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Tumor Discovery

ORIGINAL RESEARCH ARTICLE

Molecular characterization of colorectal cancer: Insights from miRNA, mRNA, and protein analysis

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Abstract

Recent research highlights the significant roles of microRNAs (miRNAs) in various diseases, particularly cancer, where they serve as diagnostic and prognostic markers. This study focuses on colorectal cancer (CRC) by examining the expression of six specific miRNAs, miR-20a, miR-21, miR-29a, miR-31, miR-92a, and miR-224, in 81 tumor samples compared to matched normal tissues. We assessed the expression levels of six target genes, *SMAD4*, *PTEN*, *TGFBRII*, *BCL2*, *KLF4*, and *RASA1*, using reverse transcription quantitative polymerase chain reaction and immunohistochemistry. Our results indicated a significant upregulation of miR-20a, miR-21, miR-29a, and miR-31 in tumor samples, alongside a decrease in *TGFBRII* mRNA expression. Correlation analyses demonstrated that high levels of miR-20a were inversely related to both mRNA and protein levels of PTEN. Elevated expressions of miR-21 and miR-224 were associated with lower mRNA and protein levels of TGFBRII. Furthermore, increased levels of miR-29a and miR-31 showed an inverse relationship with mRNA and protein levels of RASA1. These findings suggest a strong link between upregulated miRNAs and downregulated target genes, indicating their significant roles in CRC carcinogenesis. Notably, the upregulation of miR-20a, miR-21, miR-29a, and miR-31 may serve as effective biomarkers for differentiating CRC from normal mucosa, potentially enhancing screening strategies in the general population.

Keywords: Colorectal cancer; microRNA; mRNA; Immunohistochemistry

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1. Introduction

Colorectal cancer (CRC) stands as a prominent gastrointestinal malignancy globally, with more than 1.2 million new cases reported annually. It holds the position of being the third most common cancer worldwide and significantly contributes to cancer-

related deaths.[1] In recent years, there has been a notable emergence of new favorable subsets of cancers of unknown primary origin, including CRC of unknown origin. This new clinical entity is treated as CRC and has contributed to the rising incidence rates of CRC. Understanding these trends is crucial, as they may influence the sociodemographic factors and clinical aspects that ultimately affect the quality of life in patients undergoing surgical treatment for CRC.[2] There is significant evidence affirming the vital role of screening and early detection in combating CRC, effectively reducing its incidence and mortality rates.[3] For example, the 5-year survival rate is as high as 93.2% for TNM stage I as compared to only 8.1% for stage IV.[4] However, existing screening tools such as (i) colonoscopy screening, which is currently the most reliable screening tool, has been hampered because of its invasive nature and high cost; and (2) the fecal occult blood test, which has low sensitivity and requires dietary restriction, impedes compliance and use. In addition, studies have investigated several molecular biomarkers for CRC detection, such as carcinoembryonic antigen (CEA), and shown that high CEA levels are associated with CRC progression. However, its utility in the disease screening is limited due to the serum level of CEA not being elevated after the tumor has invaded the serosa membrane.[5,6]

Given these factors, there is a pressing need for specific molecular markers to improve the diagnosis of CRC. Recently, researchers have concentrated on microRNAs (miRNAs) because of their involvement in essential cellular processes such as development, cell cycle advancement, differentiation, proliferation, and apoptosis.[7] Aberrant expression of miRNAs, which function as either tumor suppressors or oncogenes, has been implicated in various cancers. Moreover, miRNAs exhibit discriminatory potential across cancer types, predicting outcomes in hematological and solid malignancies.[8] Notably, the differential expression of miRNAs between CRC tumors and adjacent normal tissue underscores their promise in early diagnostic and prognostic applications.[9]

Biomarker testing is now a standard part of CRC investigation, particularly regarding major genetic mutations like RAS, which are associated with tumor aggressiveness and chemotherapy response. In addition, growing evidence indicates that inflammation contributes to CRC progression, prompting studies on the predictive and prognostic roles of various blood-based inflammatory markers, such as the neutrophil–lymphocyte ratio, lymphocyte–monocyte ratio, and platelet–lymphocyte ratio. Furthermore, miRNAs act as both tumor suppressors and oncogenes, with ongoing research exploring their diagnostic, prognostic, and predictive implications. For instance, FOLFOX resistance in advanced CRC is linked to the altered expression of serum miRNAs, such as miR-19a. In metastatic CRC, miR-126 upregulation has been associated with resistance to bevacizumab, while overexpression of miR-31, miR-100, and miR-125b, along with miR-7 downregulation, correlates with resistance to cetuximab.[10]

