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Tissue and/or Serum Expression levels of miRNA-31 and miRNA-141 as potential diagnostic markers for Colon Cancer in Egyptian Patients.

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ABSTRACT:
Cancer colon is the most common cancer of the digestive tract representing the third most common cancer. MiRNAs regulate 30% of human genes. They can act as oncogenes and tumor suppressors. MiRNA-31 expression level is altered in many cancers; it is increased in squamous cell carcinoma, and hepatocellular carcinoma, but significantly decreased in gastric, breast, and prostate cancer. MiRNA-141 is an important member of the miR-200 family regulates different cellular processes, such as proliferation and angiogenesis. The study aimed to determine the tissue and serum expression of miR-31 and miR-141 in Egyptian patients with CC. Fifty subjects were enrolled in this study. They were divided into 2 groups; Group I: 25 newly diagnosed stage II and III primary CC patients, and Group II: 25 healthy controls. Increased expression of miRNA-31 in cancerous tissue more than adjacent non-cancerous tissue was observed. Regarding miRNA-141; there was a significant increase in serum miRNA-141 in cancer colon group than the control group. A ROC curve showed that AUC for serum miRNA-141 was 0.869. This suggests the possible use of serum miRNA-141 in diagnosis of CC.

Keywords: MiRNA-31, MiRNA-141, cancer colon

INTRODUCTION

Cancer colon (CC) is a major source of cancer morbidity and mortality. Worldwide, it is the third most common cancer, and the third leading cause of cancer-related death (Bray, 2024). In Egypt, it constitutes about 6.5% of all cancers and 14% of all patients subjected to colonoscopy. Furthermore, the National Cancer Institute Registry in Cairo University declared that CC was among the most common cancers registered in Egypt (Ibrahim et al,2014).

It is a complex disease regarding its anatomic position and molecular background. Its etiology is multifactorial with many risk factors both modifiable and non-modifiable. Modifiable risk factors include diet, physical activity, parasitic infections especially cryptosporidium spp., and obesity (Abd El-Latif et al., 2023). Non-modifiable risk factors such as age, gender, race, and hereditary mutations (Xi & Xu, 2021). Genetic factors have an important role in the etiology of CC both in the familial and sporadic types. CC involves multiple signaling pathways that influence the development of the disease (Manne et al., 2010; Marx et al., 2023).

There are different modalities for screening and diagnosis of CC. Colonoscopy is the preferred screening method for CC, but it is expensive, inconvenient, and invasive. Therefore, there is an urgent need for detecting new diagnostically specific, sensitive, and non-invasive markers to improve the diagnosis of CC (Zhang et al., 2017). Epigenetic changes including changes in Rat sarcoma (RAS) pathway play important role in development of CC (Bardhan & Liu, 2013; Jung et al., 2020).

Micro-ribonucleic acids (miRNA) are a class of small, evolutionary conserved, single stranded, endogenous non-coding RNAs composed of 19-25 nucleotides (You et al., 2019). They regulate the expression of genes at the post-transcriptional level through binding to target mRNA to prevent protein production by either mRNA degradation or translation repression (Macfarlane & Murphy, 2010). MiRNAs are found in intra- and extra-cellular spaces such as plasma and other body, urine, and saliva. It was clearly established that specific miRNAs promote cell proliferation, transformation, and tumorigenesis via behaving as targets..

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of genomic lesions which are related with either stimulation of oncogenes or suppression of tumor suppressor genes in malignant cells. Furthermore, the pro and anti-tumorigenic behaviors of particular miRNAs were described both in vitro and in vivo (Macfarlane & Murphy, 2010; Smolarz et al., 2022).

Circulating miRNAs are stable and are recognized as effective molecular markers for many tumors including solid tumors (Brunet Vega et al., 2013). Research on circulating microRNAs revealed their potential use for cancer diagnosis, chemo- or radio-resistance monitoring, tumor subtype classification and outcome prognosis (Kandil et al., 2022; Ohtsuka et al., 2021; Wang et al., 2017).

The miRNA-31 is an important regulator of immune system function, embryonic implantation, development and muscle and bone homeostasis. Its expression level is elevated in some types of cancer, but it is significantly decreased in others. Furthermore, previous research revealed that on certain occasions, miR-31 can act as either a tumor suppressor or an oncogenic miRNA(Stepicheva & Song, 2016; Yu et al., 2018).

