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Role of circulating miRNA-17-3P as a potential diagnostic biomarker for sporadic colon cancer in Egyptian cohort

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Abstract

Background: Cancer colon is one of the leading causes of death and MiRNAs are incorporated in biological pathways that affect tumorigenesis as proved by multiple studies. The present study aimed to investigate whether miR-17-3p is elevated in the plasma free samples of colon cancer patients in correlation with other tumor markers (CEA, CA19.9).

Results: MiRNA 17-3P plasma free sample levels were significantly elevated in the plasma free samples of colon cancer patients compared with healthy controls ($P < 0.001$); on the other hand, serum levels of CA19.9 were significantly higher in colon cancer patients compared to healthy controls while serum levels of CEA were found to be of no statistical significance.

Conclusion: The detection miRNA-17-3p may be considered of clinical value for the detection of colon cancer; also, CA19.9 detection was found to significantly increase the sensitivity and specificity of a colon cancer diagnosis.

Keywords: miR-17-3p, Carcinoembryonic antigen, CA19.9, Colon cancer, Diagnostic biomarker

Background

All over the world, colon cancer is estimated to be the 2nd most common type of cancer in females (614,000 cases representing ~ 9.2% of all cancers) and the 3rd most common type in males (746,000 cases representing ~ 10.0% of the total). Early detection of patients with cancer has a favorable outcome with treatment, with the 5-year survival rates exceeding 70%. For several decades, colonoscopy has been used as the first choice for screening of early-stage colon cancer; but because of its invasive character, patients show low compliance in the clinical setting. Thus, cost-effective and non-invasive biomarkers with high sensitivity and specificity are essentially required to help early detection of colon cancer [1].

Recently detected microRNAs (miRNAs) are endogenous, small, non-coding evolutionarily conserved RNA (~ 22–25 nucleotide length), also referred as micro-coordinators of gene expression that have been revealed to be an efficient method to study the biology of various diseases and to be used as a novel diagnostic and prognostic biomarkers with a great level of specificity and sensitivity. Additionally, miRNAs are characterized by a high degree of stability so they could be isolated from body fluids and tissues and can be measured easily [2].

MiRNAs are incorporated in various biological pathways that may affect tumorigenesis and progression and also may function as oncogenes or tumor suppressors, based on the downstream effects produced by the affected miRNA. Although the miRNA function is not completely understood yet, they have been accompanied by various important molecular pathways that have been related to carcinogenesis and tumor progression as proved by multiple experimental models and clinical

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studies that were carried out on different types of cancer, including colon cancer [2].

MiRNAs have been assumed to be a potential reliable biomarker for screening of cancer because they are highly stable and can be measured easily using the common laboratory methods. In 2015, Kara et al. [3] conducted a study aiming to evaluate the expression profile of some cancer-related genes and their regulatory miRNA molecules. In this study, they used a high-throughput real-time PCR method on 54 colon cancer patients and normal colon tissue samples of 42 healthy controls. Their results revealed that a wide range of miRNA types, including miRNA-17-3p were significantly deregulated in colon cancer. They strongly concluded the possible involvement of novel cancer-related genes and their regulatory miRNAs in colon cancer physiopathology along with the probability of using this miRNA as a biomarker for early detection of colon cancer.

A lot of studies have thrown the light on the role of various biomarkers in the progression and diagnosis of colon cancer. These markers include CEA and CA19.9. Recently, the gene encoding CEA has been categorized as a member of the immunoglobulin supergene family that includes gene encoding for adhesion molecules as intercellular adhesion molecule 1(ICAM-1), major histocompatibility (MHC) antigen, and lymphocyte function-associated antigen [4].

The application of CEA as a marker for the diagnosing of colon cancer is limited because it is neither sensitive nor specific, mainly at the onset of the disease. At a cutoff of 2.5 µg/L, the range of sensitivity is about 30–80% according to the stage of the disease. But, as concluded by Fletcher, in symptomatic patients, the sensitivity of CEA is more elevated than asymptomatic subjects as the symptomatic group usually has more advanced disease [5].