In this study, we have selected a panel of six miRNAs (miR-20a, miR-21, miR-29a, miR-31, miR-92a, and miR-224), previously implicated in CRC and proposed as diagnostic and prognostic markers. These miRNAs have shown promise in CRC research, with multiple studies highlighting their dysregulation and potential clinical significance.[11-16] For instance, Sun *et al.*[17] showed that miR-31 significantly contributes to the activation of the RAS signaling pathway by inhibiting the translation of RASA1. This action enhances the growth of CRC cells and promotes tumor development. MiR-92a has been found to be upregulated in CRC and associated with tumor growth, migration and invasion via suppression of PTEN.[18] MiR-29a has been associated with CRC progression and poor prognosis, acting by downregulating genes involved in apoptosis and extracellular matrix remodeling.[19] Our experiments suggest that miR-20a disrupts the homeostasis of the colonic epithelium by interfering with the regulation of Myc/p21 by transforming growth factor beta (TGF-β), a process that is crucial for malignant transformation.[20] Colorectal tumors with elevated levels of miR-21 exhibited microsatellite instability and demonstrated a diminished response to 5-fluorouracil-based chemotherapy,[21] Our investigation aims to elucidate whether this miRNA panel: (i) orchestrates the activation of common signaling pathways pivotal in CRC carcinogenesis, as gauged by mRNA and protein expression levels of genes targeted by these miRNAs, and (ii) holds potential as screening biomarkers for CRC.

2. Methods

2.1. Selection criteria

In this study, we utilized paired formalin-fixed paraffin-embedded (FFPE) cases, consisting of primary CRC tissues and corresponding normal mucosa samples obtained from 81 patients. The normal tissue specimens comprised margin blocks and tissue immediately adjacent to the tumor, exclusively sourced from mucosal tissue. All patients underwent surgical procedures at Queen's Medical Centre (QMC) in Nottingham, United Kingdom, between 2012 and 2014. Case selection criteria included the availability of comprehensive clinicopathological data and the presence of at least 50% tumor cells in the tumor block. Notably, all tissue samples analyzed in this study

originated from adenocarcinomas and were acquired through surgical interventions.

2.2. Macrodissection

To mitigate potential confounding effects from stromal cells, tumor specimens underwent macrodissection following evaluation by a pathologist to ensure a minimum of 50% tumor tissue content, as recommended in literature.[22] Two 20 μm-thick serial sections were excised from each paraffin block. After identifying tumor regions on unstained sections from hematoxylin–eosin-stained slides, macrodissection was carried out. Total RNA and miRNA were subsequently isolated using the miRNeasy FFPE kit (Qiagen, Hilden, Germany).

2.3. Quantitative reverse-transcription polymerase chain reaction (RT-qPCR)

After synthesizing cDNA with the miScript II RT Kit and the QuantiTec Reverse Transcription Kit (Qiagen, Hilden, Germany) for miRNA and mRNA, respectively, the targeted genes were quantified using the miScript SYBR Green PCR kit (Qiagen) on a 7500 Fast Real-Time PCR System (Applied Biosystems, Thermo Fisher, USA). The primer sequences and their efficiencies, determined through assay optimization, are provided in Supplementary File. The ΔΔCt method was employed for relative quantification of mRNA, comparing miRNA and mRNA expression in normal versus CRC tissues. RNU6B and HPRT were used as reference genes for miRNA and mRNA, respectively.[23,24]

2.4. Evaluation of protein expression

2.4.1. Immunohistochemistry

2.4.1.1. TMA

After validating antibody specificity and determining optimal concentrations, we assessed protein expression in CRC tissue using Tissue Microarray (TMA) sections. Western blotting analysis confirmed the specificity of the antibodies (Supplementary file Figure S1). TMAs facilitate high-throughput evaluation of biomarker expression across numerous tissue samples, comprising paraffin blocks with minute tissue specimens arranged in an array configuration. The antibodies employed are listed in Supplementary File. TMA sections were prepared at the Nottingham Health Science Biobank, QMC, Nottingham, UK. We stained 4-μm paraffin-embedded CRC TMA sections using Novolink Polymer Detection Systems (Leica Microsystems, Germany) with anti-SMAD4, anti-KLF4, anti-RASA1, anti-PTEN, anti-TGFBRII, and anti-BCL2 antibodies. Each run included positive and negative controls to validate experimental success. Detailed immunohistochemistry staining procedures are provided in Supplementary File.

2.4.1.2. Assessment of protein expression

Following initial validation under a light microscope, TMA slides were scanned at 20× magnification using a Nanozoomer Digital Pathology scanner (Hamamatsu Photonics, Japan). Protein expression in tumor cells was assessed semi-quantitatively using the H-score method, which combines the percentage of positive tumor cells with staining intensity (0 for negative, 1 for weak, 2 for moderate, and 3 for strong staining).[25] To ensure consistency, all slides underwent independent evaluation by a second scorer, and the intraclass correlation coefficient (ICC) was employed to assess inter-scorer agreement.