MicroRNA-141 is an important member of the miR-200 family. Dysregulated miRNA-141 has been demonstrated in many types of cancers depending on their types. MiRNA-141 regulates different cellular processes, such as proliferation, migration, angiogenesis, epithelial mesenchymal transition, chemosensitivity and apoptosis by modulating multiple targeting (Gao et al., 2016). MiR-141 acts a dual role in tumorigenicity, also it can modulate cellular motility. It was found to be upregulated in various cancers such as ovarian, lung, colorectal, nasopharyngeal, bladder and prostate cancers, but it was downregulated in gastric, breast and pancreatic cancers, hepatocellular and primary peritoneal carcinoma (Bracken et al., 2008; Wang et al., 2022).

The aim of the present work was to determine the tissue and serum expression of miR-31 and miR-141 in Egyptian patients with CC and to study their possible association with the different clinicopathological features

Materials and Methods

Patients

After Approval of the Ethical Committee of the Medical Research Institute, this study was based on collecting samples from subjects in the Surgical Endoscopy Unit and Internal medicine Endoscopy unit in Medical Research Institute, Alexandria University, after taking their consent, to determine tissue and serum expressions of miR-31 and miR-141. Approval serial number: E/C.S/N.T48/2020.

Fifty subjects were enrolled in this study. They were divided into 2 groups; Group I consisted of 25 newly diagnosed stage II and III primary CC patients, and Group II consisted of 25 healthy controls as proven by colonoscopy or CT scan. Three milliliters of whole venous blood samples were withdrawn from both groups (controls and patients) under aseptic technique. Multiple fresh punched biopsies were taken from CC patients both from cancerous and non-cancerous colon tissues after leaving at least 15 cm as a safety margin.

Exclusion criteria included subjects who were receiving or received any form of cancer treatment, those who suffered from any other malignancies, those who had recurrent CC, or those who suffered from any inflammatory bowel disease e.g ulcerative colitis and Crohn's disease.

Total RNA Extraction:

Total RNA extraction from both tissues and serum samples were done using Qiagen® miRNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany - Cat. no. 217004).

RNA quantitation and quality assessment:

The concentration and purity of RNA were assessed using NanoDrop Spectrophotometer (Thermo Scientific, USA), where the absorbance at 260nm (A260), at 280 (A280) and 230 (A230) were measured. The ratios of (A260/A280) and (A260/A230) were used to indicate the protein contaminants and organic compounds for the extracted RNA, respectively (Desjardins & Conklin, 2010).

Complementary deoxyribonucleic acid (cDNA) synthesis from RNA (Reverse transcription)

It was done by a commercially available reverse-transcription (RT) kit with miRNA-specific primers (Applied Biosystems by Thermo Fisher Scientific Baltics, Vilnius, Lithuania Cat. n. 4366596). The reverse-transcription master mix composed of deoxynucleotide triphosphate (dNTPs) with deoxythymine triphosphate (dTTP), MultiScribe TM reverse transcriptase, buffer, RNAase inhibitor and nuclease-free water.

Thermocycling was carried out using PCR thermocycler (Applied Biosystems). The cycling conditions for the reverse-transcription were 30 minutes at 16 °C and another 30 minutes at 42°C, then finally 5 minutes at 85°C (Cui, 2019).

Quantitative real-time PCR (qPCR)

This was done on Bio-Rad CFX connect real-time PCR instrument. The reagents used included TaqMan® Universal Master Mix II, no UNG (Applied Biosystems by Thermo Fisher Scientific Baltics, Vilnius, Lithuania Cat. n. 4440043), TaqMan®miR-31 (Cat.no.4427975, id: 002279), miR-141Assay (Cat.no. 4427975, id: 000463), TaqMan® miRNA-16 Assay (Cat.no.4427975, id: 000391) as internal control for serum samples, and U6 (Cat.no. 4427975, id: 001973) as internal control for tissue samples. The PCR reaction mix consisted of TaqMan miRNA Assay (20×), TaqMan 2× Universal PCR Master Mix, No AmpErase UNG and Nuclease-free water. PCR reaction for each miRNA was performed in separate tubes.

For each reaction well 18.67µL from the PCR reaction mix in case of tissue samples, and 17µL in case of serum samples were pipetted to each reaction well, then 1.33µL of each cDNA in case of tissue samples, and 3µL of each cDNA in case of serum samples were added to its corresponding reaction well and mixed by several pipetting. The No Template Control (NTC) containing nuclease-free water instead of cDNA was included in each run (negative control) to ensure that PCR products were not due to contamination by genomic DNA. Thermocycling was done using Bio-Rad CFX connect real-time PCR instrument. The first cycle was the Initial enzyme activation step and lasted for 10 minutes at 95°C, then the Denaturation, Annealing/extension steps and repeated for 40 cycles at 95°C and 60°C for 10 minutes, 15 seconds respectively (Cui, 2019).

