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Clinical utility of hsa-miR-181a-5p expression level in Egyptian colorectal cancer patients

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ABSTRACT

Background: Colorectal cancer (CRC) is considered one of the most aggressive malignancies worldwide, with high mortality and morbidity rates because of its asymptomatic nature at the early stages. Further, CRC is affected by ethnicity and environmental factors. MicroRNAs (miRNAs) dysregulation has a high impact on CRC development and progression, thus this study was conducted to explore hsa-miR-181a-5p expression level in the sera of Egyptian CRC patients and assess its clinical utility.

Methods: Relative hsa-miR-181a-5p expression level was determined using quantitative real-time PCR in 192 specimens collected from 160 CRC patients and 32 healthy subjects.

Results: Hsa-miR-181a-5p relative expression was significantly up-regulated in CRC patients compared to controls and was found to be associated with increased susceptibility to CRC. Moreover, the expression level of hsa-miR-181a-5p had a powerful diagnostic performance to detect CRC, although it is still inferior to that of carcinoembryonic antigen (CEA) and cancer antigen 19.9 (CA 19.9).

Conclusion: Over-expressed hsa-miR-181a-5p may contribute to the pathogenesis of CRC, and it seems to be a valuable diagnostic biomarker to discriminate between CRC patients and healthy subjects.

1. Introduction

Colorectal cancer (CRC) represents a life-threatening and therapeutically challenging disease ranking as the third most common malignancy and the fourth prime cause of cancer mortalities worldwide [1].

Diagnosing CRC at an early stage is not easy, as cancer is often asymptomatic. However, several markers, including carcinoembryonic antigen (CEA) and cancer antigen (CA 19.9), have been recognized and are accepted in routine clinical practice [2].

Although considered good markers, CEA and CA 19.9 are not specific to a particular histological type of carcinoma and the organ which they come from. CEA concentration may be elevated in various benign conditions, including hepatitis, pancreatitis, obstructive pulmonary disease, and inflammatory bowel disease, while CA 19.9 is used in diagnosing pancreatic, colorectal, and gastric cancers [3]. Genetic and epigenetic alterations in colon and rectum cells are one of the fundamental mechanisms driving CRC tumorigenesis [4].

MicroRNAs (miRNAs) play vital roles in several physiological and pathological processes in human beings. They function as post-transcriptional gene regulators via binding to sequences in the 3'-untranslated region (3'UTR) of their target mRNAs, leading to mRNA degradation and/or translational suppression. Therefore, miRNAs have an impact on various cellular processes, including cell growth, differentiation, proliferation, and apoptosis through the activation of different signaling pathways and, in turn, they can be classified into oncogenic (OncomiR) or tumor suppressor miRNAs according to their role [5]. Race and ethnicity strongly affect the expression of miRNAs in CRC, as evidenced by the dysregulation patterns of miRNAs' expression observed in different populations [6].

MicroRNA 181a (miR-181a) belongs to the miR-181 family, and its oncogenic potential can be recognized in its up-regulated expression reported in various cancer types [7-9]. On the other hand, its expression was down-regulated in non-small cell lung cancer tissues and was found to suppress cell migration, invasion, and angiogenesis in breast cancer [10, 11]. MiR-181a-5p is a mature single strand of miR-181a, while miR-181a-3p is a passenger strand. MiR-181a-5p influences several tumor properties, including cell proliferation, metastasis, angiogenesis, epithelial-mesenchymal transition, and autophagy. It is important to note that the expression of miR-181a-5p is specific to certain tissues and can simultaneously target multiple genes, potentially playing dual roles. The function of miR-181a-5p is not reliant on a specific target but rather on the collective impact of its targets, which may encompass both tumor suppressor genes and oncogenes [12, 13]. As controversy about the miR-181a role exists, this study was designed to explore the expression level of hsa-miR-181a-5p in the sera of Egyptian CRC patients and evaluate its clinical utility.

2. Subjects and methods

2.1. Study population

This is an observational study conducted on 192 subjects admitted to the Department of Medical Oncology, Faculty of Medicine, Tanta University, Egypt, in the period between 2015 and 2021 and underwent endoscopic biopsy. Subjects were classified according to the standard clinical, radiological, endoscopic, and histological diagnosis into 160 patients with CRC and 32 subjects with a normal colon. The exclusion criteria included patients suffering from immune disorders or malignant diseases primarily arising from other organs, hereditary non-polyposis or familial adenomatous polyposis, as well as those who received any treatment before surgery. The clinical data were obtained from patients' records. Stages and pathologic features of primary tumors were defined according to the American Joint Commission on Cancer criteria.

