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# Biochemical value of microRNA-223 in colon cancer Egyptian patients

**Sara M. Muhammad[1*], Abeer I. Abd El-Fattah[1], Abeer Ismail[2], Muhammad S. Mostafa[3], Ola E. Elazazy[4]**

[1] Biochemistry and Molecular Biology Department, Faculty of Pharmacy (Girls), Al-Azhar University
[2] Clinical and Chemical Pathology Department, National Cancer Institute (N.C.I.), Cairo University.
[3] Medical Oncology Department, Faculty of Medicine, Cairo University
[4] Biochemistry Department, Faculty of Pharmacy, Badr University in Cairo (BUC).

***** Correspondence: saramahmoudmuhammad@gmail.com

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**Abstract:** One of the highly prevalent cancers worldwide is Colorectal cancer (CRC). MicroRNAs (miRNAs) are small non-coding molecules. A candidate miRNA whose expression was correlated with tumor aggressiveness and poor prognosis in colon cancer patients is miR-223. Furthermore, miR-223 could be used as a diagnostic marker for CRC and a therapeutic target as well. The present study aimed to examine the potential of miR-223 to diagnose CRC in Egyptian patients and correlate its level with carcinoembryonic antigen (CEA), carbohydrate antigen 19.9 (CA 19-9), and different stages of colon cancer. The study included 60 individuals selected from the National Oncology Institute; the subjects were classified into two groups: 20 healthy control group volunteers and 40 colon cancer patients group, which were subdivided into 3 sub-groups according to severity: Low-risk colon cancer group which included stages (zero-one): 15 subjects, Intermediate-risk colon cancer group which includes stage (two): 13 subjects and High-risk colon cancer group which includes stages (three-four): 12 subjects Evaluation of serum levels of CEA and CA 19.9 was done through sandwich ELISA and miR-223 was done through RT-PCR. There was a significant increase in miR-223 expression levels in patient groups compared to the control group. There was a strong positive significant correlation between the expression levels of miR-223 and different degrees of risk. Moreover, the serum levels of miR-223 had an Area under the Curve (AUC) value of 0.997, with 100% sensitivity and specificity of 90% at $p<0.05$. Thus, miR-223 could be used as a diagnostic and prognostic marker in CRC.

**Keywords:** CRC; miR-223; miRNA; CEA; CA 19-9; biomarker.



## 1. INTRODUCTION

Colorectal cancer (CRC) ranks third among all cancer types and is one of the leading causes of mortality worldwide [1,2]. CRC risk factors include genetic history, age, and several chronic illnesses. While a family history of CRC accounts for a quarter of total cases and inherited colorectal syndromes for 10%, over 65% of total cases develop spontaneously in average-risk individuals [3]. Nevertheless, research has shown that adhering to healthy behaviors and habits can lower CRC risk by half [4]. Moreover, the slow development of CRC into invasive end-stage cancer makes prevention and early diagnosis crucial. Thus, early cancer detection by screening allows for a better prognosis since the precancerous growths can be removed [5]. As a result, developing novel, unconventional methods for CRC detection may reduce cancer prevalence and improve disease outcomes and patients' quality of life [6]. Thus, there is a never-ending need for optimizing a diagnostic and prognostic biomarker that can characterize carcinogenesis genetically and epigenetically [7].

MicroRNAs (miRNAs) are one of the non-coding ribonucleic acids extensively studied in the past decade; they have powerful regulatory roles and are associated with several pathologies and malignancies [8]. They can regulate gene expression before, during, and after transcription [9]. Apoptosis, angiogenesis, differentiation, and the cell cycle are only a few of the many essential activities that miRNAs govern [10]. Altered miRNA expression is a signature of illness and could occur due to epigenetic effects [11]. Thus, miRNAs are a promising target in



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diagnosing and staging CRC; also, their high tissue specificity and pivotal involvement in oncogenesis render them useful in predicting treatment outcomes [12]. MiRNA-223, amyeloid-derived miRNA, was reported as onco-miRNA in various cancers such as ovarian [13], hepatocellular carcinoma [14], and bladder cancer [15]. It was found that miR-223 targets the survival and death-related genes, and the miR-223 knockdown decreased CRC cell proliferation, migration, and invasion [16]. Also, miR-223 in immune cells was found to facilitate cell fate decisions [17].

Current screening methods for early identification of CRC including the carcinoembryonic antigen (CEA) [18] and cancer antigen 19-9 (CA19-9) [19], are useful but hampered mostly by poor specificity and sensitivity [20], that accounts for the importance of a specific and sensitive biomarker to identify vulnerable population. Therefore, this study was done on Egyptian patients who have colon cancer to assess the diagnostic value of miR-223 in colon cancer, and correlate its levels with CEA, CA 19-9 and with different stages.