Relative quantification (RQ) of miRNA expression $2^{-\Delta\Delta Ct}$

Calculation of serum relative expressions of miR-31 and miR-141 (Rao et al., 2013) was done using the comparative cycle threshold method ($2^{-\Delta\Delta Ct}$), after normalization for the expression of endogenous control (Rao et al., 2013).

Statistical analysis

Micro-RNA expression was expressed as median and range and its ratio in tumor tissue to adjacent normal one was calculated. We used IBM SPSS software package version 20.0. and 29.0 (IBM Corp). Categorical data were represented as numbers and percentages. For continuous data, they were tested for normality by the Shapiro-Wilk test. Quantitative data were expressed as range (minimum and maximum), mean, standard deviation and median. Mann Whitney test was used to compare two groups for not normally distributed quantitative variables. Wilcoxon signed ranks test was used for abnormally distributed quantitative variables, to compare between tumor and normal tissue expression of miRNAs in the same patient. Kruskal Wallis test was used for abnormally distributed quantitative variables, to compare between more than two studied groups. Spearman coefficient was used to correlate between two distributed abnormally quantitative variables. Significance of the obtained results was judged at the 5% level. P-value of less than 0.05 was considered statistically significant (Arnold & Emerson, 2011).

Results

Demographic and clinical data in cancer colon patients:

The patients group consisted of 25 CC patients. The demographic and clinical data are shown in table (1).

Table (1): Descriptive analysis of basic and clinical data of CC patients.

Variables			n	%
Gender	M		13	52
	F		12	48
Age (y)	≥56		14	56
	<56		11	44
Family history	Yes		1	4
	No		24	96
BMI	Obese		14	56
	Non obese		11	44
Smoking	Yes		5	20
	No		20	80
Alcohol	Yes		0	0
	No		25	100
Diet	Meat	<3 times/week	16	64
		>3 times/week	9	36
	Fatty food	yes	23	92
		no	2	8
	Fibers	yes	25	100
		no	0	0
NSAIDs	Yes		0	0
	No		25	100
Clinical presentation	Abdominal pain		14	56
	Constipation		11	44
	Diarrhea		13	52
	Bleeding per rectum		7	28
Age (y)	Median(min-max)		60 (31-76)	
BMI (kg/m^2)	Mean ± SD.		25.39 ± 3.58	

n: number y: years
BMI: Body Mass Index, M: male, F: female.
NSAIDs: non-steroidal anti-inflammatory drugs, SD: Standard deviation.
Min: minimum, max: maximum.

Radiological, colonoscopy and pathological findings of CC patients:

Fifteen patients (60%) were presented by left-side tumor, where 10 patients (40%) were presented by right-side tumor. The main site was the sigmoid in 10 patients (40%), ascending colon in 7 patients (32%), splenic flexure and descending colon in 4 patients (16%), transverse colon in 3 patients (12%) and finally caecum in one patient. Table 2.

The size of the tumor ranged from 2.0 – 13.0 with median 6 cm. Regarding TNM staging of the included patients, all patients were M0 either stage II or stage III. Stage III was the most common represented in 16 patients (64%), while the rest 9 patients (36%) were stage II. Table 2.

Regarding T stage, T3 was the most common 18 patients (72%) followed by T2 in 6 patients (24%), while T4 was the least one in only 1 patient (4%). Lymph node metastasis (N) was classified according to TNM into 3 categories. N0: no regional lymph node metastasis, was found in 9 patients (36%), N1: metastasis in less than 4 LNs, was found in 7 patients (28%), N2: metastasis in 4 or more lymph nodes, was found in 9 patients (36%). Seventeen patients (68%) had positive vascular invasion, while 8 patients (32%) had a negative vascular invasion. Table 2.

Adenocarcinoma was the dominant pathology type in 21 patients (84%). Four patients (16%) had mucoid adenocarcinoma. According to tumor differentiation (grading); 18 patients (72%) were moderately differentiated (grade 2), 5 patients (20%) were well differentiated (grade 1) and 2 patients (8%) were poorly-differentiated (grade 3). Table 2.

Table (2): Descriptive analysis of radiological, colonoscopy and pathological findings.

