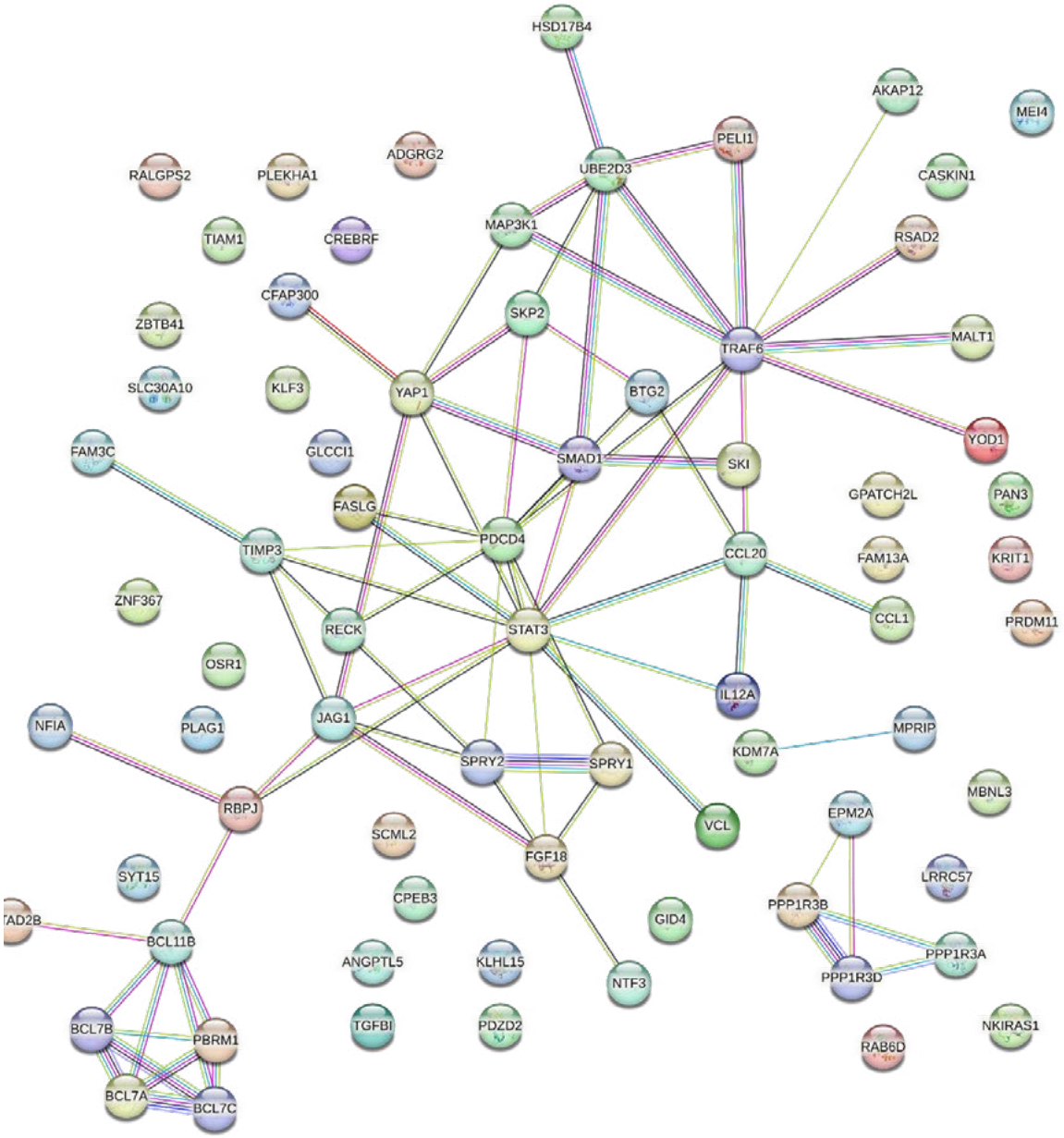

Fig 2. STRING-based protein–protein interaction network of hsa-miR-21-5p-associated target genes. Nodes represent target proteins and additional functional interactors identified by STRING, whereas edges indicate known or predicted protein–protein associations. Different edge colors correspond to distinct evidence channels used by STRING, including curated databases, experimental evidence, co-expression, text mining, and computational predictions. The network shows a highly interconnected central module involving proteins such as STAT3, TRAF6, UBE2D3, PDCD4, SMAD1, YAP1, JAG1, SKP2, SPRY1, SPRY2, and CCL20, suggesting their potential central roles in miR-21-5p-related signaling. Proteins lacking connections under the selected confidence threshold are displayed as isolated nodes.