## 2. METHODS

### 1.1 Study population:

Sixty individuals of both sexes were enrolled into the study, selected from the National Oncology Institute, between April 2018 and January 2019, after receiving the approval on the study protocol from Al-Azhar University's Faculty of Pharmacy (Girls) ethical committee (No. 152), and in compliance with the Helsinki Declaration, the subjects were assigned into four groups after the nature of the study was explained and written informed consents collected then divided into; Control group (20 systemically healthy individuals), CRC group subdivided into three group according to their severity; Low-risk colon cancer group (15 patients in stages zero-one), Intermediate-risk colon cancer group (13 patients in stage two), High-risk colon cancer group (12 patients in stages three-four). Patients suffering from any other inflammatory disease which can affect the gastrointestinal tract, another type of gastrointestinal and autoimmune disease, were excluded.

### 1.2 Serum collection

Five ml of venous blood from each subject was collected in a red–top BD vacutainer blood collection tube and left to coagulate for 30-60 minutes. Serum was separated on the same day of blood collection, within 2-4 hours of blood withdrawal. Centrifugation of the collected samples was performed at 4500 rpm for 10 minutes at 4 ° C in a *"Heraeus™ Megafuge™ 8R"* centrifuge (Thermofisher Scientific, USA) using a swinging bucket rotor. Serum supernatants were then carefully removed. Finally, serum samples were kept at – 40°C in aliquots until further processing. All serum processing was performed in the laboratory´s blood processing area using the standardized procedure applied to clinical specimens. Hemolyzed serum samples (determined by visual inspection) were excluded.

### 1.3 Analysis of miRNA

Firstly, miRNA was extracted from the serum samples via a miRNA extraction kit (mirVanaTM PARISTM Kit, Ambion, USA) then purified. The absorbance of extracted RNA was determined using a Nanodrop® spectrophotometer at 260 nm, 280 nm, and 230 nm. Two-step RT-PCR was used for quantification with the TaqMan® miRNA assays; during the reverse transcription (RT), cDNA was reversely transcribed from the extracted total RNA via TaqMan® MicroRNA Reverse Transcription Kit. Then, using the TaqMan® MicroRNA Assay and the TaqMan ® Universal PCR Master Mix, PCR products were amplified from cDNA samples according to the manufacturer's procedures. Target cDNA generated from RNA samples was amplified using sequence-specific primers from TaqMan Assay Plates by the AmpliTaq® Gold DNA polymerase during the target amplification step. Step-one real-time PCR was used for cDNA amplification (Applied Bio systems). It was held at 50°C for 2 minutes, enzyme activation at 94 °C for 10 minutes, subsequently 40 cycles of denaturation (95 °C for 15 s), annealing and extension (60°C for 60 s). The current gold standard for correcting any biases in RNA input or RT efficiency is normalization to endogenous control genes. Thus, the data was normalized to snRNA U6 and $2^{-\Delta\Delta ct}$ Method was used to calculate relative changes in miRNA expression [21].

The cycle threshold (CT), the cycle at which the reactions' fluorescence signal reaches a threshold, was identified, and used in quantifying the relative expression of miRNA (Rq). Δ CT values were calculated as follows:

Using the $2^{-\Delta\Delta CT}$ method, the findings are presented as the fold change (FC) in normalized miRNA expression.

$\Delta CT = CT_{(microR-223)} - CT_{(endogenous\ control)}$

$\Delta\Delta CT = \Delta CT_{(patient)} - \Delta CT_{(control)}$

$FC\ (or\ Rq) = 2^{-\Delta\Delta CT}$

### 1.4 Determination of CEA & CA19.9

Both CEA and CA19.9 were detected using commercial sandwich ELISA kits where a microtiter pre-coated plate with the respective antigen was incubated with samples then washed, and an enzyme conjugate was added to the plate, the color developed is proportional to the respective antigen.

### 1.5 Statistical analysis :

The statistical package SPSS® version 28 (IBM® Corp., Armonk, NY, USA) was used in the analysis. When applicable, numerical data was presented as a mean, standard deviation, median, and range. Percentages and frequencies were used to represent qualitative data. Relationships between qualitative variables were examined via Fisher's exact test or Pearson's Chi-square test. Numeric data were tested for normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests. Three group comparisons were done using Kruskal-Wallis test (non-parametric ANOVA) then a post-Hoc test was used to test for inter-group significance. Correlations were assessed using Spearman-rho coefficient. The Receiver Operating Characteristic (ROC) curve was used to predict cut-off values of miR-23. The diagnostic value of miR-223 was evaluated by calculating sensitivity, specificity, positive predictive values (PPV), negative predictive value (NPV) and accuracy. All performed tests were two-tailed. A *p-value* < 0.05 was considered significant.