CA19-9 is a Sialylated Lewis antigen of the MUC1 protein [6]. The American Society of Oncology does not encourage the use of CA19-9 because it is less sensitive in asymptomatic, non-advanced cases of colon cancer giving false negative results. Also, it is present in non-cancer cases (false positive) [7].

CA19-9 is less specific in colon cancer as it may be found in various types of tumors such as pancreatic, gastric cancers and hepatocellular carcinoma, as well as in benign cases such as bile duct diseases as obstruction of the bile duct. Besides, subjects who do not have the Lewis antigen “(a blood type antigen on RBCs) who represent approximately 10% of the Caucasian population,” do not express CA19-9, even if they have large tumors. This is due to a deficiency of a fucosyl transferase enzyme which is required for the production of CA19-9 and the Lewis antigen [7]. Both CEA and CA19-9 biomarkers are considered of prognostic value better than a diagnostic tool.

The present study aimed to investigate whether miR-17-3p is elevated in the blood of colon cancer patients with different stages and assess their role as a novel biomarker for this type of cancer, and if there is any role of this biomarker in staging in correlation with other well-known tumor markers (CEA, CA19.9).

Methods

Patients

Peripheral blood samples were obtained from 50 newly diagnosed colon cancer Egyptian patients who were attending the outpatient clinics at the Alexandria Main University Hospital, Gastroenterology unit (Alexandria, Egypt) between 2016 and 2018. An additional 25 peripheral blood samples were obtained from age- and sex-matched healthy volunteers as control group patients were classified based on TNM and pathological staging system into two groups (group A: 25 patients with stage II/III and group B: 25 patients with stage VI), and patients did not receive any line of treatment before sample withdrawing.

Sample collection

Peripheral blood samples were collected into plain vacuum tubes. Immediately after collection of the samples, a 2-step centrifugation protocol was performed at room temperature (1800 g for 10 min, then 3000 g for 10 min) to get platelet-poor, cell-free plasma. Aliquots were frozen in – 80 °C till further processing, and miR-16 was used as the endogenous control.

Relative quantification of microRNA- 17-3-p expression

Total RNA including miRNA was purified from the cell-free plasma samples and was done using the miRNeasy extraction kit (Qiagen, Germany). QIAzol. Single-stranded cDNA was produced from purified samples of RNA using the TaqMan® MicroRNA RT Kit with miRNA primers specific for miRNA-17-3-p (ID 000392) and miR-16 (ID 000391) (Applied Biosystems, USA) according to the manufacturer's protocol. Real-time PCR (260) for relative quantifications of miRNA 17-3P was performed on the Stratagene mx3005p qPCR system. PCR reaction mix was prepared as summarized in Table 1.

Table 1 qPCR reaction mix

PCR reaction mix components	Volume (µL)/ 20-µL Reaction	Final concentration
TaqMan universal master mix II, with no UNG, 2×	10.0	1×
TaqMan MicroRNA Assay (20×)	1.0	1×
cDNA/DNA template + RNase free water	9.0	1 to 100 ng
Total volume	20.0	–

Data analysis

The cycle threshold (Ct) values were assessed using the SDS 2.0.1 software (Applied biosystems). The average expression levels of miR-17-3p were normalized via miR-16 (Applied biosystems) as a reference gene, and subsequently, the $2^{-\Delta\Delta CT}$ method was applied.

Serum CEA determination

CEA was assayed using an immune radiometric assay (ADVIA Centaur XP (Siemens Diagnostics, Tarrytown, NY, USA), and the threshold for a positive result was 10 ng/ml. The assay was performed based on the manufacturer's instructions.

