


# Potential diagnostic role of circulating MiRNAs in colorectal cancer

Dina A Abo-elela<sup>1</sup>, Ahmed MH Salem<sup>1</sup>, Menha Swellam<sup>2</sup> and Marwa GA Hegazy<sup>1</sup> 

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## Abstract

**Objectives:** Colorectal cancer (CRC) is the third most common and fourth most deadly cancer worldwide despite its various screening method. Thus, the search for novel and better markers is continuous. This study aimed to assess the combined expression levels of miR-133a, miR-574-3p, and miR-27a in early diagnosis of colorectal cancer in comparison to traditional tumor markers (CEA and CA19.9).

**Methods:** miR-133a, miR-574-3p, and miR-27a were assessed in sera of 120 participants categorized into healthy control group ( $n = 20$ ), benign group ( $n = 30$ ) and malignant group ( $n = 70$ ) using real-time PCR.

**Results:** miR-133a, miR-574-3p, and miR-27a expressions showed significant difference among different staging, grading and tumor size of CRC. The sensitivities of the three miRNAs whether combined or individually used were better than routinely used tumor markers (CEA and CA19.9) leading to more accurate and faster diagnosis of CRC.

**Conclusion:** Synergetic detection of miRNA-133a, miRNA-574-3p, and miRNA-27a may serve as better noninvasive biomarkers with higher combined sensitivity for early diagnosis of CRC than individual detection of miRNAs.

## Keywords

Colorectal cancer, miR-133a, miR-574-3p, miR27a

## Introduction

Colorectal cancer (CRC) is the third most common cancer as well as the fourth most lethal cancer worldwide. CRC accounts for around 10% of all new cancer cases universally, remaining the second most common cause of cancer-related deaths.<sup>1</sup> In Egypt, the expected percent of CRC is 6.5% of all cancers.<sup>2</sup>

Obesity, diabetes, Irritated bowel disease, precancerous colonic lesions, and family history of CRC were the highly considerable risk factors.<sup>2</sup> Extensive research has recommended that nutrition may play both a causal and protective role in the development of colon cancer.<sup>3</sup>

MicroRNAs (miRNAs) are regulatory RNAs with 18–25 endogenous non-coding nucleotides that show post-transcription gene regulation. MiRNAs regulate gene expression and contribute to development, differentiation,

inflammation, and carcinogenesis.<sup>4</sup> Dysregulation of mature miRNA biogenesis steps can cause alterations in miRNA expression in cancer.<sup>5</sup>

MiR-133a has been confirmed as a tumor suppressor in many cancers by suppressing cellular proliferation, migration, and invasion.<sup>6</sup> Human miR-133a is encoded by 2

<sup>1</sup>Biochemistry Department, Faculty of Science, Ain Shams University, Cairo, Egypt

<sup>2</sup>Biochemistry Department, Biotechnology Research Institute, High Throughput Molecular and Genetic laboratory, Central Laboratories Network and the Centers of Excellence, National Research Centre, Cairo, Egypt

### Corresponding author:

Marwa GA Hegazy, Department of Biochemistry, Faculty of science, Ain Shams University, Abassia, Cairo 11566, Egypt.  
Emails: [marwa\\_hegazy@sci.asu.edu.eg](mailto:marwa_hegazy@sci.asu.edu.eg); [marwa\\_hegazy2000@yahoo.com](mailto:marwa_hegazy2000@yahoo.com)



different genes, miR-133a-1 and miR-133a-2, which are processed into matching mature sequence. miR-133a-1 and miR-133a-2 are implanted in the MIB1 gene on chromosome 18 and C20orf166 on chromosome 20, respectively.<sup>7</sup>

MiR-133a has been shown to regulate diverse target genes in numerous cancer cells. Among them, eukaryotic translation initiation factor 4A1 (eIF4A1) acts as a serious effector of miR-133a. eIF4A1, an adenosine triphosphate (ATP)-dependent RNA helicase, is vital for assembly of the translational active ribosome.<sup>8</sup> MiR-133a represses the luciferase activity of Luc-eIF4A1-3'UTR by targeting the 3'UTR of eIF4A1 mRNA. The inhibitory effect of miR-133a on cell proliferation can be abolished by overexpression of eIF4A1.<sup>9</sup> Tumor growth inhibition by miR-133a could be attributed, at least in part, to activating p53/p21 pathway.<sup>10</sup>