2.5. Statistical analysis

Statistical analysis was conducted using the SPSS version 22 software package (IBM acquired SPSS Inc. USA). Categorical data were assessed for statistical significance using the Chi-square test, while continuous data were analyzed for differences between datasets using the Wilcoxon test. Fisher's exact test was employed to explore associations between unpaired tumor groups. Spearman's correlation was used to detect correlations between targets. For multiple corrections testing, the Bonferroni step-down (Holm) correction was applied. In both statistical analyses, $P < 0.05$ was considered statistically significant. In this study, we used the Wilcoxon test, a non-parametric method robust to outliers and suitable for non-normally distributed data. Outliers, identified as data points outside the 10th and 90th percentiles in Figure 1, were retained in the analysis without additional transformation or removal. This approach provides an accurate representation of the dataset, accommodating natural data variation without the influence of strict distributional assumptions.

3. Results

3.1. MiRNA quantification

3.1.1. Cutoff point for miRNAs detection

Before analyzing mRNA expression levels, we sought to establish a cutoff point to distinguish high and low expression levels. Initially, RNA was extracted from 20 individual pure normal colon tissues and pooled with equal volumes. Subsequently, the expression levels of all miRNAs and mRNAs were assessed in each normal colon tissue sample compared to the pooled sample. On average, the minimum fold expression for all mRNAs in normal colon tissues was 0.6, while the maximum was 1.8. Downregulation was defined as <0.6 fold, and upregulation as >1.8 fold. This method provided a robust benchmark for distinguishing between high and low mRNA expression in subsequent analyses. For the miRNAs, the average minimum fold change in expression for all miRNAs in

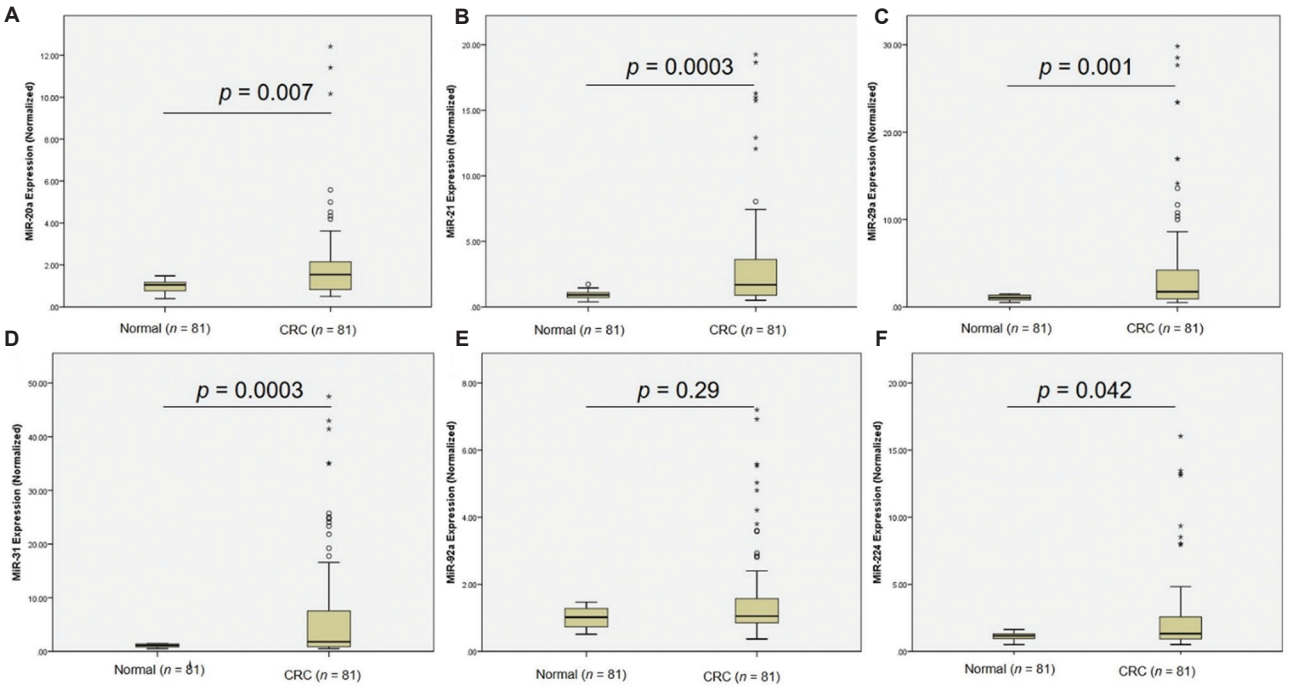


Figure 1. miRNA selection and validation by quantitative reverse-transcription polymerase chain reaction analysis. Box plots of plasma levels of (A) miR-20a, (B) miR-21 (C) miR29a, (D) miR-31, (E) miR-92a and (F) miR-224 in healthy normal subjects (n = 81) and patients with colorectal cancer (n = 81). Expression levels of the miRNAs (log10 scale at y-axis) are normalized to *RNU6B*. The lines inside the boxes denote the medians. The boxes mark the interval between the 25th and 75th percentiles. The whiskers denote the interval between the 10th and 90th percentiles. Filled circles indicate data points outside the 10th and 90th percentiles. Statistically significant differences were determined using Wilcoxon tests. Open circle and asterisks represent data points that are outside the 10th and 90th percentiles. These are often referred to as outliers, indicating individual measurements that fall significantly outside the typical range for the respective groups.
Abbreviation: miRNA: MicroRNA.

normal colon tissue was 0.5, while the maximum was 1.5 (with values below 0.5 indicating downregulation and those above 1.5 indicating upregulation).

