Study of microRNA expression profiling as biomarkers for colorectal cancer patients in Lebanon

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Abstract. The high incidence and mortality rates of colorectal cancer (CRC) reveal its hazardous effect globally. Thus, it is important to diagnose CRC at an early stage to decrease its burden and improve survival rates. Previous studies have investigated the role of short non-coding microRNAs (miRNAs or miRs) in numerous types of cancer, including CRC. Previous studies have been performed to investigate the role of miRNAs as biomarkers in diagnosis, prognosis and prediction of CRC development. The aim of the present retrospective study was to identify the expression levels of miR-31, miR-145, miR-146b and miR-186 to highlight their role in CRC diagnosis and progression at different stages of the disease (precancerous polyp, adenoma and adenocarcinoma) in a Lebanese population. The expression levels of miRNAs was revealed using TaqMan reverse transcription-quantitative PCR on formalin-fixed paraffin-embedded tissues from Lebanese patients at different stages; their diagnostic value was determined using a receiver operating characteristics curve. Compared with healthy controls, miR-31 was upregulated ($P<0.0001$) at all stages. By contrast, miR-145, miR-186, and miR-146b were significantly downregulated at all stages ($P<0.0001$, $P=0.0009$ and $P=0.0241$, respectively). Of the four miRNAs studied, miR-31 and miR-145 were identified as potentially useful diagnostic factors, with an area under the curve of 0.7771 and 0.8269 and diagnostic accuracy of 71.3 and 78.5%, respectively. These data suggested that miR-31 and miR-145, upon further clinical validation, may be used as potential diagnostic biomarkers for the early detection of CRC at the polyp stage.

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Introduction

Worldwide, colorectal cancer (CRC) is considered one of the most common malignancies and a significant threat to health (1,2). In 2020, CRC was ranked the fourth most common type of cancer and the third most common cause of cancer deaths worldwide with an incidence rate of 24.8 and a mortality rate of 12 cases per 100,000, according to the World Health Organization (3,4). In Asia, the incidence rate is increasing, making CRC a notable health burden (5). In the Lebanese population, CRC ranked fourth among men for the most common types of cancer with an expected incidence rate of 17.5 cases per 100,000 and ranked second among women with an expected incidence rate of 10.6 cases per 100,000 in 2020, making it one of the highest reported types of cancer in the country (6).

CRC is initially characterized by polyps (Ps), which are classified according to their growth pattern as adenomatous or serrated. Ps progress to the adenoma (Ad) stage within 10-20 years, followed by invasive adenocarcinoma (ADC). Since there is a slow progression from the precancerous P to invasive ADC stage, early detection can decrease the mortality rate and increase the chances of recovery and survival (7,8). A study demonstrated that early stage diagnosis in patients with CRC led to a decrease in the CRC mortality rate in Australia (9). Furthermore, in the United Kingdom, diagnosis at early stages has been shown to increase the survival rate compared with diagnosis at later stages (9).

A number of screening tools have been utilized for early detection of CRC; these can be invasive and non-invasive. Usually, CRC is identified via endoscopic imaging and histopathological examination of removed specimens (biopsies) (10). Colonoscopy is an invasive visual screening tool used to visualize and detect Ps. Other screening tools include fecal occult blood and immunochemical tests, which detect blood in the stool (8,11,12). However, these screening tools have limitations in terms of sensitivity, specificity, cost and degree of

invasiveness. Therefore, the development of novel diagnostic biomarkers is crucial for early diagnosis of CRC (13,14).

CRC is considered to be a multifactorial disease that is characterized by a sequence of genetic and epigenetic alterations in the different stages. These alterations are classified into three classes: Chromosomal and microsatellite instability and CpG island methylation phenotype (13,15). In addition, epigenetic regulators, including long non-coding RNAs and microRNAs (miRNAs or miRs), have been implicated in different types of cancer, including CRC (15). miRNAs, short non-coding RNAs composed of 18-24 nucleotides, are known as gene regulatory molecules for their ability to suppress translational activity by using other miRNAs as cleavage targets, thereby altering the function of protein-coding genes (13,16). miRNAs can be characterized as oncogenic or tumor suppressor miRNAs. Oncogenic miRNAs are upregulated in cancer and mediate downregulation of tumor suppressor genes. By contrast, tumor suppressor miRNAs are downregulated in cancer, which is accompanied by upregulation of oncogenes (17,18). These oncogenes are highly expressed and contribute to cancer development by increasing cellular proliferation and tumor progression (19).