MiR-27a exerts numerous regulatory functions in different kinds of cancer.<sup>11</sup> MiR-27a was reported to be located on chromosome 19 and played a vital role in tumor development.<sup>12</sup> MiR-27a acts as a tumor suppressor. Studies have recognized sphingosine-1-phosphate phosphatase 1 (SGPP1) and Smad2 as two new goals of miR-27a, which is related to signal transducer and activator of transcription 3 (STAT3) to control cancer cell proliferation, apoptosis and migration.<sup>13</sup>

MiR-574-3p was found to act as tumor suppressor in several cancers.<sup>14</sup> Genes encoding miR-574-3p are located on chromosomes 4p (4p14 and 4p15) with a tumor inhibitory miRNA function.<sup>15</sup> It has been reported that miR-574-3p blocked the epithelial-mesenchymal transition (EMT) and promoted cisplatin sensitivity through inhibiting the activity of zinc finger E-box binding homeobox transcription factor 1 (ZEB1) in GC cells.<sup>16</sup> A study potentiated the role of miR-574-3p in blocking the cell proliferation of human CRC cells *in vitro* through targeting Cyclin D2 (CCND2), which increases cell apoptosis.<sup>17</sup>

About 15% of CRCs are diagnosed in metastatic stages (stage IV). Despite the existing screening methods and prognosis factors, there are still an excessive number of patients that are facing therapeutic failure and metastasis.<sup>18</sup> Thus, this study aims to evaluate the expression levels of miRs-133a, 27a and 574-3p as early diagnosis biomarkers of CRC and associate them with clinicopathological features of CRC patients and benign polyps patients.

## Subjects and methods

### Enrolled participants

Upon obtaining ethical approval from the Research Ethics Committee (faculty of medicine Ain shams university, R173/2022), this case-control study enrolled 120 subjects and categorized into three groups, healthy control (20 subjects), benign group (30 subjects), and malignant group (70 subjects). All participants have signed informed consent.

Patients' inclusion criteria were newly diagnosed CRC patients before having colorectal operations, receiving radio- or chemotherapy and have no other malignancies or distant metastasis, while patients did not achieve these criteria were excluded from the study. The clinicopathological data were collected from patients' clinical sheets.

### Sample size calculation

Sample size is 94 or more measurements/surveys are needed to have a confidence level of 95% that the real value is within  $\pm 5\%$  of the measured/surveyed value

### Samples collection and processing

Patients attending oncology unit at faculty of medicine Ain Shams University during period from February 2018 to December 2019 were enrolled. Blood samples collected from enrolled individuals were allowed for clotting 30 min at room temperature. Centrifugation at 10,000 g for 10 min at 4°C were followed by aliquoting and storing at  $-80^{\circ}\text{C}$  for tumor markers assessment and miRNA expression analysis.

### Determination of serum tumor markers

The quantitative measurement of human carcinoembryonic antigen (CEA) and human cancer antigen 19.9 (CA19.9) in serum was performed by the solid phase enzyme-linked immunosorbent assay using available commercial ELISA kit (Catalog No.C29-333) (Immunospec Corporation, Netherland).

### Reverse transcription (RT-q) PCR

- MiRNA extraction: Circulating miRNAs were extracted from the serum samples using miRNeasy Mini kit (Catalogue # 217004, Qiagen, Germantown, MD), as recommended by the manufacturer's instructions: miRNA extraction was carried out using 100  $\mu\text{L}$  of serum sample lysed in 500  $\mu\text{L}$  of QIAzol Lysis Reagent. The RNA was eluted using RNase-free water in a final volume 30  $\mu\text{L}$ . The purity and the concentration of the purified miRNA were detected using nano-drop spectrophotometer (Quawell, Q-500, Scribner, Staten Island, NY) and stored at  $-80^{\circ}\text{C}$  till further assessments.
- Reverse transcription and cDNA preparation: As recommended in the manufacturer's instructions using MiScript II reverse transcription kit (CAT number # 218161, Qiagen, USA) cDNA was synthesized at  $37^{\circ}\text{C}$  for 60 min in total volume of 20  $\mu\text{L}$  of reverse transcription reaction components (4  $\mu\text{L}$  MiScript HiFlex buffer, 2  $\mu\text{L}$  nucleic mixture,

2  $\mu$ L MiScript RT mixture, RNase-free water (variable depending on the volume of the added template miRNA) and template of purified miRNA with adjusted concentration 100 ng/mL). Complementary DNA concentration and purity were detected using nano-drop spectrophotometer (Quawell, Q-500, Scribner, Staten Island, NY) and stored at  $-20^{\circ}\text{C}$  till performing qPCR.

