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ORIGINAL ARTICLE

Expression of specific microRNAs in tissue and plasma in colorectal cancer

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Background: MicroRNAs (miRNA/miR) play significant roles in the regulation of cell differentiation, cell cycle progression, and apoptosis. They become dysregulated during carcinogenesis and are eventually released into the circulation, enabling their detection in body fluids. Thus, this study compared the miRNA expression in tissue and plasma samples of colorectal cancer (CRC) patients and clinically healthy controls and determined miRNA expression as a potential CRC biomarker. **Methods:** Using quantitative reverse transcription polymerase chain reaction (RT-qPCR), miR-21-5p, miR-29a-3p, miR-92a-3p, miR-135b-5p, miR-196b-5p, and miR-197-3p, expression was analyzed and compared between the malignant (n = 41) and the adjacent neoplasm free mucosal tissues (n = 41) of CRC patients. The findings were validated in plasma samples (n = 36) collected from the same CRC patients prior to surgery or any form of treatment and compared to plasma from their age and sex-matched controls (n = 36). **Results:** MiR-21-5p, miR-29a-3p, miR-92a-3p, and miR-196b-5p were upregulated and miR-135b-5p was downregulated in CRC malignant tissues compared to their expression in adjacent neoplasm-free tissue. This was further observed in the plasma of the same CRC cases compared to controls. MiR-92a-3p showed itself the most sensitive (0.93; $p < .001$) and most specific (0.95; $p < .001$) in detecting CRC in tissue. In plasma, miR-196b-5p was the most sensitive (0.97; $p < .001$) and specific (0.94; $p < .001$) in detecting CRC. Plasma miR-92a-3p and miR-196b-5p were the most sensitive (0.95; $p < .001$) and specific (0.94; $p < .001$) in the early detection of CRC. **Conclusions:** Results show that specific miRNAs dysregulated in malignant tissues are released and can be detected in the circulation, supporting their potential as non-invasive biomarkers of CRC.

Key Words: microRNA; Colorectal neoplasm; Liquid biopsy; Biomarker

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Colorectal cancer (CRC) is a potentially preventable disease due to its slow development, unlike other solid tumor malignancies [1]. However, it remains a major cause of cancer-related deaths worldwide since approximately 90% of CRC patients are diagnosed only after presentation of signs and symptoms and are already in the more advanced stage of the disease [2]. GLOBOCAN reported over 1.9 million new CRC cases and 935,173 deaths in 2020 [3].

A screening program for CRC is probably the best way to mitigate the disease. It is estimated that over 95% of CRC cases would benefit from curative surgery if diagnosis is made at an early or premalignant polyp stage [4]. CRC screening programs worldwide are targeted at adults from 50–55 years of age, a population at increased risk of adenomas and cancer. Screening is based on several methods with varied advantages and drawbacks and different economic impact. Existing screening modalities to diagnose CRC are most often impractical due to invasiveness and cost (e.g., colonoscopy) or insufficient diagnostic accuracy (e.g., fecal-based occult blood tests) [5,6].

The discovery of notable numbers of non-coding microRNAs (miRNA/miR) that are stable in tissues and body fluids such as plasma, serum, stool, and exosomes could lead to development of a novel approach to CRC detection using circulating miRNAs as early diagnostic biomarkers for CRC. MiRNAs are small non-coding RNAs involved in gene regulation and cancer development. These miRNAs modify cell proliferation, apoptosis, and

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metastasis through their interaction with intracellular signaling networks [7]. Numerous studies [8,9] have discovered aberrantly expressed miRNAs in several human malignancies. Wikberg et al. [10] observed that plasma miRNA levels significantly increased approximately three years prior to presentation of symptoms and CRC diagnosis. This finding supports the potential of circulating miRNA to improve existing CRC risk prediction and screening strategies.

Up- and downregulation of miRNA expression are seen in CRC. Several miRNAs exhibit tissue-specific or development-stage-specific expression patterns and play potential roles in maintaining tissue identity and function. Similarly, miRNAs play a vital role in malignancy as tumor suppressors and oncogenes. Thus, discovery of novel miRNAs and validation of specific signature miRNAs in a specific population can potentially change the landscape of CRC diagnostics.

Numerous miRNAs have been identified as candidate biomarkers of CRC in several independent studies through sequencing and polymerase chain reaction (PCR) techniques [11-22]. Among the miRNAs, miR-21-5p, miR-29a-3p, miR-92a-3p, miR-135b-5p, miR-196b-5p, and miR-197-3p were validated in other populations; hence, these were tested in a group of Filipino CRC patients and clinically healthy controls.