Variable	n	%
Tumor location		
RT colon	10	40
LT colon	15	60
Tumor site:		
Ascending colon & Hepatic flexure	7	28
Transverse colon	3	12
Splenic flexure & descending colon	4	16
Sigmoid	10	40
Cecum	1	4
Max. Tumor diameter (cm)		
Min. – Max.	2.0 – 13.0	
Median (IQR)	6.0 (4.0 – 6.50)	
T stage		
T2	6	24.0
T3	18	72.0
T4	1	4.0
N stage		
N0	9	36.0
N1	7	28.0
N2	9	36.0
Stage		
II	9	36.0
III	16	64.0
Vascular invasion		
No	8	32.0
Yes	17	68.0
Histopathological type		
Adenocarcinoma	21	84.0
Mucoid Adenocarcinoma	4	16.0
Grade of differentiation		
Grade 1	5	20.0
Grade 2	18	72.0
Grade 3	2	8.0

Cm: centimeter, Min: minimum, Max: maximum, n: number, SD: standard deviation. IQR: interquartile range, Rt: right, Lt: left, LN: lymph node

Results of tissue samples:

Comparison between relative expression of miRNA-31 and miRNA-141 in cancerous tissue and adjacent non-cancerous tissue of CC patients:

The median miRNA-31 expression in normal tissue of CC patients was 0.81 (0.07 – 74.34), while its median expression in cancer tissue was 5.49 (0.13 – 96.44). Thirteen non-cancerous tissues had levels above or equal the median and 12 non-cancerous tissues had level below the median. Thirteen cancer tissue had levels above or equal the median and 12 cancer tissue had levels below the median.

The median miRNA-141 expression in normal tissue of CC patients was 1.03 (0.01 – 27.64), while its median expression in cancer tissue was 1.04 (0.10 – 3.12). Thirteen non-cancerous tissues had levels above or equal the median and 12 non-cancerous tissue had levels below the median. However, 13 cancer tissue had levels above or equal the median and 12 cancer tissue had levels below the median.

Statistical analysis using Wilcoxon signed ranks test showed that there was statistically significant increase in miRNA-31 expression in cancer tissue more than in adjacent non-cancerous tissue of the same patients (Z=3.027, p=0.002), but there was no statistically significant difference in miRNA-141 expression between cancer and adjacent non-cancerous tissue of the same patients (Z=1.466, p=0.143). Table 3.

Table (3): MiRNA-31 and miRNA-141 expression in cancerous and adjacent non-cancerous tissue of CC patients.

	miR-31 expression		**miR-141 expression**	
	Cancer tissue	**Adjacent non-cancerous tissue**	**Cancer tissue**	**Adjacent non-cancerous tissue**
Median	5.49	0.81	1.04	1.03
Min-max	0.13 – 96.44	0.07 – 74.34	0.10 – 3.12	0.01 – 27.64
Z value	3.027*		1.466	
P value	0.002*		0.143	

Z: Wilcoxon signed ranks test, Min: minimum, Max: maximum.
p: p value for comparing between cancerous and non-cancerous tissue, *: Statistically significant at $p \leq 0.05$.

Relation between tissue miRNA-31 and miRNA-141 expressions and clinicopathological criteria of CC patients.

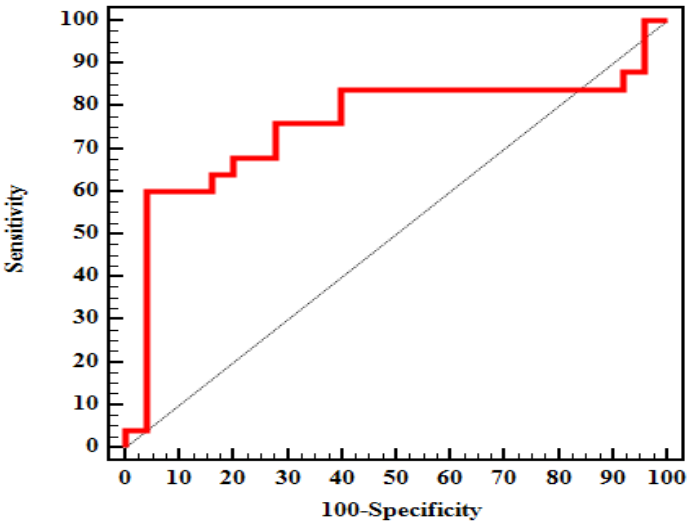
No statistically significant difference in miRNA-31 and miRNA-141 expression in tumor tissues and any demographic or clinicopathological criteria (p >0.05) except the significant increase in tissue miRNA-31 expression in mucoid adenocarcinoma than adenocarcinoma (U= 12, p=0.025).

No significant correlations were found between tissue expressions of both miRNAs with each other in CC patients.

Diagnostic utility for the use of tissue miRNA-31 expression

The receiver operating characteristic (ROC) curve analysis of tissue miRNA-31 expression and cancer colon was done. It showed that the Area Under ROC curve (AUC) for tissue miRNA-31 was 0.757. A cut off value of (≥0.867) was sufficient to detect the presence of CC with sensitivity of 84% and specificity of 60%, confidence interval (0.610 – 0.904), positive predictive value 67.7 and negative predictive value 78.9 as seen in figure (1).