Serum CA19.9 determination

CA19.9 was assayed with an immunoradiometric assay (ADVIA Centaur XP (Siemens Diagnostics, Tarrytown, NY, USA)), and the threshold for a positive result was 37 ng/ml. The assay was carried out based on the manufacturer's instructions

Statistical analysis of the data

Data were fed to the computer and analyzed using the IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). Qualitative data were described using the number and percentage. Quantitative data were described using range (minimum and maximum), mean, standard deviation (SD), and median. The significance of the obtained results was judged at the 5% level.

Results

Table 2 shows the comparison between patients and controls as regards demographic data. There were no differences between patients and controls regarding gender distribution, as both had 56% females and 44% males. The mean age was 50.18 ± 13.24 for the patient's group; however; it was 48.24 ± 15.06 for the control group. There were no significant statistical differences between patients and controls as regards gender or age distribution.

Table 2 Baseline characteristics of the study population

	Patients (n = 50)		Control (n = 25)		Test of sig.	p
	No.	%	No.	%		
Gender						
Male	22	44.0	11	44.0	$\chi^2 = 0.00$	1.000
Female	28	56.0	14	56.0		
Age (years)						
Min.–max.	25.0–77.0		20.0–81.0		t = 0.571	0.571
Mean ± SD	50.18 ± 13.24		48.24 ± 15.06			
Median	50.0		50.0			

χ^2 Chi-square test, t Student's t test, p p value for comparing between the studied groups

Table 3 Levels of miR-17-3p, CEA, and CA19.9 in studied population (patients and control)

miRNA	Patients (n = 50)	Control (n = 25)	U	p
Min.–max.	1.74–32.45	0.27–3.41	5.00*	> 0.001*
Mean ± SD	9.07 ± 7.25	0.95 ± 0.63		
Median	6.85	0.88		
CEA				
Min.–max.	0.10–110.0	0.10–14.0	465.50	0.073
Mean ± SD	8.63 ± 20.83	2.94 ± 3.24		
Median	2.90	2.20		
CA19.9				
Min.–max.	0.25–700.0	5.0–30.0	302.50*	< 0.001*
Mean ± SD	39.40 ± 97.61	9.56 ± 5.20		
Median	20.45	8.0		

Using the Mann-Whitney U test, the microRNA 17-3P levels were found to be significantly elevated in the cell-free plasma samples of colon cancer patients compared with healthy controls ($P < 0.001$), (Table 3); also, miR-17-3p exhibited significantly different expression levels ($P < 0.001$) between stage II/III and stage VI colon cancer patients (Table 4). On the other hand, serum levels of CA19.9 were also found to be significantly higher in colon cancer patients compared to healthy controls ($P < 0.001$) (Table 3) along with significant differences between the cell-free plasma sample level of the same marker between stage II/III and stage VI colon cancer patients, yielding an assumed role in staging ($P < 0.001$) (Table 4); but on the contrary, serum level of CEA was found of no statistical significance either between colon cancer patients and control or between patients of different stages (Tables 3 and 4).

Using the ROC curve analysis to predict patients from control, the AUCs for miR-17-3p were 0.996 (95% CI

Table 4 Levels of microRNA 17-3P, CEA, and CA19.9 among group A and group B patients

miRNA	Group A (stage II + III) (n = 25)	Group B (stage IV) (n = 25)	U	P
Min.–max.	1.74–29.86	2.71–32.45	1.0	< 0.001*
Mean ± SD	7.48 ± 6.92	10.66 ± 7.37		
Median	5.28	8.69		
CA19.9				
Min.–max.	0.25–63.0	0.62–700.0	93.0	< 0.001*
Mean ± SD	19.95 ± 17.25	58.86 ± 135.5		
Median	12.0	32.0		
CEA				
Min.–max.	0.70–41.0	0.10–110.0	296.0	0.749
Mean ± SD	5.48 ± 8.09	11.78 ± 28.28		
Median	2.90	2.70		

