

Circulating Micro RNA- 21 and - 92a as Biomarkers of Colorectal Cancer

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Background and study aim: Colorectal cancer (CRC) is a major cause of cancer-related deaths in both men and women. Colonoscopy is the most reliable tool for CRC diagnosis, but its complexity and costs hamper its wide application. There is a pressing need for new non-invasive biomarkers to improve early diagnosis of CRC. Aim was to assess serum micro RNA (miR) -21 and miR-92a for diagnosis of CRC.

Patients and Methods: This comparative cross sectional study was carried out on 50 subjects. The cases group comprised 35 consecutive treatment naive patients with sporadic CRC proved by colonoscopy and histopathology of the biopsied specimens as well as abdominal CT which all together helped in tumor staging applying TNM, Duke's and MAC Coller stages. Serum carcinoembryonic antigen (CEA) and cancer antigen (CA) 19-9 were also assessed. Fifteen matched healthy subjects (with normal colonoscopy) served as the control group.

Results: Serum levels of miR-21 and miR-92a were significantly higher ($P < 0.001$) in cases compared to the control (5.53 ± 0.17 vs 4.82 ± 0.20 and 7.01 ± 0.234 vs 6.56 ± 0.20 log RU, respectively). Serum miR-21 and miR-92a levels revealed a significant positive relation with tumor size and TNM and MAC Coller stages ($P < 0.05$). No significant relationship was detected between either serum miR-21 or miR-92a levels with age or sex. Applying ROC curve, at a cutoff value of ≥ 5.25 log RU, serum miR-21 was 94.3% sensitive and 93.3% specific for detection of CRC with an AUC= 0.99 and serum miR-92a level at a cut off value ≥ 6.75 log RU, was 91.4% sensitive and 80% specific for detection of CRC with AUC= 0.91. When both markers were combined, the sensitivity and specificity were 97.1% and 93.3% respectively.

Conclusion: Serum miR-21 and miR-92a levels represent a sensitive and specific tool for CRC diagnosis, with higher accuracy of miR-21.

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer diagnosed in both men and women [1]. The lifetime risk of developing CRC is about 1 in 21 (4.7%) for men and 1 in 23 (4.4%) for women. Many factors increase the risk including: obesity, diet high in red meat (such as beef, pork, lamb, or liver) and processed meat, smoking, heavy alcohol use, history of inflammatory bowel disease, family history of CRC or adenomatous polyps and diabetes mellitus [1].

The CRC mortality rates can be decreased by early diagnosis through screening. However, the present CRC screening techniques [colonoscopy, faecal occult blood test (FOBT), and carcinoembryonic antigen (CEA) test] are limited by their difficulties and costs beside uncertain or delayed results [2].

Although colonoscopic screening for CRC is the most reliable tool; its difficulties and costs have hampered its wide application. On the other hand, FOBT has the limitation

of low sensitivity and dietary restrictions [3]. Thus, there is a pressing need for new non-invasive biomarkers to improve the early detection of CRC [4].

The discovery of micro RNAs (miRNAs), that play important roles in oncogenesis, has opened new opportunities of non-invasive tests for the early diagnosis of cancers [5].

MiRNAs are a family of small, noncoding RNAs (19-22 nucleotides) which post-transcriptionally regulate gene expression. In general, miRNAs are transcribed as a group called the pri-miRNA complex, which is cleaved in the nucleus to form the pre-miRNA which is then translocated to the cytoplasm where they undergo final maturation into a functional miRNA [6]. Studies have shown that profiles of miRNA expression differ between normal- and tumour- tissues and vary among different tumour types [7]. Aberrant miRNA expression profiles have been identified and emerged as potential screening biomarkers for CRC [8,9].

Although most previous studies on miRNA expression have been performed on tissue specimens, some studies have shown diagnostic and prognostic potential for circulating miRNAs because tumor-derived miRNAs can be present in blood and appear to be stably protected from endogenous ribonuclease activity in the circulation [10].