Figure (1): ROC curve of tissue miRNA-31 expression.



Results of serum samples:

Demographic and clinical data in control group:

Serum control group consisted of 25 subjects, 14 controls (56%) were males, and 11 controls (44%) were females. The median age of serum control group was 48 years, and it ranges from 35 to 72 years. None of controls had a positive family history of CC.

There was no statistically significant difference between both serum levels of CC and control groups regarding gender ($\chi 2$=0.081, p = 0.777), age (t= 1.431, p= 0.159), family history of cancer colon (FE=1.020, p=1.000), BMI (t=0.803, p=0.426), smoking ($\chi 2$=0.439, p= 0.508) and dietary habits ($\chi 2$=0.333, p= 0.564).

Abdominal pain was the main complain presented in the control group as 16 controls (64%), bleeding per rectum occurs in 15 controls (60%), while diarrhea was presented in 7 controls (28%) and constipation was found in 6 controls (24%). Regarding clinical presentation only bleeding per rectum was significantly higher in serum control group than CC patients ($\chi 2$= 5.195, p = 0.023).

Comparison between serum miRNA-31 and miRNA-141 expressions in CC patients and control group.

The median miRNA-31 expression in the serum of control group was 1.28 (0.08 – 11.76), while its median expression in serum of CC patients was 1.06 (0.02 – 8.70), revealing no significant difference in serum expression of miRNA-31 between CC cases and control group using Mann Whitney test (U=311.0, p=0.977).

The median miRNA-141 expression in the serum of control group was 0.77 (0.17 – 8.97), while its median expression in serum of CC patients was 21.91 (0.27 – 366.68), showing a significant increase in serum expression of miRNA-31 between CC cases and control group using Mann Whitney test (U=82.0, p<0.001). Table 4.

Table (4): MiRNA-31 and miRNA-141 expressions in serum of CC patients and control groups.

No	miR-31 expression		miR-141 expression	
	CC patients	Control	CC patients	Control
Median	1.06	1.28	21.91	0.77
Min-Max	0.02 – 8.70	0.08 – 11.76	0.27 – 366.68	0.17 – 8.97
U value	311.0		82.0*	
P value	0.977		<0.001*	

n: number, Min: minimum, Max: maximum.
U: Mann Whitney test, p: p value for comparing between the studied groups

Relation between serum miRNA-31 and miRNA-141 expressions and clinicopathological criteria of CC patients.

There was no statistically significant difference in serum miRNA-31 expression in relation to age, gender, BMI, smoking, meat intake, tumor location, tumor staging, vascular invasion, histopathological type and grade of differentiation ($p > 0.05$).

Serum miRNA-141 expression was significantly higher in old CC patients than those equal or more than 56 years (U=25.00, P=0.003), while there was no statistically significant difference in its serum expression and other clinicopathological conditions.

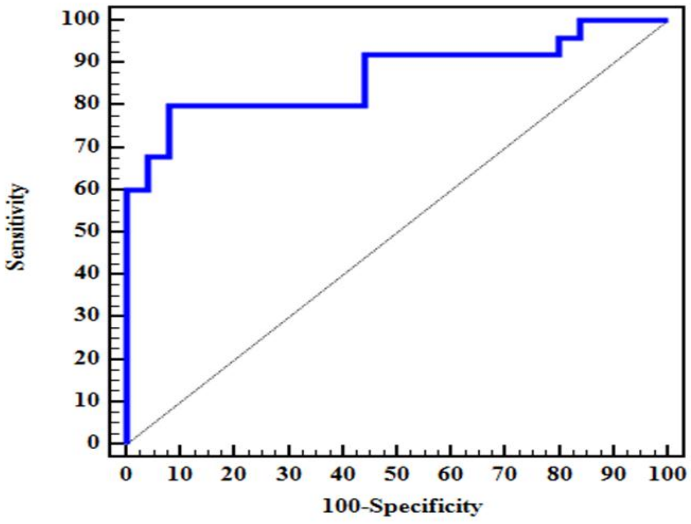
Correlation between tissue and serum expression of both microRNAs in CC patients.

There was no significant correlation between tissue and serum expression of any of microRNAs and between each other in CC patients ($p > 0.05$).

Diagnostic utility of serum miRNA-141 expression

The receiver operating characteristic (ROC) curve analysis for serum miRNA-141 expression and CC was done. It showed that the Area Under the curve (AUC) for serum miRNA-141 was 0.869. A cut off value of (≥3.4388) was sufficient to detect the presence of CC with sensitivity of 80% and specificity of 92%, confidence interval (0.763 – 0.974), positive predictive value 90.9 and negative predictive value 82.1. Figure (2).