Table 5 Agreement (sensitivity, specificity) to predict patients vs control

	AUC	p	95% CI		Cutoff	Sensitivity (CI)	Specificity (CI)	PPV (CI)	NPV (CI)
			L.L	U.L					
miRNA	0.996*	< 0.001*	0.989	1.003	> 1.61	100 (100–100)	96 (88.3–100)	96.2(94–100)	100 (100–100)
CA19.9	0.758*	< 0.001*	0.650	0.866	> 9.3	70.0(57.3–82.7)	68.0 (49.7–86.3)	81.4(69.7–93.0)	53.1 (35.8–70.4)
CEA	0.628	0.073	0.491	0.765	> 2.29	56 (42.3–69.7)	52 (32.4–71.5)	70 (55.8–77.3)	37.1 (43.4–65.9)

AUC area under a curve, p value probability value, CI confidence intervals, NPV negative predictive value, PPV positive predictive value

*Statistically significant at $p \leq 0.05$

0.989–1.003) and for CA19.9 was 0.785 (95% CI 0.650–0.866), respectively. At a threshold of 1.6 ng/ml for miRNA, the optimal sensitivity and specificity were 100% and 96%, respectively, in separating colon cancer patients from normal controls. At a threshold of 9.3 for CA19.9, the sensitivity and the specificity were 70% and 68%, respectively, denoting a significant role for both markers in the detection of colon cancer (Table 5) (Fig. 1).

Using the ROC curve analysis to predict patients with stage V from those with stage II/III, the AUCs for miR-17-3p were 0.713 (95% CI 0.859–0.566) and for CA19.9 was 0.682 (95% CI 0.832–0.531), respectively. At a threshold of 6.87 ng/ml for miRNA, the optimal sensitivity and specificity were 68% and 72%, respectively, in separating colon cancer patients with stage V from stage III. At a threshold of 12 ng for CA19.9, the sensitivity and the specificity were 84% and 52%, respectively, denoting a promising role for both markers for staging and prognosis of colon cancer. On the other hand, CEA was

proved to be of no statistical significance in staging with AUC of 0.526 (95% CI 0.360–0.693) (Table 6, Fig. 2).

Discussion

Colon cancer should be screened and diagnosed as early as possible. Early diagnosis has a great influence on the therapeutic plan along with the outcome. Some reports have revealed the existence of non-coding (non-translational) RNAs (ncRNAs) that do not carry any genetic information concerning protein synthesis. Multiple ncRNA subtypes have been discovered. Cancer cell-derived exosomes may serve to promote both tumorigenesis and metastasis by influencing other types of cells in the tissue microenvironment [8]. Exosomes have been determined as the major carriers for miRNAs in serum [9]. They can be extracted from different body fluids such as serum, urine, milk, lymph, bile, and saliva and remain stable for long-term storage and expression profiling.