3.1.2. miRNA quantification by real-time RT-qPCR

To measure the expression of miRNAs including (miR-20a, 21, 29a, 31, 92a and 224), the study screened miRNA levels in 81 CRC samples and matched normal mucosa through RT-qPCR assay, normalized to *RNU6B*. All assays were done in triplicate and the cycle threshold (Ct) value of all targets in all samples were <27 (range 16.1 – 26.8) with standard deviation (SD) < 0.5 between replicates Ct value. The miRNAs with significantly different expression in the CRC samples compared with the normal mucosa were identified by Wilcoxon test (because data are not normally distributed), with an expression fold >1.5. Among the studied miRNAs, four exhibited notably higher expression levels in CRC samples compared to normal mucosa (Figure 1): miR-31 demonstrated an average 10.83-fold higher expression in CRC than in adjacent normal colon tissue (fold range: 0.52 – 161.69, P = 0.0003); miR-29a showed an average 8.11-fold higher expression in CRC compared to adjacent normal colon tissue (fold range: 0.52 – 108.36, P = 0.001); miR-21 displayed a 6.42-fold higher expression in CRC than in normal tissue (fold range: 0.5 – 63.84, P = 0.0003); and miR-20a exhibited a 3.27-fold higher expression in CRC than in normal tissue (fold range: 0.53 – 109.16, P = 0.007). In addition, miR-92a showed a 2.2-fold higher expression in CRC than in normal tissue (fold range: 0.37 – 34.8, P = 0.2), and miR-224 exhibited a 2.68-fold higher expression in CRC than in normal tissue (fold range: 0.51 – 19.35, P = 0.042). However, after applying the Bonferroni correction for multiple testing, miR-224 lost statistical significance (P = 0.22), while significance persisted for miR-20a (P = 0.04), miR-21 (P = 0.001), miR-29a (P = 0.006), and miR-31 (P = 0.001) (Table 1).

3.1.3. Association of the expression of biomarkers and clinicopathological variables

Pearson's Chi-square test was applied to identify association between miRNAs and clinicopathological features, and the results showed that normal miRNA92a expression was associated with grade two (χ^2 = 7.037, d.f. = 2, P = 0.03). High miRNA21 expression was associated with Duke's B stage (χ^2 = 6.115, d.f. = 2, P = 0.04). However,

Table 1. Expression profiles of candidate microRNAs

Gene	Fold of change	*P*	*P*-value of Bonferroni correction test
miRNA20a	3.27	0.007	0.04
miRNA21	6.42	0.003	0.001
miRNA29a	8.11	0.001	0.006
miRNA31	10.83	0.003	0.001
miRNA92a	2.2	0.29	0.87
miRNA224	2.68	0.042	0.22

following multiple correction testing using the Bonferroni correction, they both failed to retain significance ($P = 0.28$ and $P = 0.36$, respectively) (Table 2).

3.2. mRNA quantification by real-time RT-qPCR

Significant differences in mRNA expression between CRC samples and normal mucosa were identified through a paired *t*-test. Downregulation was defined as <0.6 fold, while upregulation was set at >1.8 fold. All assays were meticulously conducted in triplicate, and replicates with a Ct standard deviation >0.5 were excluded to ensure data integrity. Among the six examined targets, *RASA1* ($P = 0.002$, fold change of 0.66 ± SD 0.74, 95% confidence interval [CI] 0.5 – 0.82) and *TGFBRII* ($P = 0.0001$, fold change of 0.46 ± SD 0.78, 95% CI 0.29 – 0.63) exhibited significantly lower expression levels in tumor samples compared to normal tissues. Conversely, *BCL2* showed a significantly higher expression level ($P = 0.05$, fold change of 3.72 ± SD 8.2, 95% CI 1.93 – 5.53). For *SMAD4* ($P = 0.12$, fold change of 0.98 ± SD 0.92, 95% CI 0.78 – 1.18), *PTEN* ($P = 0.22$, fold change of 0.97 ± SD 0.91, 95% CI 0.77 – 1.17), and *KLF4* ($P = 0.8$, fold change of 1.16 ± SD 1.6, 95% CI 0.81 – 1.51), mRNA expression levels were relatively similar in tumor and normal tissues. However, after meticulous multiple correction testing using the Bonferroni correction, *RASA1* and *TGFBRII* retained significance ($P = 0.01$ and $P = 0.006$, respectively), highlighting their robust association with CRC. Conversely, *BCL2* failed to maintain statistical significance ($P = 0.2$) (Table 3).