Furthermore, there is an increasing evidence that certain miRNAs are dysregulated in cancer (17,18). Studies have revealed that numerous miRNAs (including miR-21, miR-17-92 cluster, miR-143 and miR-145) are deregulated in patients with CRC and serve an important role in its initiation, development, and progression (20-22). One of the most commonly upregulated miRNAs in CRC is miR-21, which plays an essential role in tumor progression (23).

The role of miR-31, miR-146b, miR-145 and miR-186 have been studied in different types of cancer. Several studies have reported the oncogenic role of miR-31 and miR-146b in different populations and types of cancer, including CRC (24-27). Accordingly, miR-31 has been shown to be upregulated in CRC and is associated with tumor progression, as well as survival rate. However, miR-145 and miR-186 are downregulated in colorectal cancer, which is accompanied by activation of oncogenic mRNAs, such as twist family bHLH transcription factor 1, Fascin Actin-Bundling Protein 1 and Zinc Finger E-Box Binding Homeobox 1 (28-30).

The aim of the present retrospective study was to investigate the expression levels of miRNAs (miR-146b, miR-31, miR-186 and miR-145) in 222 formalin-fixed paraffin-embedded (FFPE) colorectal tissue samples taken from Lebanese patients with different precancerous (P) and cancer stages (Ad and ADC) and to identify miRNAs that may be used for diagnosis, particularly at P stage.

Materials and methods

Colon tissue specimens. The present study was approved by the Medical Committee at Bahman Hospital (Beirut, Lebanon; approval no. 27). Retrospective FFPE specimens from 222 patients with CRC at different stages [P (n=68), Ad (n=78), ADC (n=76)], as well as specimens of normal healthy controls (N; n=81) were obtained from the Anatomy and Pathology Department, Bahman Hospital, Haret Hreik, Lebanon from patients that underwent colonoscopy and resection between 2011 and 2019, excluding patients with hereditary disease.

Total RNA extraction. Total RNA, including small RNA fraction, was extracted by processing four 20 μm thick ribbons from each FFPE tissue sample using a RecoverAll Total Nucleic Acid Isolation kit (Ambion; Thermo Fisher Scientific, Inc.; cat. no. AM1975) according to the manufacturer's instructions. Briefly, deparaffinization of FFPE tissue was performed using xylene at 50˚C followed by washing with ethanol twice to remove xylene. Proteins were digested by incubating the samples with protease enzyme for 15 min at 50˚C and 15 min at 80˚C. Total RNA was isolated in glass-fiber filter cartridges using an isolation additive mixture and washing with high ethanol-wash buffers. DNA digestion was then performed using DNase and RNA was purified by washing and elution using reagents provided in the kit. RNA concentration and quality were detected using a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, Inc.) by measuring the optical density at 260 and 280 nm, then samples were stored at -80˚C. Only samples of high quality and integrity (A260/A280 ratio of 1.8-2.1) were used for subsequent experiments.

miRNA reverse transcription (RT) and expression level analysis. A total of 10 ng total RNA was reverse transcribed using TaqMan® MicroRNA RT kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's recommendations. The hsa-miR-146b (cat. no. 001097), hsa-miR-186 (cat. no. 002285), hsa-miR-145 (cat. no. 002278), hsa-miR-31 (cat. no. 002279), small nucleolar RNA RNU48 (cat. no. 001006) primers and probes were used as part of the TaqMan Small RNA Assays™ kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). RT was performed in a multiplex reaction setup using two miRNA primers with an endogenous control (for example, miR-31 and miR-145 primers were used together with the endogenous control RNU48).