Based on tumor differentiation, CRC patients were classified into grade I (well differentiated), grade II (moderately differentiated), and grade III (poorly differentiated). Before the start of the study, all participants provided written informed consent. The study protocol was approved by the Research Ethics Committee of Faculty of Medicine, Tanta University, Gharbia, Egypt (IRB0010038 with a Federal Wide Assurance number of FWA00022834) under the approval code of 36264MD9/1/23, and the work was conducted according to The Declaration of Helsinki. In dry tubes, 3ml of venous blood were collected from each participant, left to clot and centrifuged at 2000 xg for 5 min to obtain sera which were immediately separated and stored frozen at -80°C until assayed.

2.2. Hsa-miR-181a-5p expression analysis

MiRNeasy Serum/Plasma Kit was used to extract total RNA, including miRNAs and other small non-coding RNAs >18 nucleotides, from sera according to the manufacturer's protocol (Qiagen, Hilden, Germany). The extracted RNA was then reverse transcribed to synthesize the first cDNA strand using TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems). TaqMan™ MicroRNA Assay (Applied Biosystems) was used to conduct the quantitative real-time PCR reactions, and the expression of hsa-miR-181a-5p was normalized to human U6 snRNA.

The final volume of the PCR reactions was 20 µl, containing 1 µl of cDNA as a template, 10 µl of 2x TaqMan® Universal PCR Master Mix (Applied Biosystems), 1 µl of 20x TaqMan™ MicroRNA Assay which contained the primers and probe for the gene of interest as part of the kit (Cat# 4427975, assay ID: 000480 for hsa-miR-181a-5p and 001973 for U6 snRNA, Applied Biosystems), the volume was finally completed with nuclease-free water. PCR reactions were performed in a MicroAmp® fast optical 96-Well reaction plate and loaded into the 7500 Real-Time PCR System (Applied Biosystems, CA, USA). The thermal profile was as follows: 10 min at 95°C for enzyme activation, followed by 40 amplification cycles of denaturation at 95°C for 15 sec and 60 sec at 60°C for annealing and extension. At last, the $2^{-\Delta\Delta Ct}$ method was used for determining the quantitative measurements [14].

2.3. Assays for CEA and CA 19.9

Elecsys® dual monoclonal antibody sandwich assays (Roche Diagnostics) based on electrochemiluminescence technology have been used to measure the levels of CEA and CA19.9 (Cat# 11731629 322 and 11776193 122, respectively) according to the manufacturer's instructions, with a normal range of 0-3.4 ng/ml for CEA and 0-39 U/ml for CA 19.9. The analyses were carried out on Cobas e 411 analyzer (Roche Diagnostics, In, USA).

2.4. Statistical analysis

The Shapiro-Wilk test was first used to verify the assumption of normal distribution of the data; the normally distributed data were expressed as mean±SD, non-normally distributed data were expressed as median and interquartile range (25^{th} and 75^{th} percentile), and categorical variables are expressed as frequencies (percentages). Continuous variables were compared between the two groups using Student's t-test or Mann-Whitney U test as appropriate. $\chi 2$ test was used to compare the differences between categorical variables.

The strength of the association between the circulating hsa-miR-181a-5p level and the risk of CRC was investigated using logistic regression analyses. The strength of the association was measured by the crude odd ratio (OR), adjusted OR for age, sex, smoking habit, the presence of co-morbidity, and the presence of family history of the disease as potential confounders and their corresponding 95% confidence interval (CI).

Receiver operating characteristic (ROC) curve analysis was performed to determine the diagnostic value of hsa-miR-181a-5p. A *p*-value <0.05 was considered statistically significant.

3. Results

3.1 Basic and clinical characteristics of the studied groups

The general characteristics of the study participants are presented in Table 1. The 2 groups were age and sex matched. However, a higher number of the patients were hypertensive, smokers, and had CRC first-degree relatives compared to controls. 80% of CRC patients complained from bleeding per rectum, which may cause anemia as a consequence of the disease. As expected, CRC patients had a significant elevation in the serum levels of carcinoembryonic antigen (CEA) and cancer antigen 19.9 (CA 19.9) compared to the control group. Most of the tumors were found in transverse colon, sigmoid colon, rectum, and ascending colon; respectively. The majority of CRC patients suffered from stage II tumors with size ≤5 cm and moderately differentiated tumor cells.