3.3. Protein evaluation

3.3.1. Optimization of primary antibodies for immunohistochemical staining

Before conducting immunohistochemical staining on CRC tissues, we validated the specificity of antibodies against SMAD4, TGFBRII, RASA1, and KLF4 through Western blotting. These antibodies target proteins with molecular weights of 65 kDa (SMAD4), 75 kDa (TGFBRII), 140 kDa (RASA1), and 55 kDa (KLF4). Western blotting confirmed the presence of the

Table 2. Association between microRNAs expression and clinicopathological variables

Variable	miRNA expression		*P*	*P*-value of Bonferroni correction test
	Normal (%)	High (%)		
miR-20a				
Tumor grade				
Well	1 (2.6)	1 (2.3)	0.8	0.2
Good	34 (89.5)	40 (93.0)		
Poor	3 (7.9)	2 (4.7)		
Nodal state				
pN 0	21 (55.3)	29 (67.4)	0.3	0.7
pN I	12 (31.6)	12 (27.9)		
pN II	5 (13.2)	2 (4.7)		
Dukes' stage				
A	5 (13.2)	7 (16.3)	0.5	0.9
B	16 (42.1)	22 (51.2)		
C	17 (44.7)	14 (32.6)		
EMVI				
0	15 (39.5)	26 (60.5)	0.1	0.3
1	22 (57.9)	16 (37.2)		
2	1 (2.6)	1 (2.3)		
miR-21				
Tumor grade				
Well	2 (6.3)	0 (0.0)	0.1	0.3
Good	29 (90.6)	45 (91.8)		
Poor	1 (3.1)	4 (8.2)		
Nodal state				
pN 0	15 (46.9)	35 (71.4)	0.08	0.2
pN I	13 (40.6)	11 (22.4)		
pN II	4 (12.5)	3 (6.1)		
Dukes' stage				
A	2 (6.3)	10 (20.4)	0.04	0.1
B	13 (40.6)	25 (51.0)		
C	17 (53.1)	14 (28.6)		
EMVI				
0	12 (37.5)	29 (59.2)	0.05	0.1
1	20 (62.5)	18 (36.7)		
2	0 (0.0)	2 (4.1)		
miR-29a				
Tumor grade				
Well	1 (2.8)	1 (2.2)	0.9	0.9
Good	33 (91.7)	41 (91.1)		
Poor	2 (5.6)	3 (6.7)		

(Cont'd...)

Table 2. *(Continued)*

Variable	miRNA expression		*P*	*P*-value of Bonferroni correction test
	Normal (%)	High (%)		
Nodal state				
pN 0	22 (61.1)	28 (62.2)	0.6	0.9
pN I	12 (33.3)	12 (26.7)		
pN II	2 (5.6)	5 (11.1)		
Dukes' stage				
A	4 (11.1)	8 (17.8)	0.6	0.9
B	18 (50.0)	20 (44.4)		
C	14 (38.9)	17 (37.8)		
EMVI				
0	19 (52.8)	22 (48.9)	0.4	0.8
1	17 (47.2)	21 (46.7)		
2	0 (0.0)	2 (4.4)		
miR-31				
Tumor grade				
Well	1 (2.9)	1 (2.1)	0.1	0.3
Good	29 (85.3)	45 (95.7)		
Poor	4 (11.8)	1 (2.1)		
Nodal state				
pN 0	22 (64.7)	28 (59.6)	0.1	0.3
pN I	7 (20.6)	17 (36.2)		
pN II	5 (14.7	2 (4.3)		
Dukes' stage				
A	2 (5.9)	10 (21.3)	0.1	0.3
B	19 (55.9)	19 (40.4)		
C	13 (38.2)	18 (38.3)		
EMVI				
0	14 (41.2)	27 (57.4)	0.3	0.7
1	19 (55.9)	19 (40.4)		
2	1 (2.9)	1 (2.1)		
miR-92a				
Tumor grade				
Well	1 (1.8)	1 (4.2)	0.03	0.2
Good	55 (96.5)	19 (79.2)		
Poor	1 (1.8)	4 (16.7)		
Nodal state				
pN 0	38 (66.7)	12 (50.0)	0.2	0.6
pN I	14 (24.6)	10 (41.7)		
pN II	5 (8.8)	2 (8.3)		
Dukes' stage				
A	9 (15.8)	3 (12.5)	0.3	0.7
B	29 (50.9)	9 (37.5)		
C	19 (33.3)	12 (50.0)		
EMVI				
0	31 (54.4)	10 (41.7)	0.3	0.7
1	24 (42.1)	14 (58.3)		
2	2 (3.5)	0 (0.0)		
miR-224				
Tumor grade				
Well	1 (2.3)	1 (2.7)	0.4	0.8
Good	39 (88.6)	35 (94.6)		
Poor	4 (9.1)	1 (2.7)		
Nodal state				
pN 0	29 (65.9)	21 (56.8)	0.6	0.9
pN I	11 (25.0)	13 (35.1)		
pN II	4 (9.1)	3 (8.1)		
Dukes' stage				
A	6 (13.6)	6 (16.2)	0.5	0.9
B	23 (52.3)	15 (40.5)		
C	15 (34.1)	16 (43.2)		
EMVI				
0	23 (52.3)	18 (48.6)	0.9	0.9
1	20 (45.5)	18 (48.6)		
2	1 (2.3)	1 (2.7)		