miRNA expression was assessed by RT-quantitative (q)PCR using Bio-Rad CFX96 Real-Time System, C1000 Thermal Cycler (Bio-Rad Laboratories, Inc.). Reactions were performed in duplicate for each miRNA probe using 5.0 TaqMan® Universal Master Mix without Amperase Uracil N-glycosylase (Applied Biosystems; Thermo Fisher Scientific, Inc.; 4324018), 0.5 20X microRNA probe, 1.0 diethyl pyrocarbonate-treated water and 3.5 μl prepared cDNA. The following probes were used: hsa-miR-31 (cat. no. 002279), hsa-miR-145 (cat. no. 002278), hsa-miR-186 (cat. no. 002285), hsa-miR-146b (cat. no. 001097), RNU48 (cat. no. 001006; all Applied Biosystems). Each plate contained the samples, no template control, no RT control and healthy tissue samples. The thermocycling conditions were 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and annealing at 60˚C for 60 sec. Only samples with average miRNA Cq<35 or endogenous control Cq of 22-29 were included. The relative expression of target miRNAs at the different stages (P, Ad and ADC) was then normalized to RNU48 and compared with N using the $2^{-\Delta\Delta Cq}$ method (31). Normalization of tumor tissues was based on N present on the RT-qPCR plate to ensure inter-run calibration.

Statistical analysis. Statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc; version 8) and SPSS version 25 software (IBM, Corp.). Patient demographics are presented as mean ± standard deviation (SD) for continuous variables and as numbers or percentages for categorical variables.