- Quantitative real-time PCR (qPCR): RT-qPCR was achieved by MiScript primer assay (Cat number 218300, Qiagen, USA) for miR-133a (Hs\_miR\_133a\_2miScript Primer Assay, MS00031423), miR-27a (Hs\_miR\_27a\_2miScript Primer Assay, MS00003241), miR-574-3p (Hs\_miR\_574-3p miScript Primer Assay, MS00032025), and reaction was performed via MiScript SYBR Green PCR kit (Cat number 218073, Qiagen, USA). The reaction for miScript primer assays was carried out by using cDNA with concentration adjusted to 2 ng/mL and a total volume of 20  $\mu$ L. PCR protocol was initial activation for 15 min at  $95^{\circ}\text{C}$  and 40 cycles of  $94^{\circ}\text{C}$  for 15s followed by annealing at  $55^{\circ}\text{C}$  for 30s and extension for 30s at  $70^{\circ}\text{C}$ . All of reactions run in triplet. Also, an endogenous control, that is, RNU6-2 (Hs\_RNU6-2\_11 miScript Primer Assay, MS00033740) was used to normalize the investigated miRNAs expression level. Fluorescence detected by Strata gene real-time PCR system (Max3005P QPCR system, Strata gene, Agilent biotechnology, Santa Clara, CA). Relative expression was normalized to an internal control (miRNA U6) and calculated according to Livak and Schmittgen<sup>19</sup> using the  $(2)^{-\Delta\Delta\text{CT}}$  method.

### Statistical analysis

SPSS was used to statistically analyze data (Statistical Program for Social Science, version 26, Inc., Chicago USA). The clinicopathological and demographic factors association with investigated miRNAs were determined using ANOVA analysis. Two-tailed  $P$  value was used to discriminate positive from negative results and considered significant if  $< 0.05$ . Spearman's correlation coefficient (CC)<sup>20</sup> were used to analyze correlations between investigated miRNAs. Receiver operating characteristic (ROC) curve was plotted between studied groups to detect the sensitivities and the specificities for the miRNAs and their diagnostic efficiency.<sup>21</sup> Area under the curve (AUC) can range from 0.5 to 1 and diagnostic tests that approach 1 designate a perfect discriminator.

### Results

In this study, 120 participants were included and divided into three groups as follows:

- Malignant group with mean age ( $48 \pm 13$ ) years
- Benign group with mean age ( $45 \pm 18$ ) years
- Healthy control group with mean age ( $45 \pm 17$ ) years

The clinical data of all enrolled participants are shown in Table 1. No significant difference was reported when relating age, gender and tumor location to clinicopathological data ( $p > .05$ ). While perfect significance was found when relating different stages to different grades and tumor size ( $p < .01$ ).

### Tumor markers levels

The mean, median, and mean rank of CEA and CA19.9 among the three groups are illustrated in Table 2. Diagnostic efficacies of CEA and CA19.9 were determined using calculated cutoff values of 5.5 ng/ml and 11.5 U/ml, respectively when plotting the ROC (Figure 1). The overall sensitivities, specificities, the positive predictive values (PPVs), the negative predictive values (NPVs) and accuracies (ACC) are shown in Table 3.

### miRNAs expression levels among investigated groups

The investigated miRNAs mean levels are described in Table 4. The malignant group showed significant lower fold change

**Table 1.** Clinical data of the studied patients' groups.

Parameter	Malignant	Benign	Control
Age			
≤47 years	55.7%	60%	60%
>47 years	44.3%	40%	40%
Gender			
Male	32 (45.7%)	11 (36.7%)	7 (35%)
Female	38 (54.3%)	19 (63.3%)	13 (65%)
Grading			
Poor	11 (15.7%)	—	—
Moderate	46 (65.7%)	—	—
Well	13 (18.6%)	—	—
Stage			
First	13 (18.6%)	—	—
Second	44 (62.9%)	—	—
Third	13 (18.6%)	—	—
Tumor size			
<5 cm	11 (15.7%)	—	—
5–9 cm	46 (65.7%)	—	—
>9 cm	13 (18.6%)	—	—
Tumor location			
Colon	18 (25.7%)	—	—
Rectum	19 (27.1%)	—	—
Both	33 (47.1%)	—	—
Lymph node metastasis			
Negative	57 (81.4%)	—	—
Positive	13 (18.6%)	—	—