## 3. RESULTS

The current study was conducted on forty patients with colorectal cancer and twenty healthy subjects were enrolled as control group.

### 3.1. Tumor markers (CA19.9 and CEA) in all patients groups.

The mean ± SD of both tumor markers CA 19.9 and CEA showed almost similar values (insignificant differences) among the various grades of disease severity, as represented in Table (1).

### 3.2. Micro RNA-223 expression levels in all Studied groups.

Expression levels of miR-223 in patient groups showed significantly higher levels than controls. Additionally, a significant increase was observed in the low-risk when compared to the high-risk groups ($p < 0.05$) regarding miR-223 levels, as represented in Table (2) and Figure (1).

**Table 1:** Mean ± SD of CA19.9 and CEA in all patient groups.

| Mean ± SD | Low-Risk Group | Intermediate-Risk Group | High-Risk Group | P-value |
|---|---|---|---|---|
| **Mean ± SD of CA19.9** | 178.22 ± 77.10 | 200.36 ± 36.25 | 107.47±23.98 | 0.37 |
| **Mean ± SD of CEA** | 7.40 ± 7.80 | 7.26± 8.49 | 26.32±12.36 | 0.25 |

*Kruskal wallis test was used to detect statistical significance, SD: standard deviation, * p <0.05*

**Table 2:** Mean ± SD of Micro RNA-223 in all studied groups.

| Micro RNA-223 | Control | Low-Risk | Intermediate-Risk | High-Risk | *p*-value |
|---|---|---|---|---|---|
| **Mean ± SD** | 1.06 ± .08 | 2.1 ± 0.63[a] | 5.08 ± 0.6 [a] | 8.43 ± 1.09 [a,b] | < 0.001* |

*Kruskal wallis test was used to detect statistical significance, SD: standard deviation, * p < 0.05 (*Significant)
a: Significant when compared to control group
b: Significant when compared with low-risk

### 3.3. Correlations of miRNA223 with CEA , CA 19.9 and degree of risk.

Spearman correlation coefficient (r) showed the absence of significant correlation between miR-223 and CEA or CA19.9 ($p > 0.05$). Nevertheless, there was a strong positive significant correlation with degree of risk, coefficient values are presented in Table (3).

### 3.4. Receiver Operator Characteristic (ROC) analysis for miR- 223.

The ROC curve for the serum miR- 223 expression levels of patient groups versus control group showed a 100% sensitivity and a 90% specificity as represented in Table (4) and Figure (2).

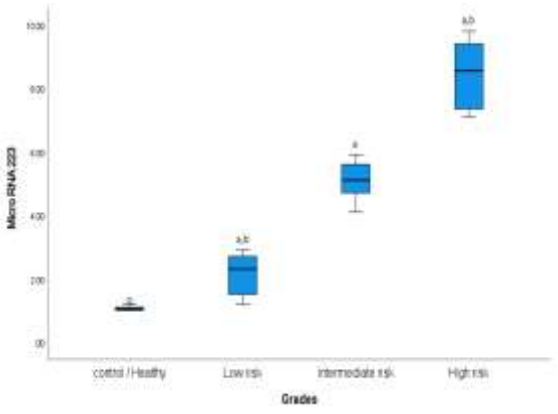


**Figure 1:** Serum levels of miRNA223 in all studied groups.

**Table 3:** Correlations of miRNA223 with CEA, CA 19.9 and degree of risk.

| | | | |
|---|---|---|---|
| **microRNA223** | CEA | r | 0.200 |
| | | p-value | 0.216 |
| | CA19.9 | r | 0.148 |
| | | p-value | 0.362 |
| | Degree of risk | r | 0.953 |
| | | p-value | <.001* |

*r: spearman correlation coefficient*, significance was considered at $p < 0.05$

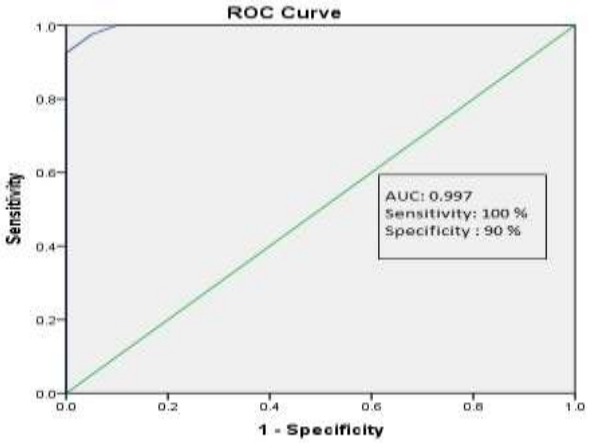


**Figure 2:** ROC curve for serum miR-223 levels in patient groups versus control group.

**Table 4:** Receiver Operator Characteristic (ROC) for miR- 223 in patients versus Control group.

| | AUC | Sensitivity | Specificity | NPV | PPV | Accuracy | p-value |
|---|---|---|---|---|---|---|---|
| **Serum miR-223** | 0.997 | 100.0% | 90.0% | 100.0% | 95.2% | 96.7% | <0.05 |