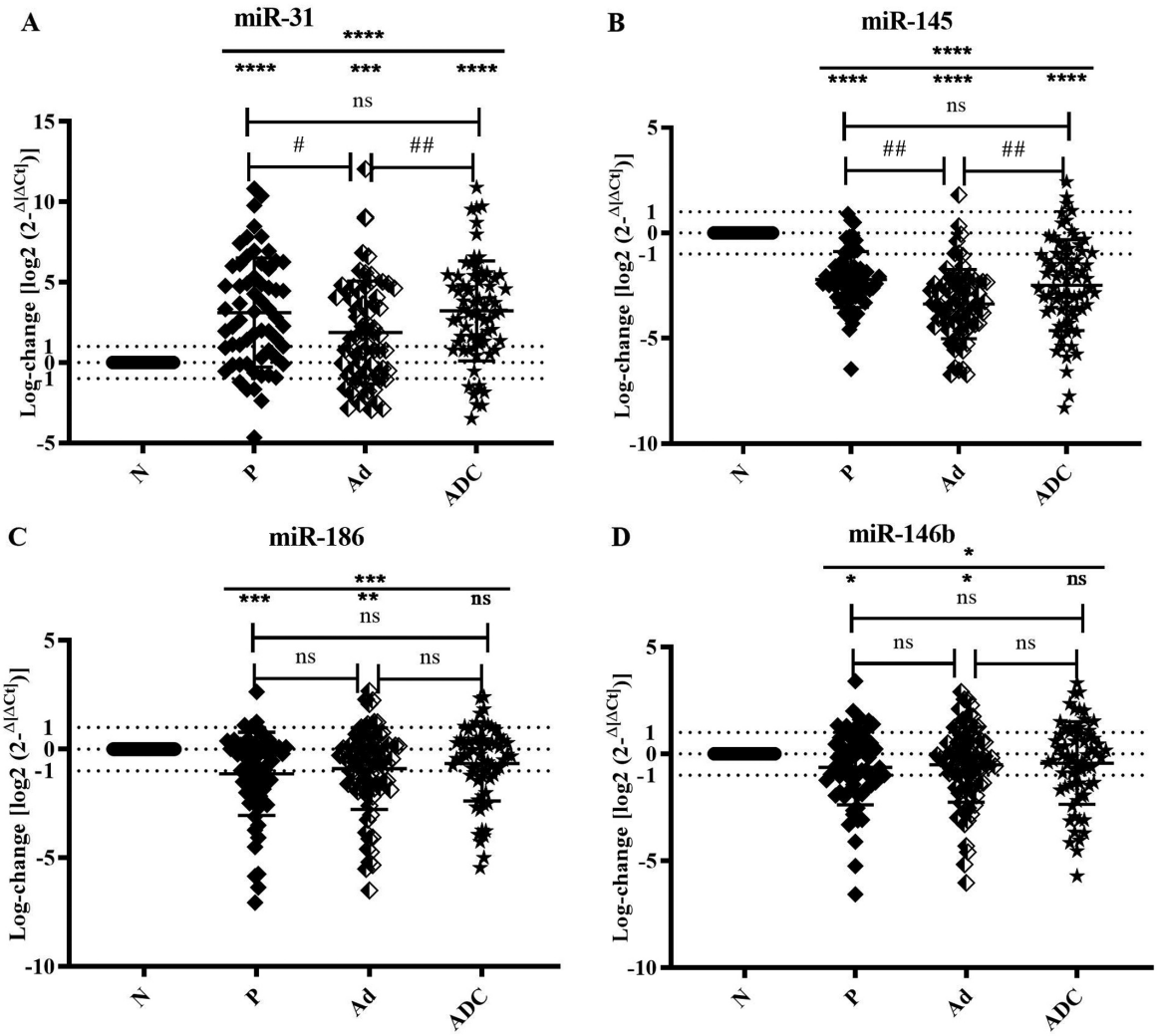

Figure 1. Dysregulation of miR-31, 145, 186 and 146b expression at different stages. Scatter plots show expression profiles of (A) miR-31, (B) miR-145, (C) miR-186 and (D) miR-146b, represented by log-changes at all stages relative to the average ΔCq value of healthy controls (measured by reverse transcription-quantitative PCR with RNU48 as an endogenous control). Data are presented as the mean ± SEM. Data were analyzed by Kruskal-Wallis (*P<0.05, **P<0.01, ***P<0.001 and ****P<0.001) and Dunn's test ($^{\#}$P<0.05 and $^{\#\#}$P<0.01). miR, microRNA; N, normal; P, polyp; Ad, adenoma; ADC, adenocarcinoma; ns, not significant.

Due to the lack of normality, Kruskal-Wallis test was performed to compare expression levels each miRNA at P, Ad, and ADC stages with healthy controls, followed by Dunn's test to perform pairwise comparison. The diagnostic value of miRNAs at P stage was detected by receiver operating characteristic (ROC) curve analysis using the ΔCq values of the samples and the predicted probability was calculated using binary logistic regression. The ROC curve was plotted to indicate the area under the curve (AUC) and P-value, as well as to calculate the positive predictive and negative predictive values and diagnostic accuracy between P and N to determine the diagnostic value of miR-31, miR-145, miR-186 and miR-146b. Youden's index was calculated in addition to the cut-off value, sensitivity and specificity of miRNAs. P<0.05 was considered to indicate a statistically significant difference. Independent set of 20 samples was also used to confirm the diagnostic accuracy of miR-31 and miR-145.

Results

Clinicopathological characteristics of patients. The features of 303 biopsies are presented in Table I, including 222 patients in the precancerous and cancerous stages with a mean age of 60.15±15.13, in addition to 81 N samples with a mean age of 57.1±18.66 years. The male to female ratio was 1.09 for patients and 0.7 for N samples. P and Ad samples were all non-malignant (hyperplastic Ps and low-grade Ads). In addition, 93.4% of ADC samples were grade 2 (moderately differentiated). A total of 0.9% had Crohn's disease while 0.45% had a family history of CRC (data not shown).

Overexpression of miR-31 at different stages of CRC. The mean expression of miR-31 across all stages was significantly upregulated (P<0.0001). miR-31 was significantly increased in the P, Ad and ADC stages compared with N (P<0.0001, P=0.0007 and P<0.0001, respectively; Fig. 1A; Table II).

In addition, miR-31 was significantly downregulated in Ad compared with P stage (P=0.0244) but significantly upregulated in ADC compared with Ad (P=0.0023). However, there was no significant change in its expression at ADC compared with P stage (P=0.4816) Therefore, miR-31 expression increased significantly at all stages with respect to healthy controls and at P and ADC stages compared with Ad (Fig. 1A).

Table I. Clinicopathological characteristics of patients.