**Table 2.** The mean, median, and rank of studied tumor markers.

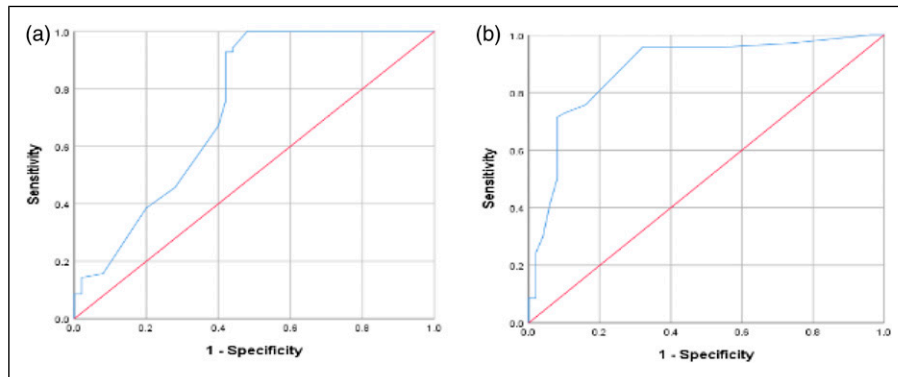
	CEA		
	Malignant	Benign	Control
Mean $\pm$ SD	10.71 $\pm$ 5.16	5.26 $\pm$ 4.14*	3.1 $\pm$ 1.07*
Median	10.5	4	3
Mean rank	79.49	41.5	22.53
	CA19.9		
Mean $\pm$ SD	13.8 $\pm$ 4.2	10.93 $\pm$ 5.58	3.1 $\pm$ 1.07**
Median	13	13	3
Mean rank	72.32	62.82	15.65

\*Perfect significance was found when relating benign and control to malignant group.

\*\*Perfect significance was found when relating control to malignant group.

level when related to the other two enrolled groups for miR-133a and miR-574-3p, while it revealed significant higher fold change level for miR-27a. ROC curve (Figure 2) plotting, showed diagnostic efficacy of miRNAs which is determined using the calculated cutoff values as 36.5-, 46.5-, and 18.5-fold change for miR-133a, miR-574-3p, and miR-27a, respectively. Areas under the curve (AUC) for miR-133a, miR-574-3p, and miR-27a were found to be 0.974, 0.975, and 0.904, respectively. The overall sensitivities, specificities, The PPV, NPV, and ACC are shown in Table 5.

As seen in Table 6 the combination of the three miRNAs (miR-133a, miR-574-3p, and miR-27a) in a single test would significantly increase sensitivities to 100%, which would be essential for early diagnosis of CRC.



**Figure 1.** Receiver operating characteristic (ROC) curve for tumor marker (a) CA19.9 (b) CEA. The best cutoff values were 11.5 U/ml for CA19.9 and 5.5 ng/mL for CEA with AUC (95% CI) 0.736 (0.639–0.834) and 0.88 (0.814–0.946) for CA19.9 and CEA, respectively, at  $p < .05$ .

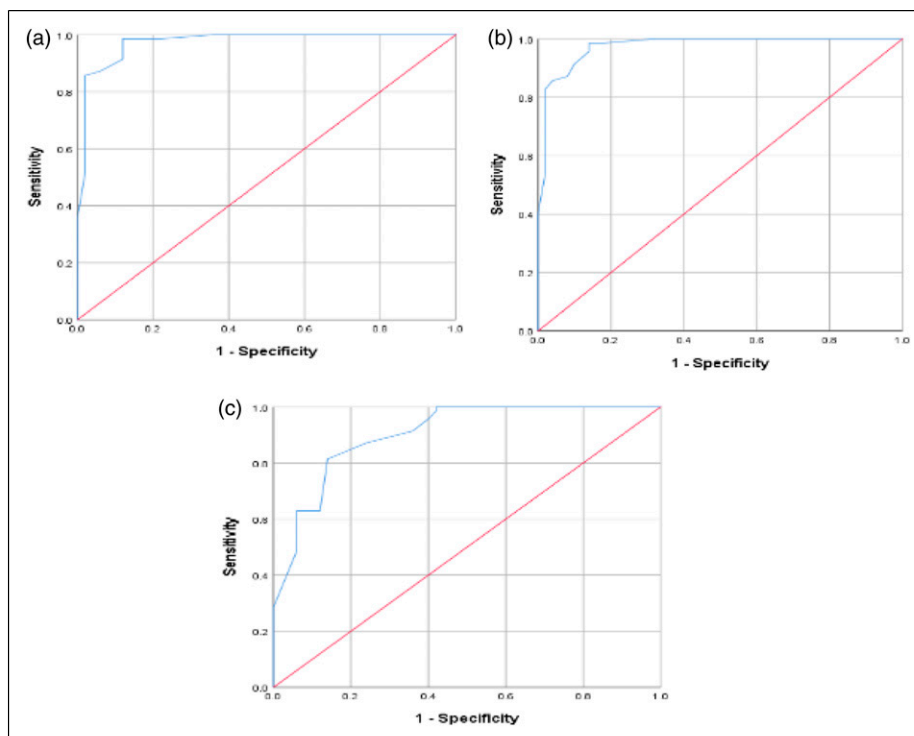
**Table 3.** ROC curve analysis for CEA and CA19.9