3.2. Hsa-miR-181a-5p expression level

Fig. 1 illustrates that hsa-miR-181a-5p was significantly over-expressed in CRC patients compared to the control group, with a median value of 5.75 vs. 1.15, respectively (p<0.001). Further, CRC patients were divided into 2 sub-groups (high vs. low) according to the median value of the relative expression level. Table 2 shows that the high hsa-miR-181a-5p relative expression level was significantly associated with advanced stage (p=0.005) and poor differentiation (p=0.004).

3.3. Relative hsa-miR-181a-5p expression as a risk factor for CRC

Table 3 demonstrates the results of the logistic regression analyses performed to test the association of hsa-miR-181a-5p relative expression with CRC. The over-expression of hsa-miR-181a-5p was associated with an increased risk of CRC after adjusting for age, sex, smoking habit, the presence of co-morbidity, and the presence of family history of the disease as potential confounders.

3.4 Efficacy of hsa-miR-181a-5p expression as a potential diagnostic biomarker for CRC

Fig. 2 illustrates the ROC curves of hsa-miR-181a-5p relative expression level in addition to CEA and CA 19.9 serum levels to discriminate between CRC patients and healthy controls.

Hsa-miR-181a-5p showed a high diagnostic value for CRC at an optimum cut-off point of 2.6368 with 91.25% sensitivity, 100% specificity, and an area under curve (AUC) of 0.982 (95% CI: 0.965-0.999, p<0.001). However, this ability was lower than the CEA and CA 19.9 diagnostic efficacy.

Table 1. General characteristics of study population

	Control (n=32)	CRC (n=160)	*p*-value
Age (Years)	53.50 (49.25-56.5)	55.00 (49.25-63.00)	0.116
Sex (M/F)	24 (75%)/8 (25%)	106 (66.25%)/54 (33.75%)	0.334
Smoking habit (Y/N)	6 (18.75%)/26 (81.25%)	74 (46.25%)/86 (53.75%)	0.004
Co-morbidity			
DM	6 (18.75%)	26 (16.25%)	
HTN	0 (0%)	32 (20%)	0.016
DM and HTN	2 (6.25%)	2 (1.25%)	
No	24 (75%)	100 (62.50%)	
Family history			
First degree relatives	4 (25%)	74 (46.25%)	<0.001
No	28 (75%)	86 (53.75%)	
Bleeding (Y/N)	0 (0%)/32 (100%)	128 (80%)/32 (20%)	<0.001
ALT (IU/L)	18.00 (14.25-26.75)	27.00 (18.00-36.75)	0.005
AST (IU/L)	31.00±8.15	33.00 (24.25-40.00)	0.705
Albumin (g/dl)	4.15 (3.83-4.88)	3.80 (3.60-4.20)	0.002
Total bilirubin (mg/dl)	0.68 (0.55-0.87)	0.66 (0.49-0.82)	0.365
Direct bilirubin (mg/dl)	0.20 (0.15-0.24)	0.20 (0.14-0.25)	0.521
ALP (IU/L)	170.00 (110.25-218.00)	230.00 (183.50-302.75)	<0.001
Urea (mg/dl)	31.19±6.67	32.50 (25.00-39.00)	0.649
Creatinine (mg/dl)	0.90 (0.70-1.10)	0.80 (0.60-1.00)	0.192
Uric acid (mg/dl)	4.44±1.10	4.75 (3.53-5.88)	0.371
RBCs count (x10^6/µl)	3.95 (3.53-4.58)	2.75 (2.50-3.00)	<0.001
WBCs count (x1000cell/µl)	7.63±1.11	9.84±1.84	<0.001
Platelet count (x1000/µl)	250.00 (192.00-299.00)	239.00 (198.00-289.00)	0.423
Hb (g/dl)	11.62±1.24	8.00 (7.33-8.90)	<0.001
HCT (%)	35.00 (32.25-38.50)	25.00 (22.00-27.00)	<0.001
CEA (ng/ml)	1.35 (0.63-2.45)	190.00 (150.00-266.75)	<0.001
CA 19.9 (U/ml)	13.50 (11.25-18.50)	295.00 (240.00-367.50)	<0.001
Tumor location			
Ileocecal	-	10 (6.25%)	
Ascending colon	-	42 (26.25%)	
Hepatic flexure	-	8 (5%)	
Transverse colon	-	20 (12.5%)	
Splenic flexure	-	6 (3.75%)	
Descending colon	-	6 (3.75%)	
Sigmoid colon	-	28 (17.5%)	
Rectosigmoid junction	-	6 (3.75%)	
Rectum	-	32 (20%)	
Anal	-	2 (1.25%)	
Tumor size			
>5cm	-	40 (25%)	
≤5 cm	-	120 (75%)	