Characteristic	Healthy control	Polyp	Adenoma	Adenocarcinoma	Precancerous and cancerous stages
Sample, n (%)	81.00 (36.40)	68.00 (30.70)	78.00 (35.10)	76.00 (34.38)	222.00 (73.26)
Age at diagnosis, years (mean ± SD)	57.10±18.66	58.06±15.34	58.46±15.22	63.47±14.55	60.15±15.13
Sex					
Male (%)	35.00 (43.20)	43.00 (63.20)	37.00 (47.40)	36.00 (47.30)	116.00 (52.20)
Female (%)	46.00 (56.70)	25.00 (36.70)	41.00 (52.50)	40.00 (52.60)	106.00 (47.70)
Colon margin (%)	81 (100)				
Colon (%)		48.00 (70.50)	73.00 (93.50)	61.00 (80.20)	182.00 (81.90)
Right sided colon (%)		13.00 (27.00)	11.00 (15.00)	14.00 (22.90)	38.00 (20.80)
Left sided colon (%)		30.00 (62.50)	57.00 (78.00)	45.00 (73.70)	132.00 (72.50)
Transverse colon (%)		5.00 (10.40)	5.00 (6.80)	2.00 (3.20)	12.00 (6.50)
Rectum (%)		20.00 (29.40)	5.00 (6.40)	15.00 (19.70)	40.00 (18.00)
Tumor grade stage (%)				G1, 3.00 (3.90); G2, 71.00 (93.40); G3/4, 2.00 (2.60)	

G1, well differentiated; G2, moderately differentiated; G3/4, poorly differentiated.

Downregulation of miR-145 in different stages of CRC. Notably, miR-145 was significantly downregulated across P, Ad and ADC stages when compared with N (P<0.0001; Fig. 1B; Table II).

miR-145 expression was significantly downregulated at Ad compared with P (P=0.0014) and at ADC compared with Ad stage (P=0.0044). However, no significant change in the expression of this miRNA between P and ADC stage (P=0.6184) was noted (Fig. 1B).

Dysregulated expression of miR-186 in different stages of CRC. miR-186 was significantly deregulated (P=0.0009) according to the Kruskal Wallis test. Significant downregulation of miR-186 was observed at P and Ad (P=0.0006 and P=0.0053, respectively) compared with N. ADC stage showed a slight but not significant downregulation of miR-186 (P=0.147; Fig. 1C; Table II).

Evaluation of the expression profile of miR-186 between the different stages showed no significant change between Ad and P (P=0.4439), ADC and Ad stages (P=0.319) and ADC and P stages (P=0.0923; Fig. 1C).

Expression profile of miR-146b in different stages of CRC. Compared with N, miR-146b demonstrated a significant decrease expression (P=0.0241). Dunn's test revealed a significant downregulation at P (P=0.026) and Ad stages (P=0.0263; Fig. 1D, Table II). No significant change was observed between expression levels of miR-146b in Ad and P (P=0.8971), ADC and P (P=0.3774) and ADC and Ad stages (P=0.4279; Fig. 1D). Therefore, miR-146b expression did not significantly change in the different precancerous and cancerous stages of the disease.

ROC for miR-31, miR-145, miR-186 and miR-146b. The positive and negative predictive value and diagnostic accuracy of miR-31, miR-145, miR-186, and miR-146b were assessed (Table III). miR-31 and miR-145 were found to significantly differentiate between P and N (AUC=0.7771; 95% CI, 0.6972-0.8570; P<0.0001 and AUC=0.8269; 95% CI, 0.7566-0.8972; P<0.0001, respectively; Fig. 2). At the optimal cut-off values of 2.533 for miR-31 and 2.857 for miR-145, the sensitivity and specificity were 76.67 and 69.74 vs. 80 and 72%, respectively. miR-31 and miR-145 exhibited a diagnostic accuracy of 71.3 and 78.5% respectively. Moreover, the diagnostic accuracy of miR-31 and miR-145 was 77.4 and 76.7% respectively, when applied on an independent set of samples (Table SI). On the other hand, miR-186 and miR-146b showed poor discrimination between P and N (AUC=0.6875; 95% CI, 0.5987-0.7763; P=0.0002 and AUC=0.6313; 95% CI, 0.5372-0.7253; P=0.0086, respectively). Therefore, miR-31 and miR-145 can be used as early diagnostic markers to differentiate between P and N tissue (Fig. 2).