	AUC	95% CI	Sensitivity, %	Specificity, %	Ppv, %	Npv, %	ACC., %	$p$
CEA	0.88	0.814–0.946	75.7	84	86	72	80.8	.00
CA19.9	0.736	0.639–0.834	67.1	60	70.4	56.6	64.1	.00

**Table 4.** Expression levels of investigated miRNAs among groups.

RQ	Groups						$P$	$\chi^2*$
	Malignant		Benign		Control			
	Median	Mean rank	Median	Mean rank	Median	Mean rank		
miRNA-133a	26.00	36.78	42.5	92.5	41.5	95.53	.00	81.837
miRNA-574-3p	36.00	42.5	52.5	92.03	53.00	96.25	.00	78.001
miRNA-27a	25.00	44.71	18.00	73.00	8.00	97.00	.00	56.865

\*Kruskal–Wallis test,  $P$  is highly significant, RQ: relative quantity.



**Figure 2.** Receiver operating characteristic (ROC) curve for (a) miR-133a (b) miR-574-3p, (c) miR-27a. The best cutoff values were 36.5-, 46.5-, and 18.5-fold change for miR-133a, miR-574-3p, and miR-27a, respectively, with AUC (95% CI) 0.974 (0.948–1.000) for miR-133a, 0.975 (.948–1.000) for miR-574-3p and 0.904 (0.849–0.958) for miR-27a at  $p < .05$ .

**Table 5.** ROC curve analysis for miR-133a, miR-574-3p, and miR-27a.

	AUC	95% CI	Sensitivity, %	Specificity, %	Ppv, %	Npv, %	ACC., %	$p$
miR-133a	0.974	0.948–1.000	98.6	88	92	100	95	.00
miR-574-3p	0.975	0.949–1.000	98.6	86	90.7	97.7	93	.00
miR-27a	0.904	0.849–0.958	87.1	76	83.5	80.8	82.5	.00

**Table 6.** Combined sensitivities and specificities of studied miRNAs.

	Sensitivity	Specificity	Combined sensitivity	Combined specificity
miR-133a	98.6%	88%	—	—
miR-574-3p	98.6%	86%	—	—
miR-27a	87.1%	76%	—	—
miR-133a and miR-574-3p	—	—	100%	88%
miR-133a and miR-27a	—	—	100%	68%
miR-574-3p and miR2-7a	—	—	98.5%	70%
miR-133a, miR-574-3p, and miR-27a	—	—	100%	68%

### Diagnostic efficacy for investigated miRNAs and tumor markers

As reported in Table 7, very significant change was reported among the investigated parameters levels in CRC patients as

compared to other two studied groups with significant difference ( $p < .05$ ) for miRNAs and biomarkers (CEA, CA19.9). The mean levels of miR-133a, miR-574-3p, and miR-27a were significantly changed ( $p < .05$ ) in CRC group as compared to benign and healthy control groups.

**Table 7.** Levels and positivity rates of investigated parameters among studied groups.

Groups markers	Control	Benign lesions	Colorectal cancer	Statistics
miR-133a	95.5 (41.5)	92.5 (42.5)	36.78 (26)	$\chi^2 = 81.837^a$ $p < .05$
Mean rank (median)	0%	20%	100%	
Positivity				
miR-574-3p	96.25 (53)	92.03 (52.5)	42.5 (36)	$\chi^2 = 78.001^a$ $p < 0.05$
Mean rank (median)	5%	20%	98.57%	
Positivity				
miR-27a	97 (8)	73 (18)	44.71 (25)	$\chi^2 = 56.865^a$ $p < 0.05$
Mean rank (median)*	0%	40%	87.14%	
Positivity				
CA19.9	3.1 ± 1.07 (3)	10.93 ± 5.58 (13)	13.8 ± 4.2 (13)	$F = 44.041^b$ $p < 0.05$
Mean ± SD (median)	0%	66.67%	67.14%	
Positivity				
CEA	3.1 ± 1.07 (3)	5.26 ± 4.14 (4)	10.71 ± 5.16 (10.5)	$F = 30.248^b$ $p < 0.05$
Mean±SD (median)	0%	26.67	75.71%	
Positivity				

<sup>a</sup>Kruskal–Wallis test, P is highly significant.

<sup>b</sup>Analysis of variance (ANOVA) test.