Tumor stage (TNM staging)		
I		
➢ T1N0M0	-	6 (3.75%)
➢ T2N0M0	-	10 (6.25%)
IIA		
➢ T3N0M0	-	54 (33.75%)
IIB		
➢ T4aN0M0	-	16 (10%)
IIC		
➢ T4bN0M0	-	28 (17.5%)
IIIA		
➢ T1N2aM0	-	46 (28.75%)
Tumor grade		
I	-	16 (10%)
II	-	98 (61.25%)
III	-	46 (28.75%)

Data are expressed as mean±SD for Gaussian data, median (interquartile range) for non-Gaussian data, and frequency (percentage) for categorical data. CRC: Colorectal cancer, DM: Diabetes mellitus, HTN: Hypertension, ALT: Alanine transaminase, AST: Aspartate transaminase, ALP: Alkaline phosphatase, RBCs: Red blood corpuscles, WBCs: White blood cells, Hb: Hemoglobin, HCT: Hematocrit, CEA: Carcinoembryonic antigen, CA: Cancer antigen, TNM: Tumor-node-metastasis.

Table 2. Association of hsa-miR-181a-5p relative expression level with clinicopathological features in CRC patients

Variable	**Hsa-miR-181a-5p relative expression level**		***p*-value**
	Low (n=81)	**High (n=79)**	
Age			
<55 years	35 (43.21%)	41 (51.90%)	0.271
≥55 years	46 (56.79%)	38 (48.10%)	
Sex			
Male	54 (66.67%)	52 (65.82%)	0.910
Female	27 (33.33%)	27 (34.18%)	
Smoking habits			
Yes	38 (46.91%)	36 (45.57%)	0.865
No	43 (53.09%)	43 (54.43%)	
Presence of co-morbidity			
Yes	34 (41.98%)	26 (32.91%)	0.236
No	47 (58.02%)	53 (67.09%)	
Family history			
First degree relatives	37 (45.68%)	37 (46.84%)	0.883
No	44 (54.32%)	42 (53.16)	
Tumor location			
Proximal colon (Ileocecal to transverse colon)	42 (51.85%)	38 (48.10%)	0.714
Distal colon (splenic flexure to sigmoid colon)	21 (25.93%)	19 (24.05%)	
Rectum (Rectosigmoid junction to anal)	18 (22.22%)	22 (27.85%)	
Tumor size			
<5 cm	18 (22.22%)	22 (27.85%)	0.411
≥5 cm	63 (77.78%)	57 (72.15%)	
Tumor stage			
I	12 (14.81%)	4 (5.06%)	0.005
II	54 (66.67%)	44 (55.70%)	
III	15 (18.52%)	31 (39.24%)	
Tumor grade			
I+II	66 (81.48%)	48 (60.76%)	0.004
III	15 (18.52%)	31 (39.24%)	

Data are expressed as frequencies (percentage).

Table 3. Binary logistic regression analysis of hsa-miR-181a-5p as a risk factor for CRC

Variable	Crude OR (95% CI)	*p*-value	†Adjusted OR (95% CI)	*p*-value
Relative expression of hsa-miR181a-5p	12.015 (4.493-32.132)	<0.001	11.649 (4.007-33.862)	<0.001

CRC: Colorectal cancer, OR: Odd ratio, 95% CI: 95% confidence interval, †: Adjusted for age, sex, smoking habit, the presence of co-morbidity, and the presence of family history of the disease as potential confounders.