## 4. DISCUSSION

Early detection with screening increases the 5-year survival rate and reduces the CRC mortality rate, as has been demonstrated [22]. In reality, CRC is often detected in its advanced stages when no symptoms have been present. Screening for CRC can be done with a number of different invasive and noninvasive techniques, such as the fecal occult blood test [19], flexible sigmoidoscopy, and colonoscopy [23], each of which has its sensitivity and accuracy. Each screening method has its own set of benefits and drawbacks [22].

Researchers have found numerous miRNAs in various human body fluids, including many with high levels of accessibility and specificity. Multiple investigations have found stable miRNAs in different cancer types in specimens from tissue, blood plasma, and serum [24]. Thus, it is suggested that they can act as promising biomarkers. According to Strubberg et al., at least 250 miRs have been found to have potential as CRC diagnostic biomarkers and prognostic indicators [25].

Invitro knockdown of miR-223 in CRC cells leads to decreased proliferation, migration, and invasion, and this onco-miRNA has been shown to target genes involved in cell survival and death. This miRNA may serve as a biological biomarker and therapeutic target in the fight against CRC [16]. Additionally, miR-223 overexpression was linked to a higher risk of disease development and could help in the early diagnosis of CRC [20].

The current study evaluated the expression levels of miR-223 in increasing the severity of CRC Egyptian patients. It was also deduced from the study at hand a significant overexpression in miR-223 among all patient groups compared to healthy controls. Moreover, miR-223 levels significantly differed between patient groups, where significantly high levels of miR-223 were reported in the high-risk group compared to the low-risk colon cancer group. These results were consistent with Mahmoud et al., who stated that miR-223 was one of the early detection markers for CRC [20].

About half of colon tumors metastasize to the lung, liver, and lymph nodes, and the 5-year survival rate for individuals with metastatic CRC is dismal, even with recent and ongoing breakthroughs in diagnosis and therapy [26]. miR-223, besides being correlated with poor prognosis, was also correlated with CRC metastasis [24], which is consistent with the current study finding of significantly higher levels of miR-223 in the high-risk colon cancer group.

Chemotherapy resistance is one of the constraints facing CRC treatment. It contributes to poor prognosis, and several studies stated that the increased levels of miR-223 are correlated with chemotherapy resistance in CRC through targeting different genes [27, 28]; thus, designing a miR-223 sponge could be a possible therapeutic target of CRC to improve sensitivity to treatment and favor better prognosis.

To assess miR-223 diagnostic potential, a ROC curve analysis was performed, where serum levels of

miR-223 had a significant AUC value of 0.997, with 100% sensitivity and specificity of 90% in the patient group versus the control group. This result agreed with Hala et al., who found through ROC curve analysis that miR-223 had an accuracy of 97%, sensitivity of 97.1%, and specificity of 96.7%, showing great diagnostic and prognostic value [20]. Moreover, Pi-Yueh et al. stated that miR-223 was detected in the plasma of CRC patients and had sensitivity and specificity of 96.8 % and 75%, respectively, and an AUC of 0.907 [29].

One of the obstacles facing the current study was the allocation of patients with different severities of CRC in the time frame designated for the study. As a result, it is recommended that future studies consider including a larger number of patients in a suitable time frame. Moreover, studies on miR-223 as a therapeutic target for CRC are highly recommended.

## 5. CONCLUSIONS

The level of miRNA 223 significantly increases with the increase of CRC grade in Egyptian colon cancer patients. Additionally, it has a valuable diagnostic and prognostic value in colon cancer patients.

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**Conflicts of Interest:** None

**Ethical Statement:** The present work was approved by the Research Ethical Committee of the Faculty of Pharmacy (Girls), Al-Azhar University, Cairo, Egypt (REC number:152).

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**List of Abbreviations: AUC:** Area under the curve; **CA19-9:** Carbohydrate Antigen 19-9; **CDNA:** Complementary DNA; **CEA:** Carcinoembryonic Antigen; **CRC:** Colorectal Cancer; **CT:** Cycle threshold; **FC:** Fold change; **miRNAs:** Micro RNAs; **NPV:** Negative predictive values; **PPV:** Positive predictive values; **r:** Relation coefficient; **ROC:** Receiver Operator Characteristic; **RQ:** Quantifying the relative expression of miRNA; **RT:** Reverse transcription; **Rt PCR:** Reverse transcriptase polymerase chain reaction; **SD:** Standard Deviation.

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