On the other hand, extremely substantial raise in tumor markers levels (CEA and CA19.9) in CRC patients when compared to the other enrolled groups.

### Relation between miRNAs, tumor markers and clinicopathological factors

Regarding correlation of miR-133a, miR-574-3p, and miR-27a expression level positivity rate to clinicopathological data, no significant difference was found when relating the three miRNAs to age, gender, and tumor location. While perfect significant difference positivity rate was shown ( $p < .05$ ) when relating them to stage, grade, tumor size and LN metastasis among CRC patients (Table 8). No significant association was recorded between CEA as well as CA19.9 with the clinicopathological factors.

When the expressions of miR-133a, miR-574-3p, and miR-27a were used to detect the CRC grade as diagnostic biomarkers, they detected low grades from high grade of CRC with a positivity of 100% for high and low grades in miR-133a, 90% and 100% for low and high grade in miR-574-3p, 27.3% and 100% for low and high grade in miR-27a (Supplementary Table 1).

The expressions of miR-133a, miR-574-3p, and miR-27a could distinguish between early, moderate and late stages with positivity rate 100% for all stages in miR-133a, 92.3% for early and 100% for moderate and late stages in miR-574-3p, 30.8% for early stage and 100% for moderate and late stages in miR-27a (Supplementary Table 2).

A significant positivity rate was detected for the whole three miRNAs in distinguishing negative node metastasis from positive node metastasis in CRC patients. Sensitivity and specificity were 100% and 100% for miR-133a, 100%

and 98.2% for miR-574-3p, 100% and 84.2% for miR-27a, respectively (Supplementary Table 3).

### Discussion

Colorectal cancer (CRC) is the third most diagnosed cancer and second deadliest malignancy for both genders. CRC has both strong environmental associations and genetic risk factors.<sup>22</sup> These environmental and genetic factors cooperate to produce colon polyps that develop to colorectal cancer. The polyp to cancer development sequence is mainly motivated at the cellular level by gene alterations and epigenetic changes and is now accepted to be a heterogeneous process.<sup>23</sup>

The routinely used tumor markers for diagnosis and prognosis of CRC aren't significant as they are also elevated in other benign conditions. CEA is elevated in cases of hepatitis, pancreatitis, obstructive pulmonary disease and inflammatory bowel disease.<sup>24</sup> In this study, CEA sensitivity and specificity was 75.7% and 84%, respectively, it showed no significant difference to tumor grade and stage, which is consistent with Gao et al, 2018.<sup>25</sup> In addition, CA19.9 has been reported to increase in many types of cancer.<sup>26</sup> In this study, CA19.9 had sensitivity of 67.1% and specificity of 60% with no significant difference when related to tumor staging, grading and lymph node metastasis. The results obtained from this study showed that these tumor markers cannot be used alone for diagnosis of CRC which is consistent with Lakemeyer et al, 2021.<sup>27</sup> So, we aimed to find more precise markers that can be used for faster diagnosis and better prognosis such as miRNAs.

Recently miRNAs are heavily investigated to provide a new way for understanding development and progression



**Table 8.** Relation between positivity rates of investigated parameters with clinicopathological data among colorectal cancer patients.