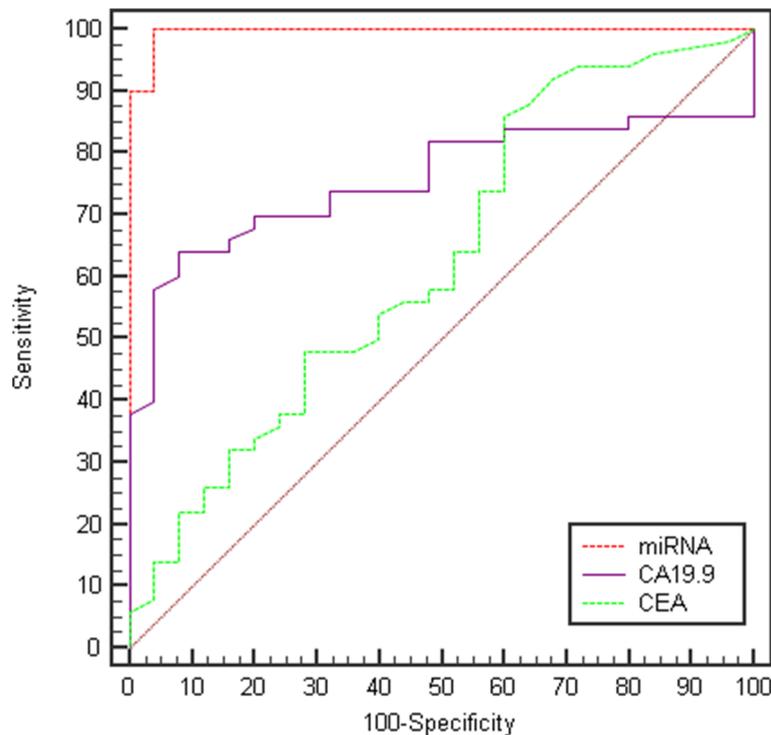


Fig. 1 ROC curve analysis to predict patients vs control

Table 6 Agreement (sensitivity, specificity) for different parameters to predict patients with stage IV from stage II + III

	AUC	<i>p</i>	95% CI		Cutoff	Sensitivity (CI)	Specificity (CI)	PPV (CI)	NPV (CI)
			L.L	U.L					
miRNA	0.713*	0.010*	0.566	0.859	> 6.87	68.0 (46.5–85.1)	72.0 (50.6–87.9)	70.8 (52.56–89.0)	69.2 (51.4–86.9)
CA19.9	0.682*	0.028*	0.531	0.832	> 12	84.0 (69.6–98.4)	52.0 (32.4–71.6)	63.6 (47.2–80.1)	76.5 (56.3–96.6)
CEA	0.526	0.749	0.360	0.693	≤ 2.7	52.0 (32.4–71.6)	56 (36.54–75.5)	54.2 (34.23–74.1)	53.8 (34.7–73.0)

AUC area under a curve, *p* value probability value, CI confidence intervals, NPV negative predictive value, PPV positive predictive value

*Statistically significant at $p \leq 0.05$

The main finding of the present results was the significant difference between the level of miRNA in patients with colon cancer and its level in healthy adults. This suggests a value of miRNA as a biomarker for colon cancer detection.

Fu et al. [10] conducted a recent study in 2018 aiming to detect candidate exosomal miRNAs that might be associated with colon cancer and its distant metastasis.

Their results revealed that 25 miRNAs had been up-regulated, and 5 miRNAs had been downregulated in exosomes purified from the SW620 culture supernatant. Then, they assessed candidate miRNAs for diagnosing colon cancer using quantitative RT-PCR in colon cancer patients and proved significantly elevated expression levels of circulating both exosomal miR-17-3p and miR-92a-3p. They had a conclusion that circulating exosomal miR-17-3p and miR-92a-3p might offer a promising non-invasive diagnostic as well as a prognostic biomarker for primary and metastatic colon cancer.

The meta-analysis of Masuda and his colleagues [11] also matched our results. They studied the recently discovered types of miRNA including miR17-3p and assess its association with colon cancer. They reported that miRNAs have considerable potential as biomarkers as well as therapeutic targets. This might be explained by the fact that miRNAs can induce and modulate tumorigenesis and tumor progression in colon cancer. Nevertheless, they confirmed that the clinical importance of miRNAs as biomarkers is not conclusive yet, and independent validation studies are required for clinical application.

Many recent and even old studies have previously revealed the correlation between CEA level and colon cancer. Su et al. [12] tried to detect whether serum levels of CEA are correlated with the presence of primary colon cancer and/or recurrent colon cancer after radical resection. Nevertheless, as a result of the limited number of cases in our study, the current results in that CEA levels