MATERIALS AND METHODS

Study participants and samples

Formalin fixed paraffin embedded (FFPE) tissue samples surgically removed from patients with histologically confirmed CRC and seen at the Mariano Marcos Memorial Hospital and Medical Center (MMMH-MC) in Ilocos Norte, Philippines from March 2018 to December 2018 were included in this study. Each FFPE tissue block positive for malignant cells was matched with neoplasm-free mucosal tissue removed from the same patient and designated as case and control, respectively. Successive 5-μm-thick tissue samples were sectioned using a microtome (Leica Biosystems, Wetzlar, Germany), with the outer sections mounted on glass slides and then stained with hematoxylin and eosin (H&E). The inner sections (approximately 5 mg) were aseptically collected in nuclease-free microcentrifuge tube. The H&E-stained slides were evaluated by a pathologist to ensure that the tissue samples designated as cases and controls were strictly positive and negative for cancer cells, respectively.

Blood samples were also collected from the same CRC patients prior to their surgery or any form of treatment (cases). These were matched with blood samples from clinically healthy volunteers (controls) of the same age (± 2 years) and sex as the CRC patients. Controls were strictly not suspected of any type of malignancy at the time of physical or clinical assessment by a physician, had not undergone any colorectal resection except for sigmoid diverticular disease and had not been diagnosed with inflammatory bowel disease (IBD), chronic ulcerative colitis, or Crohn's disease. All blood samples were collected in K_2EDTA tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and centrifuged immediately at 2,000 × g for 10 minutes. at 4°C. Hemolyzed blood samples and turbid plasma were excluded from the study. Plasma was separated and aliquoted into nuclease-free cryovials and then stored in a freezer at –80°C until analysis.

RNA isolation from FFPE tissues and plasma samples

Total RNA including miRNAs were isolated from FFPE tissues utilizing miRNeasy FFPE kit according to manufacturer's instructions (miRNeasy FFPE Handbook June 2015, http://www.qiagen.com). FFPE tissue sections were deparaffinized and then digested by proteinase K, followed by heat treatment. After centrifugation, the supernatant was collected and treated with DNAse. After mixing with ethanol and buffer, the lysate was transferred into a RNeasy MinElute spin column where total RNA are bound. After washing twice, the RNA was eluted.

Plasma miRNA was isolated using the miRNeasy serum/plasma kit total RNA (Qiagen, Hilden, Germany) according to manufacturer's instructions. QIAzol was added to 100 μL of plasma, incubated, and miRNeasy serum/plasma spike-in control *Caenorhabditis elegans* miR-39 miRNA mimic (1×10^8 copies/μL) (Qiagen) was added to each sample. Aqueous and organic phase separation was achieved using chloroform. The aqueous phase was extracted after centrifugation and addition of absolute ethyl alcohol. The mixture was transferred to RNEasy mini spin columns and centrifuged. Buffers RWT and RPE were used respectively on two consecutive steps to wash the spin columns with centrifugation. The total RNA including miRNA was eluted using RNAse-free water applied directly at the center of the mini spin column silica membrane and centrifuged at full speed. The RNA eluates from tissue and plasma were stored at –20°C until subsequent analysis.

Reverse transcription of miRNA and preamplification of cDNA

MiRNAs from colorectal tissues and plasma were polyadenylated and reverse transcribed using the miScript II RT kit and miScript HiSpec buffer (Qiagen) following the manufacturer's procedure. RNAse-free water (40 μL) was added to the synthe-

sized cDNA (10 µL), and the mixture was aliquoted into PCR tubes and stored at –20°C until analysis.

MiScript PreAMP PCR Kit (Qiagen) was used to pre-amplify the cDNA target templates following manufacturer instructions. MiR-16 was analyzed to determine the optimal dilution for real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR). Samples that generated Ct values between 10 and 24 needed no further dilution for target miRNAs using RT-qPCR. Efficiency of reverse transcription was measured using miRTC assay. Efficient reverse transcription for the miRTC primer assay was set at Ct values between 14 and 20.

Quantification of selected miRNAs by qRT-PCR

SNORD61 and *C. elegans* miR-39 primers for normalization of tissue and plasma miRNA, respectively, and 10× miScript primer assay mixes for hsa-miR-21-5p, hsa-miR-196b-5p, hsa-miR-135b-5p, hsa-miR-92a-3p, hsa-29a-3p, and hsa197-3p were ordered from Qiagen. Two microliters of pre-amplified cDNA was pipetted into a 96-well PCR plate, and 23 µL of reaction mix (12.5 µL 2× QuantiTect SYBR Green PCR master mix, 2.5 µL 10× miScript universal primer, 2.5 µL 10× miScript primer assay mix, and 5.5 µL RNAse-free water) was added. Amplification was performed under the following conditions: initial activation step at 95°C for 15 minutes, denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 70°C for 30 seconds for 40 cycles. All assays were performed in duplicate.