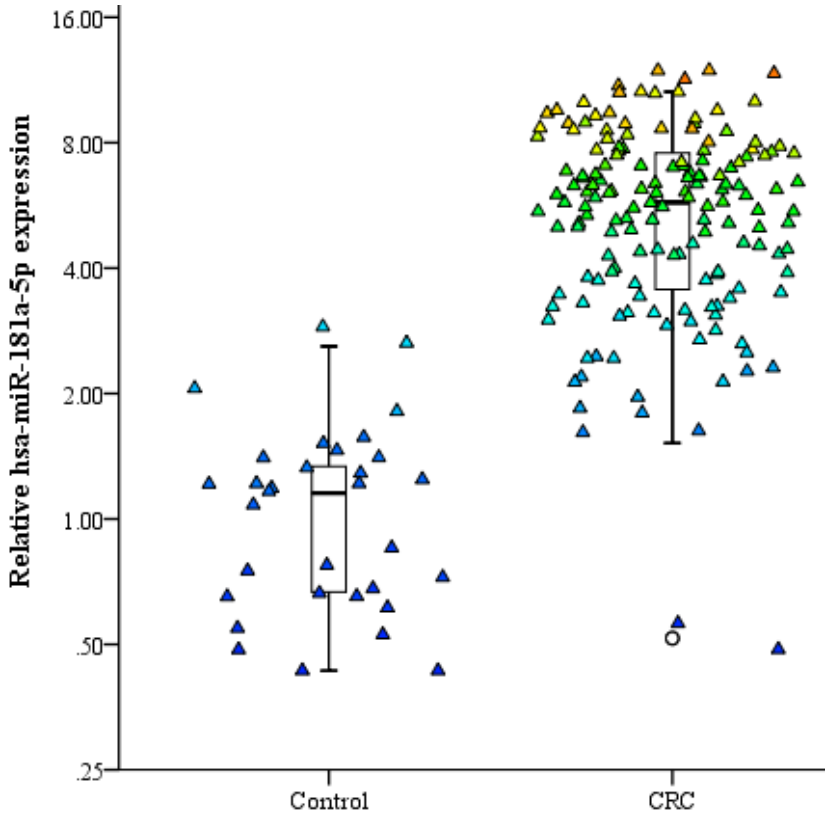
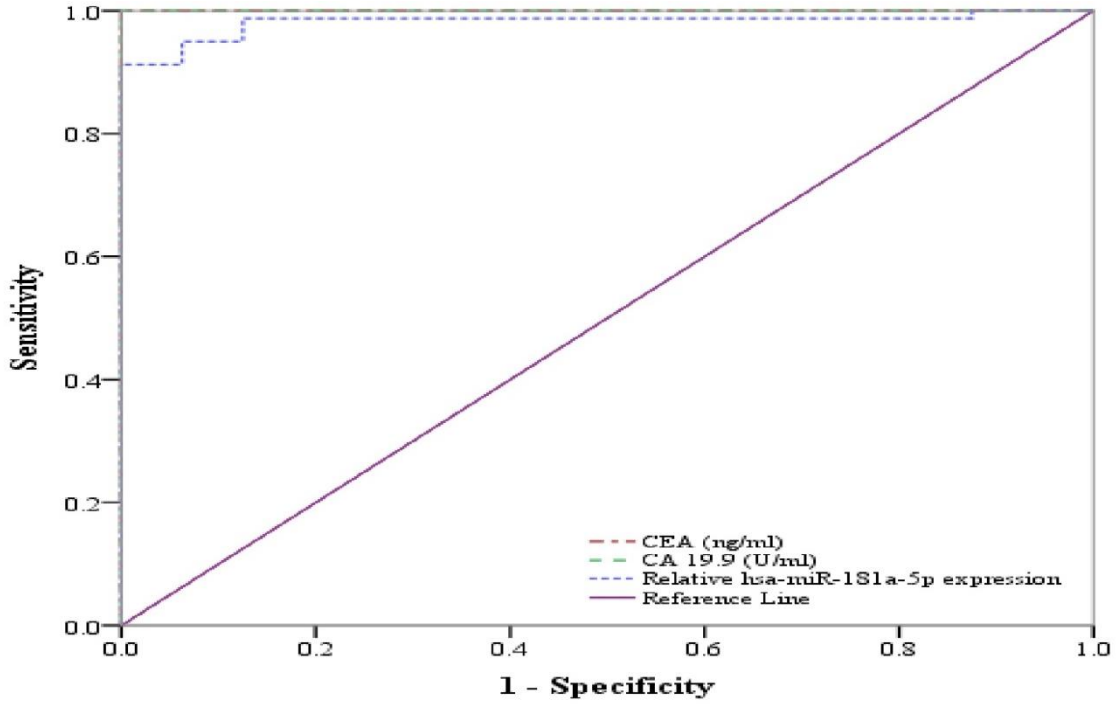


Fig. 1. Relative expression level of hsa-miR-181a-5p in the studied groups. CRC: Colorectal cancer. Expression level of hsa-miR-181a-5p in each participant is depicted by ▲ and the direction from blue towards red color indicates higher expression. Outliers between 1.5 and 3 box lengths are depicted by "o".