Abbreviation: EMVI: Extramural vascular invasion.

expected bands for each antibody in specific cell lysates: SMAD4 in SW480, RASA1 in HT29 and Lovo, KLF4 in HT29, and TGFBRII in HT29 and SW480 (Figure S1 in Supplementary File). These results validated the specificity of the antibodies for staining target proteins within CRC TMAs via immunohistochemistry. Other antibodies used in our study, sourced from our group and the histopathological department at Nottingham QMC, underwent similar validation procedures. In addition, we optimized the concentration of each antibody for immunohistochemical staining. For example, anti-SMAD4 at 1:100 exhibited optimal staining without background, while concentrations of 1:50 and 1:200 were either too high or too low for detection. Similarly, optimal concentrations were determined for TGFBRII (1:400), RASA1 (1:40), and KLF4 (1:100). Staining for BCL2 and PTEN was performed separately by the histopathology department at QMC. Further details on the staining optimization process can be found in Table 1 and Figure S2 (in Supplementary file).

3.3.2. Protein expression of target genes

In a primary operable invasive CRC patient cohort, we conducted an evaluation to delve deeper into the expression of miRNA target genes, including SMAD4, PTEN, TGFBRII, BCL2, KLF4, and RASA1. Staining patterns for all markers displayed heterogeneity both within and between tumor cores, ranging from weak to intense staining (Figure 2). Three cores per case were stained, and the average scores of these cores were used for subsequent analysis. The H-score, representing staining intensity, was determined for each marker. SMAD4 exhibited a median H-score of 85 (range: 0 – 300), PTEN had a median H-score of 95 (range: 0 – 300), TGFBRII had a median H-score of 70 (range: 0 – 225), BCL2 had a median H-score of 125 (range: 0 – 300), KLF4 had a median H-score of 90 (range: 0 – 300), and RASA1 had a median H-score of 65 (range: 0 – 200).

Table 3. Expression profiles of candidate target genes of microRNAs

Gene	Fold of change of tumor tissue versus normal tissue	*P*	*P*-value of Bonferroni multiple correction
SMAD4	0.98	0.12	
PTEN	0.97	0.22	
BCL2	3.72	0.05	0.2
TGFBRII	0.46	0.001	0.006
KLF4	1.18	0.8	
RASA1	0.66	0.002	0.01

As previously mentioned, excellent concordance between scorers was observed, as indicated by the single-measure ICC for SMAD4, PTEN, TGFBRII, BCL2, KLF4, and RASA1, which ranged from 0.71 to 0.82. Specimens were categorized into low and high expression groups based on mean scores. For instance, 63% of CRC cases exhibited low expression of SMAD4, while 37% displayed high expression relative to normal mucosa. Similarly, 83% of CRC cases demonstrated low expression of PTEN, with 17% characterized by high expression. For TGFBRII, 80% of CRC cases were categorized as low expression, while 20% showed high expression compared to normal mucosa. In the case of BCL2, 58% of CRC specimens had low expression, whereas 42% exhibited high expression. For KLF4, 44% of CRC cases exhibited low expression, while 56% displayed high expression. Lastly, 75% of CRC cases demonstrated low expression of RASA1, and 25% exhibited high expression compared to normal mucosa. Chi-square test was conducted to assess the correlation between mRNA and protein level of the markers used in this study. Results demonstrated a significant correlation between mRNA and protein levels of SMAD4 (r = 0.466,