Data processing and analysis

The relative expression levels of the miRNAs interrogated were normalized to those of SNORD61 and cel-miR-39 and determined by the $2^{-\Delta\Delta Ct}$ method. Using GraphPad Prism 8.3 for Windows (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com) and XLSTAT 2019.3.2 (Addinsoft, Paris, France), intergroup comparisons were analyzed by paired t tests and Mann-Whitney tests. Spearman correlation coefficient was used for correlation analysis. A p-value less than .05 (two-tailed) was considered statistically significant. The receiver operating characteristic (ROC) and the area under the ROC curve (AUC) with 95% confidence interval (CI) were generated, and the number of outliers per tissue and plasma group were determined by Robust Regression and Outlier Removal (ROUT) [23]. These discriminating performance calculations were carried out using GraphPad Prism 8.3 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Clinicopathological characteristics of CRC patients

A total of 41 FFPE malignant tissues and their corresponding adjacent neoplasm-free mucosal tissues (n = 41) were collected from histologically confirmed Filipino CRC patients. EDTA-treated plasma samples from the same CRC patients (n = 36) were collected prior to surgery or any form of treatment and from their age and sex-matched clinically healthy controls (n = 36). Table 1 shows the clinicopathologic characteristic of cases included in this study.

Expression patterns of selected miRNAs in CRC tissue and plasma

Table 2 shows the relative expression and fold regulation of the

Table 1. Clinicopathologic profile of cases

Characteristics	No (%) (n = 41)
Age	
<60	18 (44)
>60	23 (56)
Sex	
Male	18 (44)
Female	23 (56)
Location of tumor	
Rectum/cecum/rectosigmoid	34 (83)
Sigmoid ascending/transverse and descending	7 (17)
Histologic type	
Adenocarcinoma	33 (80)
Mucinous cell carcinoma/signet ring cell carcinoma	8 (20)
Histologic grade	
Well-differentiated	26 (63)
Moderately to poorly differentiated	15 (37)
TNM stage	
Stage I/II	20 (49)
Stage III/IV	21(51)
Lymph node metastasis	
N0–N1	29 (71)
N2	12 (29)
Distant metastasis	
M0–M1	28 (68)
Mx	13 (32)
Tumor size	
<5 cm	18 (44)
>5 cm	23 (56)
Depth of tumor infiltration	
Muscularis propria	18 (44)
Muscularis propria into the pericolic adipose tissue and outside	23 (56)
Perineural invasion	
Absent	39 (95)
Present	2 (5)

Table 2. Relative expression, fold change, and fold regulation of selected miRNAs in tissue and plasma

miRNA	Mean expression in tissue			Mean expression in tissue after outlier removal			Fold change in tissue (n=41)	Mean expression in plasma			Mean expression in plasma after outlier removal			Fold change in plasma (n=36)	Fold regulation in tissue and plasma
	Malignant tissue (n=41)	Adjacent neoplasm-free tissue (n=41)	p-value	Malignant tissue[a]	Adjacent neoplasm-free tissue[a]	p-value		CRC cases (n=36)	Clinically healthy controls (n=36)	p-value	CRC cases[a]	Clinically healthy controls[a]	p-value		
miR-21-5p	9.35±8.28	5.49±6.44	<.001	8.76±7.44	1.43±1.25	<.001	4.23	5.84±6.56	2.05±4.15	<.001	5.82±6.65	0.44±0.47	<.001	8.40	Upregulated
miR-196b-5p	4.39±5.09	2.19±3.52	.026	2.61±2.79	0.96±1.06	.010	2.45	16.60±12.09	3.13±2.31	<.001	19.20±13.65	2.58±1.40	<.001	4.75	Upregulated
miR-92a-3p	4.45±3.56	0.79±1.35	<.001	3.38±2.02	0.59±0.57	<.001	7.41	7.63±8.29	2.24±2.93	<.001	5.18±4.32	1.43±1.11	<.001	2.50	Upregulated
miR-29a-3p	3.41±2.53	1.27±1.84	<.001	3.40±2.52	0.56±0.33	<.001	3.56	3.15±3.79	0.80±0.86	<.001	2.35±1.71	0.59±0.43	<.001	2.89	Upregulated
miR-135b-5p	1.06±1.64	2.64±2.58	<.001	0.32±0.43	2.47±2.35	<.001	0.17	0.89±0.99	3.98±4.06	<.001	0.74±0.69	2.96±2.23	<.001	0.44	Downregulated
miR-197-3p	3.33±3.36	4.80±7.38	.676	2.61±2.79	2.30±2.07	.681	0.78	3.86±3.04	3.45±3.50	.112	3.15±1.89	2.88±1.14	.656	1.32	Not significant

[a]The number of samples after the outlier removal from each miRNA varied depending on the number of samples (outlier) removed during the Robust Regression and Outlier Removal analysis.

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miRNAs in malignant colorectal tissue. MiR-21-5p, miR-29a-3p, miR-92a-3p, and miR-196b-5p were upregulated in CRC tissues in relation to their adjacent neoplasm-free tissue. MiR-92a-3p showed the highest fold change (FC = 7.41, p < .001) in CRC tissue, followed by miR-21-5p (FC = 4.23, p < .001) and miR-29a-3p (FC = 3.56, p < .001). MiR-135b-5p was downregulated (FC = 0.17, p < .001) in CRC tissue compared to its adjacent neoplasm-free tissue. The relative expression of miR-197-3p in CRC tissue was not significantly different (FC = 0.78, p = .676) in relation to its adjacent neoplasm-free tissue.