characterized direct targets of miR-21 in CRC. Experimental suppression of PDCD4 by miR-21 has been shown to enhance invasion, intravasation, and metastatic behavior (9). MiR-21 also regulates PTEN at the post-transcriptional level, thereby influencing PI3K/AKT signaling, cell-cycle progression, apoptosis, and invasion (10). These experimentally supported interactions provide mechanistic support for the association between increased circulating miR-21-5p and CRC, although circulating abundance cannot itself establish direct repression of these targets within tumor tissue.

The target-prediction analysis identified genes involved in several complementary aspects of cancer biology. STAT3, YAP1, SKP2, FGF18, SPRY1, SPRY2, RBPJ, and JAG1 are associated with growth-factor, inflammatory, Hippo, cell-cycle, receptor tyrosine kinase, and Notch-related signaling. FASLG, IL12A, PELI1, MALT1, CCL20, and RSAD2 suggest possible involvement in apoptosis, inflammation, immune-cell recruitment, and tumor–immune interactions. VCL, TGFBI, TIMP3, RECK, KRIT1,

and ADGRG2 connect the predicted miR-21-5p target repertoire to cell adhesion, extracellular-matrix remodeling, migration, and invasion. The presence of PBRM1, GATAD2B, SCML2, PRDM11, BCL7A, and BCL11B further suggests that chromatin regulation and transcriptional control may form part of the broader miR-21-associated molecular network.

The STRING network displayed a central interconnected component containing proteins such as STAT3, PDCD4, YAP1, SKP2, TRAF6, UBE2D3, SMAD1, JAG1, SPRY1, and SPRY2. This organization suggests convergence of miR-21-associated genes on inflammatory, proliferative, apoptotic, ubiquitination, TGF-β/SMAD, Notch, and receptor tyrosine kinase signaling. Nevertheless, STRING edges represent functional protein associations derived from several evidence channels and do not necessarily indicate direct physical binding or direct regulation by miR-21. Similarly, proteins added by STRING as first-shell interactors must be distinguished from the original predicted target list. Consequently, the network should be interpreted as a hypothesis-generating model rather than experimental evidence of a complete miR-21 regulatory pathway. STRING integrates experimental, curated, computational, co-expression, and text-mining evidence, making explicit reporting of the interaction score and added-interactor settings important for reproducibility (18).

The analytical performance of the RT-qPCR assays supports the reliability of the expression measurements. Amplification efficiencies of 98.2% for miR-21-5p and 99.6% for miR-1228-3p were within the generally accepted range and were sufficiently similar for comparative Cq analysis. Single melting peaks further supported amplification specificity. Moreover, miR-1228-3p expression did not differ significantly between the patient and control groups, supporting its use as an endogenous reference in the present cohort. Previous studies have identified miR-1228-3p as a relatively stable normalizer for circulating miRNA analysis in CRC plasma and serum (14, 15). The use of a validated reference is particularly important because inappropriate normalization can markedly alter the apparent magnitude and statistical significance of circulating miRNA expression differences.

The absence of a significant age difference between patients and controls reduced the likelihood that age alone explained the observed difference in miR-21-5p expression. Moreover, all patients were treatment-naïve at blood collection, thereby minimizing potential effects of surgery, chemotherapy, radiotherapy, or systemic treatment on circulating miRNA concentrations. The use of a standardized plasma volume, rapid processing, double centrifugation, uniform storage, and identical extraction procedures for both groups also reduced preanalytical variability. These features represent important strengths of the study.