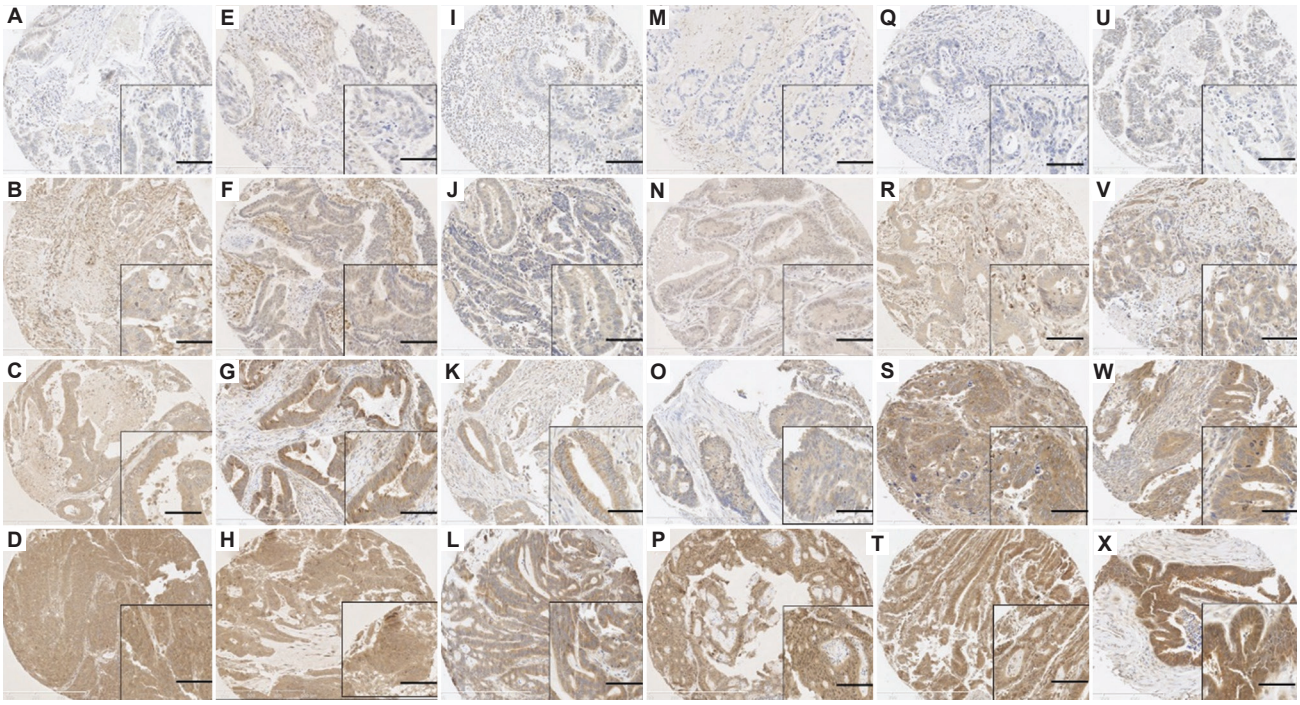


Figure 2. Representative photomicrographs of various staining intensity of markers in colorectal tissue: (A-D) SMAD4, (E-H) PTEN, (I-L) BCL2, (M-P) TGFBRII, (Q-T) KLF4, and (U-X) RASA1. Photomicrographs were viewed at ×100 magnification, whereas images at the inset boxes were at ×200 magnification; scale bar = 100 μm.

$P < 0.0001$), TGFBRII I (r = 0.708, $P < 0.0001$), BCL2 (r = 0.623, $P < 0.0001$), and RASA1 (r = 0.728, $P < 0.0001$). No correlation was observed between mRNA and protein levels of PTEN (r = −0.085, $P = 0.450$), and KLF4 (r = 0.114, $P = 0.313$).

3.4. Association the expression of biomarkers (target genes) and miRNAs

Spearman's rank-order correlation was performed to evaluate the relationship between the markers examined in this study. High expression levels of miR-21 and miR-224 were found to be associated with lower levels of *TGFBRII* mRNA (r = −0.358, $P = 0.001$; and r = −0.276, $P = 0.01$, respectively) as well as TGFBRII protein (r = −0.328, $P = 0.003$; and r = −0.319, $P = 0.004$, respectively). Furthermore, increased expression of both miR-29a and miR-31 showed an inverse correlation with *RASA1* mRNA levels (r = −0.217, $P = 0.01$; and r = −0.276, $P = 0.01$, respectively) and RASA1 protein levels (r = −0.222, $P = 0.004$; and r = −0.209, $P = 0.01$, respectively) (Table 4). A significant correlation was also observed between miR-20a and miR-29 (r = 0.380, $P = 0.0001$) as well as miR-31 (r = 0.403, $P = 0.0001$). In addition, a notable correlation was found between miR-21 and miR-29a (r = 0.526, $P = 0.0001$) and miR-31 (r = 0.285, $P = 0.01$). Moreover, a strong correlation was noted between miR-29a and miR-31 (r = 0.275, $P = 0.01$) and miR-92a (r = 0.324, $P = 0.003$). Significant correlations were also observed between miR-31 and miR-224 (r = 0.328, $P = 0.003$) and between miR-92a and miR-224 (r = 0.382, $P = 0.0001$) (Table 5).

Table 4. Association between mRNAs and target genes (mRNA and protein)

Variable	*TGFBRII* mRNA	TGFBRII protein	*RASA1* mRNA	RASA1 protein
miR-21 CC	−0.358	−0.328		
P	0.001	0.003		
miR-29a CC			−0.217	−0.222
P			0.01	0.004
miR-31 CC			−0.276	−0.209
P			0.01	0.01
miR-224 CC	−0.276	−0.319		
P	0.01	0.004		

Abbreviation: CC: Correlation coefficient; mRNA: microRNAs.