To determine whether the dysregulated miRNAs in CRC tissue were released into the circulation, plasma from the same CRC patients was collected and tested for the specific miRNA under study prior to any form of treatment. Interestingly, expression patterns of miR-21-5p, miR-29a-3p, miR-92a-3p, miR-135b-5p, miR-196b-5p, and miR-197-3p in plasma of CRC cases were similar to plasma from clinically healthy individuals (Table 2). MiR-21-5p showed the highest expression (FC = 8.40, p < .001) in CRC plasma, followed by miR-196b-5p (FC = 4.75, p < .001) and miR-29a-3p (FC = 2.89, p < .001). Similar to CRC tissue, miR-135b-5p was downregulated (FC = 0.44, p < .001) in CRC plasma compared to clinically healthy controls. The relative expression of miR-197-3p in CRC plasma was not significantly different (FC = 1.32, p = .112) in relation to control plasma.

To determine if the significant differences in miRNA expression levels in tissue and plasma were attributable to the presence of outliers, ROUT was performed. The residuals of the robust fit were analyzed to identify any outlier according to the false discovery rate approach for testing multiple comparisons. The ROUT coefficient was set at Q=1%. Table 2 shows that, upon outlier removal, the p-values between malignant and adjacent

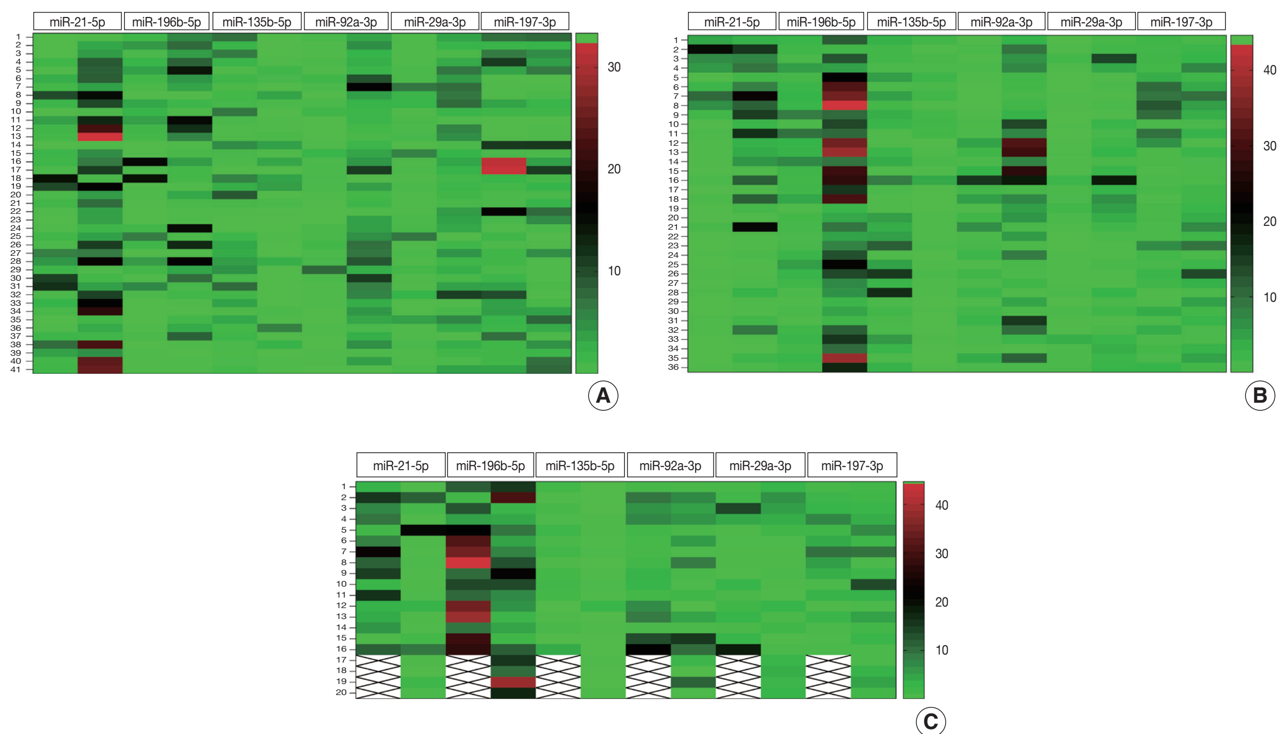

Fig. 1. Heatmap analysis showing differential expression of selected miRNA. (A) Heatmap of selected miRNA in malignant and neoplasm-free colorectal tissues. Each row (1-41) represents a tissue sample. (B) Heatmap of selected miRNA in colorectal cancer (CRC) plasma and clinically healthy control plasma. Each row (1-36) represents a plasma sample. (C) Heatmap of selected miRNAs in plasma of early (I/II) and late stages (III/IV) of CRC. Each row (1-20) represents a plasma sample. The first and second columns for each miRNA in each heatmap represent respective malignant and adjacent neoplasm-free tissues (A) plasma from CRC patients and healthy controls (B) and early (n = 16) and advanced (n = 20) stages (C). The color scale shows saturation and brightness based on the mean values of the data set. The largest mean values are shown in red (> 40), while the lowest mean values are shown in green (< 10). The mean intensities of miR-21-5p, miR-196b-5p, miR-92a-3p, and miR-29-3p are stronger in malignant colorectal cancer tissue and plasma of CRC patients compared to controls. In contrast, miR-135b-5p has a lower intensity in malignant colorectal cancer tissue and plasma of CRC patients. The mean intensities of miR-21-5p, miR-196b-5p, miR-92a-3p, and miR-29-3p are stronger in early stages compared to late stages of CRC. MiR-135b-5p and miR-197-3p have lower mean intensity in early stages compared to late stages of CRC.

neoplasm-free tissue and CRC and clinically health control plasma remained significantly different.

Ability of selected miRNAs to discriminate malignant from neoplasm-free mucosal tissue and plasma from CRC patients from clinically healthy controls