Figure (2): ROC curve of serum miRNA-141 expression



Discussion

Since colonoscopy is the preferred screening method for CC, which is invasive, inconvenient, and expensive, so there is an urgent need for identifying new specific, sensitive, and non-invasive diagnostic biomarkers to improve the detection of CC such as miRNAs (Zhang et al., 2017).

MiRNAs regulate the expression of many genes implicated in important cellular functions, such as cell differentiation, multiplication, apoptosis, and tumor development (Bedewy et al., 2017; Machida et al., 2015; Smolarz et al., 2022; You et al., 2019). The expression levels of miRNAs could provide a potential diagnostic, prognostic, and therapeutic approach for CC (Wu & Yan, 2020). It was reported that many miRNAs are aberrantly expressed in CC and regulate multiple targets. Among the several miRNAs studied in CC two were adopted in our study (miRNA-31 and miRNA-141).

The first one was miR-31. Its expression level is altered in various types of cancers indicating its ability to act as a tumor suppressor gene or oncogene. So, it is downregulated in some cancers like prostate, stomach, ovarian, hepatocellular and breast cancer. It acts as a tumor suppressive gene by targeting specific genes in different pathways such as retinoblastoma tumor suppressor protein/ E2 transcription factor (RB/E2F) and tumor suppressor protein 53 (TP53) pathways. However, it is highly expressed in other types of cancers, such as squamous cell carcinoma, head and neck, lung and CC. So, its increased expression in these types of cancers promotes tumorigenesis and progression of malignancies (Yu et al., 2018).

Altered expression of miR-141 also, relies on the tumor type. It can act either as a tumor suppressor or oncogene. It was found to be upregulated in some types of cancer such as ovarian, lung, colorectal, nasopharyngeal, bladder and prostate cancers as it acts as an oncogene. However, it is downregulated in gastric, breast and pancreatic cancers, hepatocellular and primary peritoneal carcinoma as it acts as tumor suppressor gene. MiRNA is abnormally expressed in CC and it is involved in various cell processes, including epithelial-mesenchymal transition (EMT), cell proliferation, migration, invasion and drug resistance (Fang et al., 2020).

The miR-141 was likely to regulate CC cell proliferation by targeting PH domain leucine-rich-repeats protein phosphatase 2 (PHLPP2). PHLPP2 was downregulated in some types of cancer leading to poor prognosis in these patients, hence

promotes cell proliferation. On the other hand, it can suppress the invasion of bladder cancer by facilitating the autophagy and degradation of matrix metalloproteinase 2 (MMP2) protein. Also, PHLPP1 can inhibit the metastasis of melanoma by repressing AKT2 activation (Fang et al., 2020).

In the present study, a significantly increased expression of miRNA-31 was found in cancerous tissues more than in adjacent non-cancerous tissue of the same CC patients (p=0.002). This result was in agreement with Brunet Vega et al. (2013). They performed their study on 12 formalin-fixed paraffin-embedded (FFPE) stage III colonic tumor tissue specimens and their surrounding non-cancerous normal mucosa that were collected from 12 colorectal cancer colon cases. They found that miR-31 was significantly upregulated in colorectal cancer tissue samples compared with non-cancerous adjacent normal mucosa (p=0.0035). Also Sun et al. (2013) performed miRNA microarray analyses and quantitative reverse transcription-PCR (qRT-PCR) on colorectal tissues and normal adjacent tissues from colorectal cancer patients to identify the key miRNAs involved in colorectal cancer tumorigenesis. They found a widespread disruption in miRNA expressions during CC tumorigenesis using both microarray and quantitative RT-PCR analysis; as compared with normal adjacent tissue samples, miRNA-31 was the most significantly dysregulated (p value = 0.00) (Sun et al., 2013). It was found that miR-31 regulates the levels of large tumor suppressor homolog 2 and protein phosphatase 2A subunit B isoform R2-α, so accelerating the growth of malignant cells, and it also blocks the increase in levels of hypoxia-inducible factors, thereby leading to the proliferation of malignant cells (Cui et al., 2019). Xu et al. (2012) conducted their study on 52 patients with colorectal cancer, fresh resected tumors and the corresponding adjacent normal mucosa tissues were obtained from surgically treated patients with colorectal cancer. They found that the expression of miR-31 was upregulated in the colorectal cancer tissues compared to normal mucosal tissues (p value=0.003). It was found that miR-31 can directly target the special AT-rich sequence-binding protein 2 gene (SATB2), which is responsible for chromatin remodeling and regulation of gene expression in cancer-associated fibroblasts (Xu et al., 2012). One of the possible explanations of increased expression of miR-31 in CC patients was discussed by Sun et al. (2013) that it targets Rat Sarcoma Protein Activator 1 (RASA1) and exerts profound effect on the cell signaling network, as RASA1 and its downstream proteins play important roles in control of cellular growth and differentiation. RASA1 serves as a suppressor of RAS function by enhancing the weak intrinsic GTPase activity of RAS proteins, leading to increase in the inactive GDP-bound form of RAS, resulting in aberrant intracellular signaling through the RAF-MEK-ERK and PI3K-Akt pathways. As the RAS-RAF-MEK-ERK pathway regulates normal cell cycle control. Down-regulation of RASA1 results in an increase in cell proliferation. Aberrant signaling via the PI3K-AKT pathway activates a cascade of pro-survival and anti-apoptotic signals" (Sun et al., 2013).