Table 5. Association between microRNAs

Variables	miR-29a	miR-31	miR-92a	miR-224
miR-20a CC	0.380	0.403		
P	0.0001	0.0001		
miR-21 CC	0.526	0.285		
P	0.0001	0.01		
miR-29a CC		0.275	0.324	
P		0.01	0.003	
miR-31 CC				0.328
P				0.003
miR-92a CC				0.382
P				0.0001

Abbreviation: CC: Correlation coefficient.

4. Discussion

CRC remains a significant global health burden, with substantial morbidity and mortality rates worldwide. Unraveling the intricate molecular mechanisms underlying CRC pathogenesis is crucial for advancing our understanding of the disease and identifying novel therapeutic targets and diagnostic biomarkers. In this comprehensive study, we employed a multidimensional approach, integrating miRNA quantification, mRNA profiling, and protein evaluation, to evaluate their potential as biomarkers for CRC detection.

The dysregulation of miRNAs, small non-coding RNAs that modulate gene expression, is a hallmark of CRC tumorigenesis.[26,27] Our study revealed abnormal expression patterns of several miRNAs in CRC tissues compared to adjacent normal mucosa. Notably, miR-31, miR-29a, miR-21, and miR-20a were significantly upregulated in CRC samples, indicating their roles as oncogenic drivers in CRC progression.[28-30] These findings support the growing evidence implicating these miRNAs in CRC pathogenesis and highlight their potential as diagnostic and prognostic biomarkers.

Despite the relatively small sample size, we explored clinical associations, though no significant correlations between miRNA expression and clinicopathological features were identified. This is contrary to findings by others, such as Schepeler *et al.*[31] who reported increased miR-31 levels in stage IV tumors, and Zhou *et al.*[32] who observed a correlation between miR-92a expression and advanced clinical stage.

In addition, our study identified potential mRNA targets of dysregulated miRNAs in CRC. Through mRNA quantification analysis, we observed differential expression patterns of genes involved in critical signaling pathways dysregulated in CRC. Specifically, *RASA1* and *TGFBRII*, tumor suppressor genes in Ras and TGF-β pathways, respectively, were significantly downregulated in CRC tissues.[33,34] Conversely, *BCL2*, an anti-apoptotic gene, showed upregulation in CRC samples, consistent with its role in promoting cell survival and chemoresistance.[35]

The immunohistochemical evaluation of target protein expression provided further insights into the molecular alterations within CRC tissues. The observed heterogeneity in protein expression patterns underscores the dynamic

nature of CRC tumors and the diverse cellular phenotypes within the tumor microenvironment. Importantly, the correlations between mRNA and protein levels for selected markers, including SMAD4, TGFBRII, BCL2, and RASA1, highlight the transcriptional regulation of protein expression in CRC.[17,36] These findings underscore the importance of integrating multiple omics approaches to comprehensively characterize the molecular landscape of CRC.

Although this study did not include functional validation experiments, the high correlation between miRNAs and their target genes suggests that the selected miRNAs could play roles in CRC carcinogenesis by regulating key signaling pathways. For example, the inverse correlations between miR-21, miR-224, and TGFBRII highlight their role in suppressing TGFBRII expression, thus potentiating TGF-β signaling dysregulation in CRC.[37] Similarly, the inverse associations between miR-29a, miR-31, and RASA1 underscore their regulatory role in modulating RASA1 expression, consequently influencing Ras signaling pathway activation in CRC.[14,38] In addition, the lack of correlation between mRNA and protein levels of PTEN, coupled with the strong correlation between miR-20a and reduced PTEN protein expression, suggests that miR-20a may regulate PTEN at the post-transcriptional level. These findings suggest that these miRNAs may play a role in CRC tumorigenesis by modulating key tumor suppressor genes, although further mechanistic studies are required to confirm these interactions.

This study has several strengths, such as the use of matched tumor and normal mucosa tissues, which minimizes the influence of non-tumorous miRNAs. However, there are also limitations, including the relatively small sample size, the lack of validation using alternative methods, and the absence of mutation screening.

5. Conclusion

Our findings suggest that upregulation of miR-20a, miR-21, miR-29a, and miR-31 may contribute to CRC progression by targeting genes involved in key signaling pathways. While these miRNAs show potential as diagnostic biomarkers, further research is needed to validate their clinical utility and explore their roles in CRC pathways. Future studies should focus on investigating the interactions between miRNAs, gene mutations, and other CRC-related pathways to better understand the molecular mechanisms driving CRC.

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Conflict of interest

The authors declare that they have no competing interests.

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Ethics approval and consent to participate

Access to tissues and ethics approval were granted by Nottingham Health Sciences Biobank, which has approval as an IRB from North West, Greater Manchester Central Research Ethics Committee (REC reference: 15/NW/0685).

Consent for publication

No patient consent was needed.

Availability of data

All datasets on which the conclusions of this paper rely have been presented in the main manuscript and in the supplementary file. In addition, raw data can be accessed on request.

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