To determine discriminatory ability of miRNAs under study, heatmap analysis was performed. Fig. 1A shows that majority of the CRC tissues exhibited greater color intensities than normal adjacent tissues with respect to miR-21-5p, miR-196b-5p, miR-92a-3p, and miR-29a-3p expression. For miR-135b-5p, the color intensity appeared lighter in CRC compared to normal adjacent tissue.

Heatmap analysis (Fig. 1B) of plasma samples show that color intensity for miR-21-5p, miR-196b-5p, miR-92a-3p, and miR-29a-3p of CRC was stronger than color intensity for control plasma. For miR-135b-5p, the color intensity appeared lighter in CRC plasma than in control plasma. This color intensity pattern exhibited in CRC plasma is similar to that shown by malignant tissue.

Relative expression of selected miRNAs in early and advanced stage CRC

Relative expressions of miR-21-5p (p = .531), miR-29a-3p (p = .473), miR-92a-3p (p = .578), miR-135b-5p (p = .611), miR-196b-5p (p = .769), and miR-197-3p (p = .879) in tissues of patients with early-stage CRC were not significantly different from those in tissues of patients with advanced stage CRC (Table 3). Conversely, levels of all miRNAs studied were useful to discriminate between early and advanced stage CRC using plasma. The relative plasma expressions of miR-21-5p (p = .006), miR-29a-3p (p < .001), miR-92a-3p (p < .001), and miR-196b-5p (p < .001) were significantly higher in early-stage CRC compared to advanced-stage CRC (Table 3). In contrast, significantly higher expression of miR-135b-5p (p < .001) and miR-197-3p (p = .007) was observed in plasma samples of patients with advanced CRC compared to early stage. MiR-197-3p, which failed to discriminate CRC plasma from clinically healthy plasma, was able to distinguish between early and advanced stages of CRC (p = .007). Heatmap analysis (Fig. 1C) showed the potential of specific miRNAs in discriminating early (I/II) from advanced (III/IV) stage CRC. The mean color intensities of miR-21-5p, miR-196b-5p, miR-92a-3p, and miR-29-3p are greater in the early stages compared to late stages of CRC. MiR-135b-5p and miR-197-3p have a lower mean color intensity in early stages compared to late stages of CRC.

Correlation of expression levels of miRNAs in tissues and plasma

Results show that expression levels of miR-196b-5p (r = 0.02, p = .921), miR-135b-5p (r = 0.14, p = .430) and miR-197-3p (r = -0.24, p = .150) in tissue and plasma were not correlated. A weak positive correlation was observed with miR-21-5p (r = 0.33, p = .049) while miR-92a-3p (r = 0.54, p = .007) and miR-29-3p

Table 3. Relative expression of selected miRNAs in tissue and plasma of patients with early (I/II) and advanced stage (III/IV) CRC

miRNA	Mean expression of miRNA in tissue		p-value	Mean expression of miRNA in plasma		p-value
	Early stage (n = 20)	Advanced stage (n = 21)		Early stage (n = 16)	Advanced stage (n = 20)	
miR-21-5p	10.13 ± 10.87	8.02 ± 7.39	.531	12.33 ± 5.33	7.14 ± 5.13	.006
miR-196b-5p	3.58 ± 2.53	5.02 ± 5.05	.769	10.76 ± 1.93	5.54 ± 2.35	< .001
miR-135b-5p	1.05 ± 1.63	0.76 ± 1.26	.611	2.34 ± 1.22	10.80 ± 3.76	< .001
miR-92a-3p	12.51 ± 11.89	14.90 ± 14.59	.578	9.92 ± 2.53	2.94 ± 2.95	< .001
miR-29a-3p	5.30 ± 3.19	5.06 ± 4.14	.473	6.67 ± 2.18	3.32 ± 2.18	< .001
miR-197-3p	1.18 ± 0.73	1.50 ± 1.49	.879	1.16 ± 1.03	2.60 ± 2.05	.007

miRNA (miR), microRNA; CRC, colorectal cancer.