Concerning serum miR-31 expression, our study did not show statistically significant difference between CC cases and control group. This was concordant with Brunet Vega et al. (2013). In their study blood samples were collected from 30 patients with stage III colorectal cancer and 26 healthy volunteers. They found that the expression level of miR-31 was not consistently measurable in serum samples from the cases. An opposing result was found in another study that was performed on 50 CC patients and 44 healthy controls. They revealed increased expression of miR-31 in the serum of patients with CC more than healthy controls ($p<0.01$) with an AUC of 0.968 (Wang et al., 2017). Further studies are needed to explain the cellular localization of miRNA-31 and to understand increased transportation of miRNA outside the cells are recommended to address the absence of circulating miRNA-31, despite its increased expression in cancerous tissues.

Regarding tissue miR-141 expression, this study showed no significant difference between cancerous and adjacent non-cancerous tissue of the same CC patients. On the other hand, Brunet Vega et al. (2013) found that miR-141 was significantly upregulated in colorectal cancerous tissue samples when compared with normal ones (p=0.04) (Brunet Vega et al., 2013). Another study performed on 12 CC and non-cancerous adjacent tissue samples reported that miR-141 was upregulated, but mitogen-activated protein kinase 4 (MAP2K4) was downregulated in colorectal cancer. The increased expression of miR-141 was associated with cancer cell proliferation and promoted growth of colorectal cancer cells by repressing the tumor-suppressor gene, MAP2K4. So, targeting miR-141 may provide effective therapeutic approach for colorectal cancer patients (Ding et al., 2017). However another study by Liang et al. (2019) suggested that miR-141 expression was down-regulated in colorectal cancer tissues and colorectal cancer cell lines compared to normal tissues and normal colon cells (Liang et al., 2019). MiR-141 can inhibit proliferation, invasion, and migration of colorectal cancer cells by direct targeting of tumor necrosis factor receptor-associated factor 5 (TRAF5), suggesting that miR-141 may act as a tumor suppressor and might be used as a potential therapeutic target for colorectal cancer. It was reported that TRAF5 plays a pivotal role in cancer progression. For example, expression of TRAF5 was upregulated in gastric cancer tissues, and its higher expression predicted worse overall survival. TRAF5 overexpression promoted migration of gastric cancer cell in vitro (Xie et al., 2019). Down-regulated of TRAF5 by overexpression of miR-26b resulted in a significant inhibition of cell proliferation and promotion of apoptosis in melanoma cell. Liang et al. (2019) found that expression of TRAF5 was upregulated in colorectal cancer tissues compared with adjacent non-neoplastic tissues and act as a target for miR-141.

The current study revealed that serum expression of miR-141 was significantly increased in CC patients group compared to the control group. This result agreed with Wang et al. (2017) who revealed that expression of miR-141 was significantly higher in the serum of CC patients compared with healthy controls ($p < 0.01$) (Wang et al., 2017). Another study with similar results that was conducted on 64 patients with CC and

64 healthy subjects reported that the expression of miR-141 in serum of colorectal cancer patients was significantly increased compared to the control group ($p<0.05$). Serum miRNA-141 expression might be involved in the occurrence and progression of colorectal cancer and could be used as biological indicator for early diagnosis of colorectal cancer (Wu & Yan, 2020). A contradictory result regarding expression of miR-141 in CC reported that its expression in serum, tumor tissues and lymph nodes were significantly decreased in colorectal cancer cases compared with the control group. Furthermore, its expression in serum, tumor tissue and lymph nodes in patients having lymph node metastasis were significantly lower than those in patients without lymph node metastasis ($p<0.05$) (Feng et al., 2016).