Variable	AUC	95% CI		*p*-value	Cut-off	Sens. (%)	Spec. (%)	PPV (%)	NPV (%)	Accuracy (%)
		Lower bound	Upper bound							
CEA (ng/ml)	1.000	1.000	1.000	<0.001	41.90	100	100	100	100	100
CA 19.9 (U/ml)	1.000	1.000	1.000	<0.001	74	100	100	100	100	100
Relative hsa-miR-181a-5p expression	0.982	0.965	0.999	0.009	2.6368	91.25	100	100	69.57	92.71

Fig. 2. Receiver operating characteristic (ROC) curves of hsa-miR-181a-5p expression level as well as CEA and CA 19.9 circulating levels to distinguish CRC patients from healthy controls. CRC: Colorectal cancer, CEA: Carcinoembryonic antigen, CA: Cancer antigen, AUC: Area under curve, CI: Confidence interval, Sens.: Sensitivity, Spec.: Specificity, PPV: Positive predictive value, NPV: Negative predictive value.

4. Discussion

Globally, CRC is one of the deadliest and frequently diagnosed cancers [1]. Accumulating evidence suggests that miRNAs are critical managers of the expression of CRC-associated genes [15]. MiR-181a-5p is a multifaceted miRNA that could function as an oncomiR or tumor suppressor in CRC. Thus, its reported aberrant expression was contradictory [16, 17]. Therefore, the current study aimed to evaluate the expression level of hsa-miR-181a-5p in sera of Egyptian CRC patients and test its potential clinical value. There is inconclusive data about the role and expression level of miR-181a in CRC. Some reported that miR-181a-5p was up-regulated in tumor tissues and plasma exosomes of CRC patients [18] as well as acted as an oncomiR to promote the growth and liver metastasis of CRC by targeting the tumor suppressor WIF-1 [19].

On the other hand, Lv et al.[20] demonstrated that miR-181a-5p expression level was down-regulated in the CRC tissues compared to the normal adjacent tissues. Further, miR-181a-5p over-expression reduced the expression of endogenous β-catenin and TCF4 and thereby inhibited the activity of Wnt/β-catenin signaling and, in turn, the proliferation of CRC cell lines HCT116 and SW480 [21]. In the current study, hsa-miR-181a-5p showed a significant over-expression in the CRC group compared to controls. In contrast to Tesolato et al. [22] who reported a down-regulation of hsa-miR-181a-5p in CRC patients compared to the control group, the findings of the current study concur with that of Zhang et al. [18] who demonstrated a significantly higher miR-181a-5p expression level in CRC plasma exosomes.

Moreover, the current study showed that the high hsa-miR-181a-5p expression in the CRC group was significantly associated with advanced stage and poor differentiation, which is supported by the results reported by Zhao et al. [23] who found that miR-181a-5p expression was significantly higher in tissues from stage III-IV tumors compared with stage I-II tumors. Additionally, high hsa-miR-181a-5p expression was associated with an increased risk of developing CRC after adjusting for age, sex, smoking habit, the presence of co-morbidity, and the presence of family history of the disease as potential confounders. Accumulating evidence demonstrated the oncogenic role of miR-181a in regulating CRC angiogenesis, cell motility, invasion, and tumor growth.

MiR-181a-5p exhibits pro-angiogenic properties, as evidenced by its ability to inhibit the expression of key regulators such as SRC kinase signaling inhibitor1 and reversion-inducing cysteine-rich protein with Kazal motifs [24]. Also, it promotes cell proliferation in CRC by inhibiting a vital tumor suppressor gene, PTEN [25]. Furthermore, miR-181a-5p regulates epithelial-mesenchymal transition, a crucial process in cancer metastasis [19]. This may provide a satisfactory explanation for the results observed in the current work. Further, the diagnostic value of the hsa-miR-181a-5p relative expression to distinguish between CRC patients and healthy subjects was examined compared to the golden standard biomarkers, CEA and CA 19.9. The results showed that CEA and CA 19.9 have superior diagnostic efficacy over hsa-miR-181a-5p relative expression.

In conclusion, this work sheds light on the expression level of hsa-miR-181a-5p in Egyptian CRC patients, indicating an over-expression of the studied miRNA in the CRC group compared to controls. Moreover, the results pointed out that up-regulation of hsa-miR-181a-5p is associated with higher susceptibility to CRC. Thus, modulating the expression of hsa-miR-181a-5p may provide novel approaches for CRC treatment; further studies are required to verify this hypothesis. Finally, hsa-miR-181a-5p appears to be a good candidate as a biomarker in clinical practice to diagnose Egyptian CRC patients.

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