Table 4. Correlation of the expression levels of selected miRNAs in tissue and plasma of CRC patients

Sample	miR-21-5p			miR-196b-5p			miR-135b-5p			miR-92a-3p			miR-29a-3p			miR-197-3p		
	Mean expression level	r	p-value	Mean expression level	r	p-value	Mean expression level	r	p-value	Mean expression level	r	p-value	Mean expression level	r	p-value	Mean expression level	r	p-value
Tissue	9.35 ± 8.28	0.33	.049	4.39 ± 5.09	0.02	.921	1.06 ± 1.64	0.14	.430	4.45 ± 3.56	0.54	.007	3.41 ± 2.53	0.52	.048	3.33 ± 3.36	–0.24	.150
Plasma	5.84 ± 6.56			16.60 ± 12.09			0.89 ± 0.99			7.63 ± 8.29			3.15 ± 3.79			3.86 ± 3.04		

miRNA (miR), microRNA; CRC, colorectal cancer.

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Table 5. Diagnostic performance of the selected miRNA for CRC

miRNA	Diagnostic performance for CRC irrespective of cancer stage using tissue samples				Diagnostic performance for CRC irrespective of cancer stage using plasma samples				Diagnostic performance for early-stage CRC using plasma samples			
	AUC	Cutoff point	Sensitivity	Specificity	AUC	Cutoff point	Sensitivity	Specificity	AUC	Cutoff point	Sensitivity	Specificity
miR-21-5p	0.78	3.56	0.80	0.83	0.78	3.89	0.81	0.83	0.77	3.69	0.80	0.81
miR-196b-5p	0.64	0.97	0.68	0.71	0.94	4.96	0.97	0.94	0.94	5.24	0.95	0.94
miR-135b-5p	0.76	2.32	0.80	0.83	0.83	1.45	0.86	0.89	0.84	2.05	0.85	0.88
miR-92a-3p	0.89	1.40	0.93	0.95	0.76	1.39	0.81	0.81	0.95	1.79	0.95	0.94
miR-29a-3p	0.81	1.23	0.85	0.83	0.83	1.21	0.86	0.83	0.87	0.41	0.90	0.88
miR-197-3p	0.52	2.41	0.56	0.63	0.60	2.06	0.61	0.67	0.76	2.41	0.80	0.81

miRNA (miR), microRNA; AUC, area under the receiver operating characteristic curve; CRC, colorectal cancer.

($r = 0.52$, $p = .048$) expressions in CRC tissues and plasma presented a moderate correlation (Table 4).

Diagnostic potential of selected miRNAs in detection of CRC in tissue and plasma

AUC values of the ROC were computed for each miRNA to evaluate their diagnostic value in detection of CRC in tissues and plasma. In CRC tissue, miR-92a-3p obtained the highest AUC (0.89; 95% CI, 0.82 to 0.97), with a sensitivity of 0.93 and specificity of 0.95 (Table 5). In detecting malignancy using plasma samples, MiR-196b-5p showed the highest AUC (0.94; 95% CI, 0.89 to 0.99) with a sensitivity of 0.97 and specificity of 0.94 (Table 5). In early detection of CRC using plasma samples, miR-92a-3p showed the highest AUC (0.95; 95% CI, 0.88 to 0.99), with a sensitivity of 0.95 and specificity of 0.94 (Table 5).

DISCUSSION

The identification of diagnostic molecular biomarkers is substantial in cancer research. Increasing evidence suggests that miRNAs play a vital role in regulating the development, differentiation, and progression of cancer. MiRNAs are well preserved in tissue even after formalin-fixation and paraffin-embedding. They can be efficiently extracted and assessed from tumors and body fluids such as serum, plasma, urine, stool, and saliva. The miRNAs analyzed in the current study have been identified in previous independent studies utilizing diverse study subjects (different races and populations) through sequencing and PCR techniques as candidate biomarkers for CRC. The differences and similarities in the expression patterns of these miRNAs in tissue and plasma across different study populations have been highlighted. Similarly, the current study determined the pattern of these miRNAs, thereby contributing to the current understanding of which specific signature miRNAs can be applied in the clinical setting.

The current study shows that miR-21-5p, miR-29a-3p, miR-92a-3p, and miR-196b-5p were significantly overexpressed while miR-135b-5p was underexpressed in malignant compared to neoplasm-free colorectal tissues. The above specific miRNAs were also dysregulated in the plasma of CRC patients but not in their matched clinically healthy controls. However, miR-197-3p expression in tumor tissue and plasma of cancer patients was not significantly different from that in controls.