Clinicopathological data	CEA		CA19.9		miR-133a*		miR-574-3p*		miR-27a*	
	<5.5 ng (negative)	>5.5 ng (positive)	<11.5 U (negative)	>11.5 U (positive)	>36.5 ng (negative)	<36.5 ng (positive)	>46.5 ng (negative)	<46.5 ng (positive)	<18.5 ng (negative)	>18.5 ng (positive)
<b>Age</b>										
≤48 years(n) = 69	10 (25.6%)	29 (74.4%)	10 (43.5%)	29 (61.7%)	0	39 (100%)	1 (2.6%)	38 (97.4%)	6 (15.4%)	33 (84.6%)
>48 years(n) = 51	7 (22.6%)	24 (77.4%)	13 (56.5%)	18 (38.3%)	0	31 (100%)	0	31 (100%)	3 (9.7%)	28 (90.3%)
	F 0.18	p > .05	F 1.169	p > .05	F 0.547	p > .05	F 0.062	p > .05	F 0.393	p > .05
<b>Gender</b>										
Male (n) = 50	11 (34.4%)	21 (65.6%)	12 (52.2%)	20 (42.6%)	0	32 (100%)	0	32 (100%)	3 (9.4%)	29 (90.6%)
Female (n) = 70	6 (15.8%)	32 (84.2%)	11 (47.8%)	27 (57.4%)	0	38 (100%)	1 (2.6%)	37(97.4%)	6 (15.8%)	32 (84.2%)
	F 0.939	p > .05	F 0.56	p > .05	F 0.611	p > .05	F 0.604	p > .05	F 0.075	p > .05
<b>Stage</b>										
First (n) = 13	3 (23.1%)	10 (76.9%)	4 (17.4%)	9 (19.1%)	0	13 (100%)	1 (7.7%)	12 (92.3%)	9(69.2%)	4 (30.8%)
Second (n) = 44	12 (27.3%)	32 (72.7%)	14 (60.9%)	30 (63.8%)	0	44 (100%)	0	44 (100%)	0	44 (100%)
Third (n) = 13	2 (15.4%)	11 (84.6%)	5 (21.7%)	8 (17%)	0	13 (100%)	0	13 (100%)	0	13 (100%)
	F 0.818	p > .05	F 0.09	p > .05	F 88.238	p < .05*	F 47.242	p < .05*	F 195.337	p < .05
<b>Grade</b>										
Poor (n) = 11	3 (27.3%)	8 (72.7%)	3 (13%)	8 (17%)	0	11 (100%)	1 (9.1%)	10 (90.9%)	8 (72.7%)	3 (27.3%)
Moderate (n) = 46	12 (26.1%)	34 (73.9%)	15 (65.2%)	31 (66%)	0	46 (100%)	0	46 (100%)	1 (2.2%)	45 (97.8%)
Well (n) = 13	2 (15.4%)	11 (84.6%)	5 (21.7%)	8 (17%)	0	13 (100%)	0	13 (100%)	0	13 (100%)
	F 1.077	p > .05	F 0.147	p > .05	F 113.031	p < .05*	F 54.034	p < .05*	F 168.959	p < .05
<b>Tumor size</b>										
<5 cm (n) = 11	3 (27.3%)	8 (72.7%)	3 (13%)	8 (17%)	0	11 (100%)	1 (9.1%)	10 (90.9%)	8 (72.7%)	3 (27.3%)
5–9 cm(n) = 46	12 (26.1%)	34 (73.9%)	15 (65.2%)	31 (66%)	0	46 (100%)	0	46 (100%)	1 (2.2%)	45 (97.8%)
>10 cm (n) = 13	2 (15.4%)	11 (84.6%)	5 (21.7%)	8 (17%)	0	13 (100%)	0	13 (100%)	0	13 (100%)
	F 1.077	p > .05	F 0.147	p > .05	F 113.031	p < .05	F 54.034	p < .05*	F 168.959	p < .05
<b>Tumor location</b>										
Colon (n) = 18	6 (33.3%)	12 (66.7%)	3 (13%)	15 (31.9%)	0	18 (100%)	0	18 (100%)	1 (5.6%)	17 (94.4%)
Rectum(n) = 19	5 (26.3%)	14 (73.7%)	9 (39.1%)	10 (21.3%)	0	19 (100%)	0	19 (100%)	3 (15.8%)	16 (84.2%)
Both(n) = 33	6 (18.2%)	27 (81.8%)	11 (47.8%)	22 (46.8%)	0	33 (100%)	1 (3%)	32 (97%)	5 (15.2%)	28 (84.8%)
	F 0.121	p > .05	F 0.1808	p > .05	F 0.707	p > .05	F 0.468	p > .05	F 0.029	p > .05
<b>LN metastasis</b>										
Negative (n) = 57	15 (26.3%)	42 (73.7%)	18 (31.6%)	39 (68.4%)	0	57 (100%)	1 (1.8%)	56 (98.2%)	9 (15.8%)	48 (84.2%)
Positive(n) = 13	2 (15.4%)	11 (84.6%)	5 (38.5%)	8 (61.5%)	0	13 (100%)	0	13 (100%)	0	13 (100%)
	F 0.488	p > .05	F 0.148	p > .05	F 38.45	p < .05	F 18.526	p < .05	F 172.040	p < .05

Statistics were done using one way ANOVA.