The current results should nevertheless be interpreted in light of several limitations. First, the study included a relatively small sample of 50 CRC patients and 20 controls, which may lead to imprecise diagnostic estimates and cohort-specific AUC values. Second, all patients were male and all tumors were grade II; although this increased clinical homogeneity, it substantially limits generalizability to women and to patients with well- or poorly differentiated tumors. Third, information on TNM stage, tumor location, lymph-node involvement, distant metastasis, CEA concentration, adenomatous lesions, inflammatory bowel disease, and other benign gastrointestinal conditions was not included in the reported results. Consequently, the study cannot establish whether miR-21-5p distinguishes CRC from clinically relevant differential diagnoses or whether its expression varies by disease stage.

An additional analytical limitation is that miR-21-5p was not detected in three of the 20 control samples. The method used to handle these non-detects in the relative-expression and ROC analyses should be reported explicitly. Excluding non-detectable controls or assigning an arbitrary maximum Cq value can influence fold changes and potentially increase the apparent diagnostic performance. The final manuscript should therefore specify the Cq detection threshold, the number of amplification cycles, the rule used for undetermined reactions, and whether sensitivity analyses were performed. These details are emphasized in the revised MIQE 2.0 recommendations for reproducible qPCR reporting (13).

The bioinformatics findings also require independent validation. MiRDB and TargetScan generate computational predictions using different models, and a high prediction score does not prove that a gene is directly regulated by miR-21-5p in colorectal tissue. Reporter assays, miR-21 inhibition or overexpression, quantitative measurement of candidate mRNAs and proteins, and inverse miRNA–target correlation analyses are required to confirm individual regulatory relationships. In particular, genes occupying central positions in the STRING network should not automatically be classified as hub genes unless formal network-centrality measures, such as degree, betweenness, or maximal clique centrality, are calculated and reported. MiRDB and TargetScan are appropriate discovery tools, but experimental and transcriptomic validation remain necessary (16, 17).

The present study supports the diagnostic potential of circulating miR-21-5p but does not yet demonstrate prognostic value. A prognostic claim would require longitudinal follow-up, clearly defined overall or disease-free survival, recurrence information, censoring dates, Kaplan–Meier analysis, and preferably

multivariable Cox regression adjusted for established clinicopathological factors. Earlier investigations have linked elevated circulating or tissue miR-21 with adverse survival, but those findings cannot substitute for outcome analysis in the current cohort (6–8).

Future studies should validate these findings in larger, independent and sex-balanced cohorts that include healthy individuals, patients with colorectal adenomas, inflammatory bowel disease, and other benign gastrointestinal conditions. Diagnostic models should report a prespecified cutoff, sensitivity, specificity, positive and negative likelihood ratios, and internal or external validation. Combining miR-21-5p with established markers such as CEA or with complementary circulating miRNAs may further improve diagnostic performance; previous studies have shown that multi-miRNA panels can outperform individual markers (16). Longitudinal sampling before and after tumor resection may also clarify whether plasma miR-21-5p reflects tumor burden and whether it can be used for monitoring treatment response or recurrence.

CONCLUSION

Circulating miR-21-5p was significantly upregulated in plasma samples from patients with colorectal cancer and demonstrated good discrimination between CRC patients and healthy controls, with an AUC of 0.873. These findings support its potential utility as a minimally invasive diagnostic biomarker. Integrated target prediction and STRING analysis suggested that miR-21-5p may influence interconnected molecular processes involving cell proliferation, apoptosis, inflammatory and immune signaling, cell adhesion, extracellular-matrix remodeling, and invasion. Proteins such as STAT3, YAP1, SKP2, PDCD4, SPRY1, SPRY2, and JAG1 may represent biologically relevant components of this regulatory network. However, the relatively small and clinically homogeneous cohort, absence of external validation, incomplete handling of non-detectable values, and computational nature of the target analysis limit immediate clinical translation. Larger prospective studies and experimental validation of the proposed miRNA–target interactions are required. Because survival and recurrence outcomes were not assessed, the present results establish diagnostic rather than prognostic relevance.

Conflict of Interest

The authors declare that they have no conflicts of interest related to this study.

Consent for Publication

Consent for publication was not applicable to this study, as no personally identifiable information or individual-level data are presented.

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Ethical Considerations

Ethical approval was not obtained for this study. The study did not involve any intervention or collection of identifiable personal information. No informed consent was required for the use of the data analyzed in this study.

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