MiR-21-5p plays an oncogenic role in the development and progression of CRC by modulating malignant processes such as proliferation, anti-apoptosis, cell cycle progression, and invasion in CRC cells through downregulation of *PTEN* protein expression [24]. Prostaglandin-endoperoxide synthase 2 (PGTS2) produces inflammatory mediator prostaglandin E2 (PGE2), which has been described as promoting colorectal tumor development. PGTS2-driven inflammatory responses induce tumor expression of miR-21-5p that can elevate PGE level by downregulating PGE2-metabolizing enzymes [25]. During CRC development, miR-21 also induces stemness by downregulating *TGFβR2* (transforming growth factor beta receptor 2) and stimulates invasion and metastasis by suppressing the *PDCD4* gene [26]. MiR-21 expression has been found to be upregulated in breast, lung, and gastric cancers including CRC and hematological malignancies [27]. This study agrees with previous findings that miR-21-5p is upregulated in tissues [27-29] and plasma or serum [29-32]. Mir-21-5p expression was 12 and 10 times higher in serum and stool, respectively, of CRC patients compared to those of healthy controls [31]. Meanwhile, Stiegelbauer et al. [32] observed a serological underexpression of miR-21-5p in CRC. Plasma miR-21-5p downregulation was also detected in invasive breast cancer [33].

It has been observed that miR-196b-5p is upregulated in acute lymphoblastic leukemia but downregulated in glioblastoma, cervical cancer, and B-cell lymphoma. This miRNA has been experimentally validated to regulate CRC cell migration and

metastasis through interaction with *GALNT5* and *H0XB7* genes [34]. Although they vary in expression levels, the miR-196 family of molecules is consistently overexpressed in oral cavity, esophageal, stomach, and intestinal cancer tissues [35]. Several investigations [36-38] support our findings that miR-196b expression levels in malignant tissues are significantly higher than in adjacent normal colorectal mucosa. MiR-196b-5p was also found to be elevated in serum exosomes of CRC and is associated with liver metastasis [35]. MiR-196b-5p is also more upregulated in serum of patients with CRC compared to that in adenoma patients or healthy individuals [36]. A few reports have shown that the miR-196 family can also act as tumor suppressors. For instance, miR-196a suppresses metastasis in breast cancers [37] and melanoma [38]. MiR-196-b has been found to be downregulated in different types of leukemia cells [39].

Upregulation of miR-29a in tissue and plasma of CRC patients was noted in the present study. Similarly, Brunet Vega et al. [40] noted that miR-29a along with 10 other miRNAs were significantly increased in malignant colorectal tissue samples compared with non-cancerous adjacent mucosa. In the same study, serum level of miR-29a was overexpressed in CRC patients but not in healthy controls. In a genome-wide miRNA profiling conducted by Giraldez et al. [41], in 63 plasma samples from newly diagnosed CRC patients, miR-29a was confirmed to be upregulated compared with controls. In contrast, downregulation of miR-29a has been observed in human lung cancer tissues. Liu et al. [42] demonstrated that expression level of miR-29a was significantly downregulated in 38 pairs of lung cancer tissues compared to adjacent normal tissue. MiR-29a-3p promotes CRC metastasis by regulating *MMP-2* gene and E-cadherin via the KLF-4 signaling pathway [43]. It has also been found to promote cell proliferation and epithelial-mesenchymal transition in breast cancer by targeting *TET-1* [44]. Conversely, miR-29a functions as a tumor suppressor by targeting the *MUC-1* in pancreatic cancer cell [45].

The miR-92 family, one of the four families that belong to the miR-17-92 cluster, has been shown to regulate formation of vascular endothelial cells and blood vessels. Aberrant expression of the miR-92a family has been observed in breast, lung, gastric, prostate, and pancreatic cancers [46]. MiR-92a-3p is implicated as a key oncogenic component in the miR-17-92 cluster during colorectal tumorigenesis [47]. Mir-92a promotes metastasis by suppressing *PTEN* gene expression and activation of the PI3K/AKT pathway [48]. The current study showed miR-92a-3p to be overexpressed in malignant tissue and plasma of CRC patients. Our study revealed that miR-92a-3p obtained the highest sensitivity and specificity in detecting CRC in tissue. Tsuchida et al. [47] found in their study that miR-92 was more significantly upregulated in both colorectal adenoma and carcinoma compared to the other five miRNAs in the cluster. Along with miR-21 and miR-29a, miR-92a has been observed to be significantly upregulated in CRC tissues compared to normal colorectal mucosal tissue. Moreover, this study agrees with the results of Ng et al. [9] that miR-92a was upregulated in both plasma and tissue samples of CRC patients in comparison with healthy controls.