\*Significant positivity rate p &lt; .05.

of malignant tumors. MiRNAs are Small non-coding RNA (18–25 nt) that can stop protein expression by breaking specific target mRNAs or by inhibiting their translation.<sup>28</sup> They mediate gene regulation by fusing with the 3-untranslated region (3'UTR) sequences of the target mRNAs, resulting in degradation of mRNA or translational silencing. MiRNAs perform vital roles in many cellular activities, involving cell proliferation, differentiation, programmed cell death, and growth. These miRNAs are considered to control the expression of about one-third of individual protein-coding genes.<sup>29</sup> Also, miRNAs are stable, reproducible, and consistent.<sup>30</sup>

Among the miRNAs, miR-133a is believed to be a tumor suppressor and a biomarker for prediction of several cancers, such as breast cancer, bladder cancer, gastric cancer, esophageal cancer (EC), osteosarcoma, and non-small cell lung cancer (NSCLC).<sup>31</sup> The results obtained from the current study showed that miR-133a is down-regulated in CRC and that it has perfect significance when related to tumor staging, grading and lymph node metastasis with sensitivity 98.6% and specificity 88% suggesting its role as important biomarker for diagnosis and prognosis of CRC consistent with Wan et al., 2014 and Caporali et al., 2021.<sup>32,33</sup>

In the current study, miR-133a expression was considerably decreased in the serum of CRC patients with late stage and low grade, consistent with earlier reports by Luo et al., 2013,<sup>34</sup> which also contradicts many studies, indicating that miR-133a is not simply an oncogene as many papers reported.<sup>31</sup>

MiR-574-3p has been verified as a tumor suppressor in several cancers such as prostate cancer, bladder cancer, gastric cancer, ovarian cancer and chronic myeloid leukemia.<sup>14</sup> Our results demonstrated that miR-574-3p is downregulated in CRC patients with sensitivity 98.6% and specificity 86% suggesting its role as valuable biomarker for diagnosis and prognosis of CRC.

It was illustrated that miR-574-3p expression positivity rate was correlated with the tumor size, clinical stage and lymph node metastasis ( $p < .05$ ). However, no correlation was found between the miR-574-3p expression level and age, gender or tumor location ( $p > .05$ ) which was consistent with the *in vitro* study of Li et al., 2019.<sup>17</sup>

Studies have shown that miR-27a showed a critical role in cancer biology, including polymorphisms, carcinogenesis, proliferation, programmed cell death, infiltration, metastasis, and angiogenesis.<sup>35</sup> MiR-27a acts as oncogenic miRNA that is upregulated and overexpressed in numerous cancers including gastric cancer, osteosarcoma, liver cancer, cervical cancer, ovarian cancer, pancreatic cancer, lung adenocarcinoma, breast carcinoma, and renal cell carcinoma. While it acts as tumor suppressor miRNA in esophageal squamous cell carcinoma, prostate cancer, and acute leukemia.<sup>36</sup> We found that miR-27a is upregulated in

CRC as demonstrated by You et al., 2021<sup>37</sup> with sensitivity 87.1% and specificity 76%.

The present study showed elevated relative expression level of miR-27a associated with tumor stage, tumor grade and lymph node metastasis in patients with colorectal cancer, which is in line with others.<sup>38</sup>

Statistical analysis of data obtained showed that combining both miR-133a and miR-574-3p together present perfect possible tumor markers for CRC with higher sensitivity (100%) and specificity (88%) than other markers used as illustrated in table (6). Combining the three miRNAs together suggests even better markers for early detection and prognosis of CRC with higher sensitivity (100%) than regular tumor markers used.

### Limitations

The unavailability of a huge quantity of patients, non-cooperation of patients and incomplete patient records, were some of the limitations of this study. For this reason, further validation studies with larger cohorts are needed, and comparability tests are required to confirm the linkage of serum miRs-133a, 27a and 574-3p with CRC and their targeted pathway.

### Conclusion

In conclusion, microRNAs are helpful in diagnosing colorectal cancer, regulating disease progression, predicting disease recurrence, and determining therapy success. Synergetic detection of miRNA-133a miR-27a and miRNA-574-3p serve as novel tumor marker with better sensitivity and specificity and help in early detection of CRC and with less invasive or surgical interference for such widely spread malignancy which could result in better prognosis and higher survival rate for CRC patients.

Also, overall, sensitivities and positive predictive values for miRNA-133a, miR-27a, and miRNA-574-3p were superior to the routinely tumor markers (CA19.9 and CEA) for early detection of CRC, especially with patients at early stage and low grade. These findings point out the value of using miRNA-133a, miR-27a, and miRNA-574-3p as early diagnostic molecular markers in CRC.

Furthermore, serum levels of miRNA-133a, miR-27a, and miRNA-574-3p can be used as a fair noninvasive method for early detection of colorectal cancer and differentiating between CRC histologic grade, stages, and lymph node metastasis.

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Ethical approval for this study was obtained from the Research Ethics Committee (faculty of medicine Ain shams university, R173/2022).

### Informed consent

Written informed consent was obtained from all subjects before the study.

### ORCID iD

Marwa GA Hegazy  <https://orcid.org/0000-0002-6949-3304>

### Supplemental Material

Supplemental material for this article is available online.

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