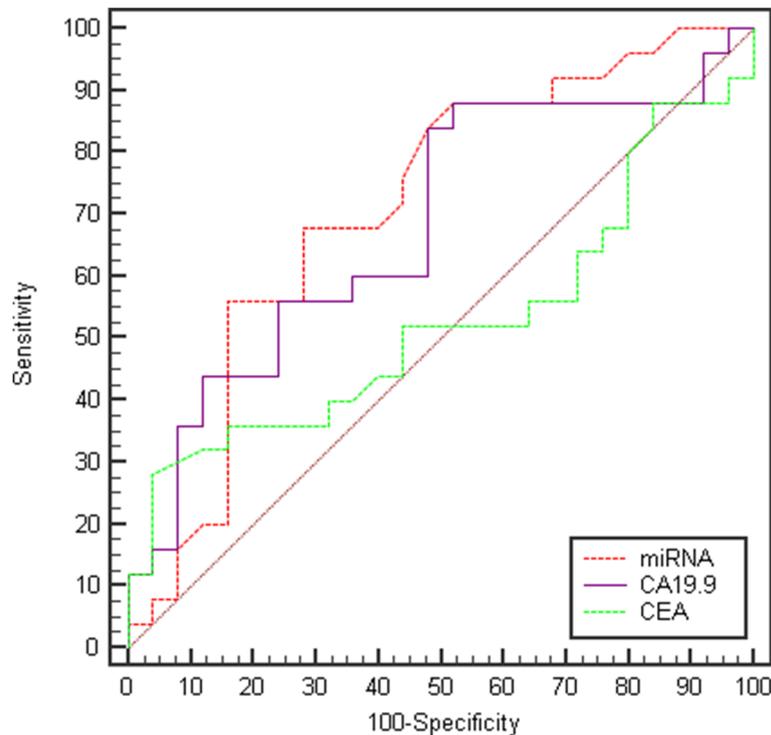


Fig. 2 ROC curve to predict stage IV from stage II + III

were not significantly higher in patients with colon cancer. Sue et al. [12] found that the overall sensitivity of CEA for the determination of primary colon cancer was 37.0%, whereas for recurrence, the sensitivity was 57%. Duffy and his colleagues [13] also reported that the CEA level was significantly higher in colon cancer patients, and that the elevated level was associated with poor prognosis.

Another essential finding of the current results was that levels of CA19.9 were significantly higher in patients with advanced colon cancer in comparison with the healthy controls. On the other hand, a significant relation was determined in advanced stages of colon cancer patients compared to the earlier stages. This was by following Vukobrat-Bijedic et al. [14] who showed that CA19.9 was a marker of advanced stages of colon cancer not for early ones. Also, Stikma et al. [15] agreed to the current results as they reported that CA19.9 was significantly higher in colon cancer patients and ensured the possibility of using it as an additional marker to follow the disease process in colon cancer patients without increased CEA level.

Conclusion

The studied miRNA 17-3P expression was found to be a sensitive and specific biomarker for the diagnosis of cancer colon with high diagnostic accuracy. It was shown that the mean value of micro-RNA-17-3p in patients with cancer colon was significantly higher than the mean value in normal healthy control ($p < 0.001$).

The mean value of micro-RNA-17-3p in patients with cancer colon stage V was higher than the mean value in patients with cancer colon stage II/III; however, it was not statistically significant ($p < 0.081$). It was also noticeable to mention that at a threshold value of 1.6 ng/ml for miRNA, the optimal sensitivity and specificity were 100% and 96%, respectively, in separating colon cancer patients from normal controls.

CA19.9 levels were found to significantly increase sensitivity and specificity in colon cancer diagnosis.

Abbreviations

AUC: Area under the ROC curve; CA19.9: Carbohydrate antigen 19.9; CEA: Carcinoembryonic antigen; CI: Confidence interval; ROC: Receiver operating characteristic

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Authors' contributions

HH study design. EA study design and data collection. AE data collection and interpretation. MA data collection and interpretation. RA laboratory work. KS writing services. All authors have read and approved the manuscript and approved the submitted version and agreed both to be accountable for the authors own contribution.

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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.

Ethics approval and consent to participate

Written informed consent was obtained from all the study participants, and the study was approved by the Ethics Committee of the Alexandria faculty of medicine. (Alexandria, Egypt) (reference number is not applicable and not available).

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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