Discussion

CRC is a major worldwide health burden with a high mortality rate (32). Despite the availability of several screening techniques for CRC diagnosis, such as colonoscopy and blood- and stool-based biomarkers, these tests are not ideal and require improvement for effective early diagnosis to increase survival rate (13,14).

Considering the contribution of miRNA in the initiation and progression of cancer, researchers have investigated their role as an early detection biomarker in several types of cancer, including CRC (20,33,34).

miRNA expression varies within populations primarily due to specific genetic variation and single nucleotide polymorphism in mature miRNA (35,36). The present study assessed the expression profile of miRNAs (miR-31, miR-145,

Table II. Expression levels of miRs-31, -145, -186 and -146b compared with healthy controls.

miRNA	Stage	Number	Mean ± SD	95% CI	P-value
miR-31	P	61	3.1150±3.3940	2.2460-3.9850	<0.0001
	Ad	67	1.8750±3.2030	1.0930-2.6560	0.0007
	ADC	70	3.2200±3.1050	2.4800-3.9610	<0.0001
miR-145	P	60	-2.2090±1.3250	-2.5510-1.8670	<0.0001
	Ad	72	-3.3740±1.6470	-3.7610-2.9870	<0.0001
	ADC	74	-2.4780±2.1590	-2.9780-1.9770	<0.0001
miR-186	P	64	-1.1270±1.9140	-1.6050-0.6491	0.0006
	Ad	78	-0.8968±1.8780	-1.3200-0.4733	0.0053
	ADC	65	-0.6588±1.7240	-1.0860-0.2316	0.1470
miR-146b	P	64	-0.6320±1.7520	-1.0700-0.1943	0.0260
	Ad	77	-0.5011±1.7560	-0.8997-0.1025	0.0263
	ADC	66	-0.4283±1.9330	-0.9035-0.04695	0.2614

P, polyp; Ad, adenoma; ADC, adenocarcinoma; miRNA, microRNA.