The role of miRNA-141 in colorectal cancer displays some contradiction, some results found that it was upregulated while others suggested that it was downregulated. Depending on our results miRNA-141 was significantly upregulated in the serum of CC patients, while there was no statistically significant difference regarding its expression between cancerous and adjacent non-cancerous tissues. This may be related to its oncogenic and tumor suppressive gene effects which together mask its upregulation effect in tissue which leads first to its tumorigenic effect and hence its level later present in serum, then its tumor suppressor gene effect started leading to decreasing its tissue level.

A previous study found that plasma levels of miR141 were substantially higher in patients suffering from advanced stages of CC and might promptly differentiate metastatic patients from those with earlier stages of the disease and from healthy controls. However, the plasma miR-141 was not differentially expressed in tumor tissues between stages of CC or between tumor tissues and adjacent non-tumor tissues in stage IV patients, indicating that the increase in the plasma levels of miR-141 in patients diagnosed in the fourth stage could be originated from several systemic reactions including inflammatory responses in these patients (Cheng et al., 2011). They discovered a number of features concerning the source of plasma miRNAs in malignancy, signifying that the increase of miR141 in plasma is not a plain sign of raised miR-141 as a result of secretion from the primary tumour. It is probable that miR-141 is merely raised in metastatic tumours where the secondaries reside. It is worth mentioning that miR-141 helps in suppressing the expression of ZEB1 and ZEB2 through acting on EGFR thus stimulating the mesenchymal-to-epithelial transition (MET) (Cheng et al., 2011).

Patients suffering from advanced stages of cancer are subjected to multiple systemic reactions that compromise the immunological system and various organs. It is possible that these responses can be the cause of variation in the levels of circulating miRNAs in cancer patients. There are many theories that rationalize the main site of secretion of extracellular miRNAs such as released either through microvesicles or directly via different protein types, or passive release from damaged cells. Another hypothesis is that circulating miRNAs arises from immune cells that are present in the tumor microenvironment (Zedan et al., 2018).

In the present study the accuracy of serum miRNA-141 expression in diagnosis of cancer colon was tested by ROC curve analysis. It showed that the AUC for serum miRNA-141 was 0.869 showing a very good diagnostic performance. A cut off value of ($\geq$3.4388) was sufficient to detect the presence of cancer colon with sensitivity of 80% and specificity of 92%. This indicates that serum miRNA-141 expression is specific and of very good sensitivity for diagnosis of CC. Based on our results miRNA-141 might be involved in the development and tumorigenesis of CC and can be used as a marker for diagnosis of CC. So, the recognition of miR-141 may signify an important improvement in the quest for important circulating markers for colon cancer that can be used in various practical implementation such as predicting disease outcome, risk stratification, assess the efficacy of treatment, and identifying cancer relapse.

In conclusion miRNA-31 was significantly upregulated in cancerous tissues more than adjacent non-cancerous tissue and thus can be used for diagnosis of cancer colon. However, its serum level was not significantly increased in cancer colon cases than control group which may be related to the small sample size. MiRNA-141 expression in tissues showed no significant difference between CC tissue and adjacent normal tissues. However, serum miRNA-141 was significantly higher in cases than control group. MiRNA-31 and miRNA-141 may have a role in the development and tumorigenesis of CC and might be potential therapeutic targets for CC, but further studies are recommended to elucidate this role in a large number of cases.

Author Contributions

All the authors were involved in the preparation of this manuscript. Study conceptualization was performed by N.S.K., T.F.M. and M.M.H. had access to raw data. N.S.K., T.F.M had full access to all data and take responsibility for the integrity of the data and the accuracy of the data analyses and had the final responsibility for the decision to submit for publication. N.S.K., T.F.M. and M.M.H. had the idea for the study. M.S.K and M.M.A selected the patients. G.A.O. and M.A.A performed statistical analyses. N.S.K., M.A.K. and M.M.H. performed the microRNA analysis. Analyses and interpretation of data were done by N.S.K., T.F.M., G.A.O, and M.A.K. N.S.K. drafted, wrote and submitted the manuscript, while all other authors were consulted with several drafts to appraise the intellectual content of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

• All patients were recruited from medical research institute teaching hospital. The study was approved by the local ethics committee of the Medical Research Institute, in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) Approval serial number:

E/C.S/N.T48/2020, for research involving humans, and written informed consent was obtained from all included patients before the acquisition of the tissues, explaining the investigational nature of this study.

• The study is approved by the Chemical Pathology Department Council, Medical Research Institute, Alexandria University.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Data Availability Statement

Data can be obtained upon reasonable request from the corresponding author.

Conflict of interest

None of the authors have any competing or conflicts of interests to disclose.

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