MiR-135b-5p, an oncogene, has a tumor-promoting effect by enabling proliferation and inhibiting apoptosis of CRC cells through negative regulation of the TGF-β signaling pathway [49]. Magalhaes et al. [50] experimentally validated that downregulation of the *APC* gene caused by miR-135b-5p led to higher transduction signaling within the β-catenin/Wnt pathway, causing a greater proliferative capacity of malignant cells. *APC* modulates β-catenin as an essential part of the multi-protein complex that marks it for proteasomal degradation. MiR-135b-5p expression in CRC tissue and plasma was downregulated in this study. Zekri et al. [51] obtained similar findings, wherein serum miR-135b-5p and miR-454 were the only downregulated miRNAs in CRC and colonic polyp groups compared to the IBD group. In contrast, Bastaminejad et al. [52] demonstrated that expression levels of miR-135b-5p in serum and stool of CRC patients were 32 and 16 times higher compared to healthy controls, respectively. While miR-135b-5p is known to be an oncogenic miRNA, it might have different binding affinities due to its numerous potential target genes [53].

Whether the miRNAs detected in circulation are products of tumor apoptosis or are secreted by the active tumor cell itself remains controversial. MicroRNAs released in the circulation are usually bound to Argonaute proteins, microvesicles, exosomes, or lipoproteins that offer different degrees of protection. Hence, circulating miRNAs exhibit half-lives ranging from minutes to hours, which might explain why their concentrations in circulation vary and why their expressions in the tumor and plasma of the same patient might not be correlated [54]. MiR-1224, for instance, is the most upregulated miRNA in CRC plasma samples, but its expression in its paired tumor sample is rather low. Several explanations have been offered regarding the non-correlation between tumor and plasma miRNA expression levels. Exosomal shifting between the tumor mass and fluid microenvironment is one possibility [55].

Cancer-associated circulating miRNAs might have originated from immunocytes in the tumor microenvironment or from some other response mediated by affected organs or system

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[56,57]. Investigators suggest that tumor cells secrete a variety of miRNAs that act on immunocytes to modulate immune responses. In response, the immunocytes secrete miRNAs that either promote or inhibit tumor proliferation, migration, and apoptosis [58]. Lastly, technical issues such as sample type, biological and racial differences, and analytical and normalization methods can significantly contribute to the lack of or weak relational expression of specific microRNAs in tissue and plasma. Researchers found that serum samples can yield lower miRNA concentrations compared to plasma primarily due to the presence of cellular contaminants particularly from platelets.

CRC has been considered an extremely heterogenous and dynamic disease characterized by multiple molecular pathways throughout its development. This can be attributed to the existence of cellular subpopulations between and within tumors of divergent genotype and phenotype expressions [59]. Chromosomal instability, microsatellite instability, aberrant DNA methylation, and DNA repair mechanisms are intricate processes involved in colorectal epithelial cell transformation and all confer on a tumor its distinctiveness [60]. This high degree of genomic diversity in CRC over time, including relationships between subpopulations within and between the tumor, inaccurately reflects the true molecular profile within the tumor tissue sample, forming a major obstacle in clinical practice [61]. Further, this tumor heterogeneity can introduce sampling bias, heightening the intricacies in validation of oncology biomarkers [62]. Molecular diagnostic studies that seek to recognize driver mutations within a tumor can be affected by the presence of multiple mutations within the same sample. Thus, it is imperative to prudently characterize tumor samples for research to avoid inexplicable results or outcomes.

There are certain limitations that need to be considered when interpreting the findings of this study. First is the small sample size in the context of miRNA-based biomarker identification and validation. Due to limited budget, the current study was only able to analyze a limited number of samples. Thus, a follow-up study to validate the current findings in a prospective set of samples in a blinded manner is recommended. Moreover, the research team has, in a separate study, applied artificial intelligence (AI) in the analysis of the data, wherein a set of samples has been used as a training set to create the AI models, and the remaining samples were used as test specimens to determine the diagnostic potential of the miRNAs. Second, this study was a single-institution study focused on a specific population in the Philippines, whose diet and lifestyle might not represent those of other ethnic regions of the country. Recruitment of CRC patients and controls from different parts of the country is suggested to determine reproducibility of findings.

In conclusion, this study presented a proof of concept that dysregulated miRNAs in CRC tissues are observed in the circulation, supporting their potential as non-invasive biomarkers for early detection of CRC. Results of this study agree with those of previous studies using samples from other populations. However, clinical translation of miRNA as a non-invasive circulating biomarker requires highly sensitive, specific, reproducible, reliable, and robust assays to enable its accurate and precise quantification in tissue, plasma, serum, and other human body fluids. Critical pre-analytical and analytical variables such as individual biological variability, sample type, sample storage, miRNA extraction and purification, detection method, and normalization all need to be optimized.

Ethics Statement

All procedures were performed in accordance with the ethical standards of institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This study complied with all applicable ethical guidelines approved by the Research Ethics Review Committee of the Mariano Marcos Memorial Hospital and Medical Center (MMMH-RERC-18-005, March 2, 2018). Study participants gave their written informed consent and were assured of the confidentiality of the results.

Availability of Data and Material

The datasets generated or analyzed during the study are available from the corresponding author on reasonable request.

Code Availability

Not applicable.

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Conflicts of Interest

The authors declare that they have no potential conflicts of interest.

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