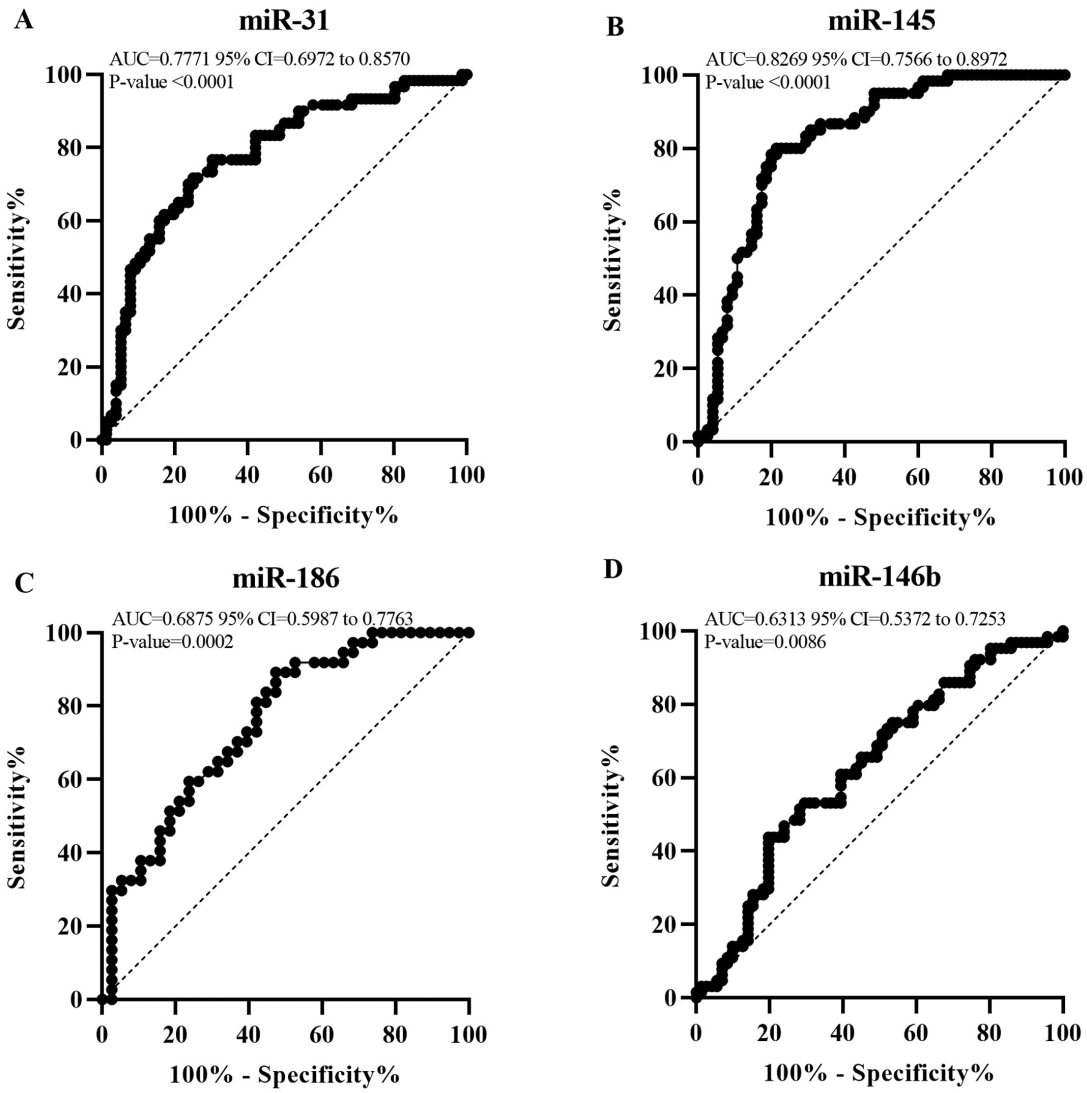

Figure 2. miR-31 and miR-145 show valuable diagnostic potential at P stage. Receiver operator characteristic curve for (A) miR-31, (B) miR-145, (C) miR-186 and (D) miR-146b expression. This graph represents the AUC for miR-31, miR-145, miR-186 and miR-146b to reveal their diagnostic value at P stage. AUC<0.5, no discrimination; 0.5≤AUC<0.7, poor; 0.7≤AUC<0.8, acceptable; 0.8≤AUC<0.9, excellent; AUC≥0.9, outstanding discrimination. AUC, area under the curve; P, polyp; miR, microRNA.

Table III. Diagnostic parameters to evaluate the diagnostic potential of miRs at polyp stage.

miR	AUC	SEM	P-value	95% CI	Sensitivity, %	Specificity, %	Cut-off value	Youden's index	PPV	NPV	DA, %
miR-31	0.7771	0.04078	<0.0001	0.6972-0.8570	76.67	69.74	2.533	0.4641	55.0	84.2	71.3
miR-145	0.8269	0.03586	<0.0001	0.7566-0.8972	80.00	72.00	2.857	0.5200	78.3	78.7	78.5
miR-186	0.6875	0.04529	0.0002	0.5987-0.7763	67.19	60.56	1.704	0.2775	48.4	78.9	64.4
miR-146b	0.6313	0.04799	0.0086	0.5372-0.7253	60.94	60.56	1.545	0.2150	43.8	76.1	60.7

AUC, area under the curve; PPV, positive predictive value, NPV, negative predictive value; DA, diagnostic accuracy; miRNA, microRNA.

miR-186 and miR-146b) in FFPE tissue of Lebanese patients at different stages (P, Ad and ADC) to identify miRNAs that are aberrantly expressed in CRC samples and highlight miRNAs that may have early diagnostic value. When the different CRC stages were compared with healthy controls, miR-31 was upregulated at all stages (P<0.0001). By contrast, miR-145, miR-186 and miR-146b were significantly downregulated at all stages (P<0.0001, 0.0009 and 0.0241, respectively). Of these four miRNAs, the present study identified miR-31 and miR-145 as potentially useful diagnostic factors with AUC=0.7771 for miR-31 and 0.8269 for miR-145, and diagnostic accuracy of 71.3 and 78.5%, respectively.

In CRC, dysregulation of miR-31 and miR-145 have been implicated in cell proliferation, invasion and migration *in vitro* and in tumorigenesis and metastasis in CRC tissue (37,38). miR-31 is reported to be upregulated in CRC tissue suppressing Special AT-rich sequence-binding protein 2 gene and regulating v-Raf murine sarcoma viral oncogene homolog B activation, serving a role in the signaling pathway downstream of epidermal growth factor receptor (39,40). Furthermore, miR-145 is downregulated in CRC tissue and induces tumorigenesis by acting on its target, Kirsten rat sarcoma viral oncogene homolog (41). Consistent with the present results, the upregulation of miR-31 and downregulation of miR-145 have been reported in CRC tissue in different populations such as the Chinese, Japanese and European populations (38,42,43).

Cui (44) reported that miR-31 upregulation has a high diagnostic value between normal and tumor stages. In addition, Peng *et al* (45) stated that miR-145 is a potential biomarker for the early diagnosis of CRC. Here, miR-31 and miR-145 significantly differentiated between P and N stages (P<0.0001; diagnostic accuracy, 71.3 and 78.5%, respectively). These results indicate that miR-31 and miR-145 detection may be valuable for the early-stage diagnosis of CRC.

miR-186 exhibits contradicting effects in certain types of cancer, including CRC, indicating its role as an onco-miRNA and tumor suppressor miRNA based on its targets (46). Islam *et al* (47) reported that miR-186 is significantly upregulated in colon tissue and cell lines in a study performed in Australia. Conversely, Li *et al* (30) reported a significant downregulation of miR-186 in colon tissues in the Chinese population and colon cancer cell lines. Here, miR-186 expression was significantly downregulated at P stage, accompanied by non-significant downregulation at Ad and ADC stages compared with N. Additionally miR-186 was significantly upregulated at ADC compared with P and Ad stages. This suggests a role for miR-186 as a potential biomarker at the early stages of multistage CRC carcinogenesis. When the diagnostic role of miR-186 was investigated using ROC analysis, it showed a poor diagnostic role between P and N stages with an AUC of 0.6875 and diagnostic accuracy of 64.4%. Hence, miR-186 was shown to be downregulated at P stage in Lebanese patients but exhibited poor diagnostic value.

miR-146b has been reported to be deregulated in different types of cancer. For example, miR-146b has been shown to be upregulated in papillary thyroid carcinoma and lung and gastric cancer (48-50). However, several studies have reported that miR-146b serves a tumor suppressor function in solid tumors, such as osteosarcoma, pancreatic and breast cancer and glioma (51-54). A study in 2012 reported that miR-146b expression is upregulated from non-neoplastic to dysplastic stage in CRC but downregulated from the dysplasia stage to the cancerous stage in Crohn's disease (27). Zhu *et al* (26) reported that elevated expression of miR-146b is associated with high prognosis of CRC at the tumor nodule metastasis stage. In the present study, there was no significant change in miR-146b expression at different stages of CRC compared with N. In addition, the ROC curve analysis showed that miR-146b cannot be used as a diagnostic biomarker between P stage and N. These results are not consistent with previous studies suggesting that miR-146b expression is variable between the Lebanese population and others (26,27). Thus, miR-146b cannot be considered a biomarker to assess the progression of CRC in the present samples.

Collectively, there is a growing appreciation for the role of miRNA expression in CRC. Among the studied miRNAs, only miR-31 was upregulated in the different stages whereas miR-145, miR-186, and miR-146b were downregulated. In addition, miR-31 and miR-145 can be considered as novel predictive tools for diagnosis of CRC at the early stages of tumorigenesis. Novel miRNA candidates can be added to established screening tools according to the expression levels and hence decrease the burden of this disease and improve survival rate.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SAS and RAM conceived and designed the study. LA provided FFPE tissue and interpreted the clinicopathological characteristics of patients. SAS, RAM, RAK analyzed and interpreted the data. ZAS and BH performed statistical analysis. RN conceived the study and revised the manuscript. SAS and RAM wrote the manuscript and confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Medical Committee at Bahman Hospital (Beirut, Lebanon; approval no. 27).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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