MiR-21 is an oncogenic miRNA that modulates the expression of multiple cancer-related target genes such as PTEN, TPM1, and PDCD and has been shown to be overexpressed in various human tumors [11]. In addition, miR-21 expression is upregulated in CRC tissues, is elevated during tumor progression, and is also associated with poor survival and response to chemotherapy [12]. Significantly elevated plasma miR-21 expression in CRC was concluded in many studies [11,13]. On the other hand, MiR-92a is part of the miR-17-92 gene cluster located at chromosome 13q13. As a known oncomir, the miR-17-92 cluster can promote cell proliferation, suppress apoptosis of cancer cells, induce tumor angiogenesis and accelerate tumor progression. Elevated expression of miR-92a has been observed in CRC, lung and thyroid cancers suggesting an important role in tumorigenesis. Many studies reported that circulating miR-92 is a potential biomarker for CRC diagnosis [13,14]. The aim of the present study was to assess miR-

21 and miR -92a expression levels as a stable blood-based biomarker for detection of CRC.

PATIENTS AND METHODS

This comparative cross sectional study was carried out on 50 subjects. The cases group comprised 35 consecutive patients with CRC (19 males and 16 females) who were attending the Departments of Hepatology, Gastroenterology and Infectious Diseases and General Surgery at Benha University Hospitals, within the period between January 2015 and October 2016. All had sporadic CRC, proved by colonoscopy and histopathology. Fifteen age and sex matched apparently healthy subjects who had normal routine laboratory investigations and colonoscopy served as healthy control. The indication for colonoscopy was unexplained abdominal pain and/or altered bowel habits. Patients with familial adenomatous polyposis or hereditary non-polyposis CRC, those who received chemotherapy or radiotherapy and pregnant female patients were excluded from the study. The study protocol was approved by the Ethics Committee of Benha Faculty of Medicine, Benha University. An informed written consent was obtained from the participants.

All the studied 35 cases were subjected to the following :

Full history taking focusing on: family history of CRC, recent- onset constipation, bleeding per rectum, significant weight loss, and/or anemia of unexplained aetiology. Thorough clinical examination including general examination focusing on: cachexia, pallor and lymphadenopathy. Local abdominal examination focusing on: palpable abdominal masses, ascites, LN and PR examination.

Laboratory investigations, including: stool analysis, complete blood count (CBC), erythrocyte sedimentation rate (ESR), random blood sugar (RBS), liver profile tests, including: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), serum albumin, serum bilirubin (total and direct) and prothrombin time (PT), serum creatinine and blood urea and Serum CEA and CA19-9.

Detection of miRNA- 21 expression level using real-time PCR technique: Extraction of total RNA including microRNA-21 from plasma samples using microRNA extraction kit according to the

manufacturer's instructions [15]. Relative quantitation of microRNA-21 level using real-time quantitative PCR (RT-PCR) according to the manufacturer's instructions [16].

Detection of miRNA- 92a expression level using real-time PCR technique: Extraction of total RNA including microRNA-92a from plasma samples using miRNA extraction kit according to [15]. Relative quantitation of microRNA-92a level using real-time quantitative PCR (RT-PCR) according to Siegel et al. [17].

Pelvi-abdominal ultrasonography and computed tomography (CT scan) were done. Complete colonoscopy was done under sedation with patient preparation through Low-volume Polyethylene glycol-based lavage solution with ascorbic acid (Low-volume PEG-ELS with ascorbic acid) administered as split-dose regimen. Multiple biopsies were taken from any suspected lesion, and were sent for histopathological examination. TNM, Duke's and MAC Coller staging systems were applied.

Statistical analysis [18] :

The collected data were tabulated and analyzed using SPSS version 16 software (SpssInc, Chicago, ILL Company). Categorical data were presented as number and percentages while quantitative data were expressed as mean \pm standard deviation, median and range. Chi square test (χ^2) or Fisher's exact test (FET) were used to analyze categorical variables. Quantitative data were tested for normality using Shapiro-Wilks test, assuming normality at $P > 0.05$. Student "t" test was used for normally distributed variables, while Man Whitney U (MWU) test, Kruskal Wallis test and Spearman's correlation coefficient (ρ) were used for not normally distributed data. ROC curve was used to determine cutoff values of miRNA-21 and miR-92a with optimum

sensitivity and specificity in prediction of patients with CRC. P value ≤ 0.05 was considered significant.

RESULTS

The studied cases included 19 males (56%) and 16 females (44%) with a mean age of 50.6 ± 15.1 ys. Age categories among the studied cases showed 26% below 40 years and 63% above 50 years.

The main presenting symptom in the studied cases was bleeding per rectum (15/35 = 42.9%) followed by recent onset constipation (13/35 = 37.1%) and significant weight loss (7/35 = 20%). Positive family history was found in (6/35=17%) of the studied cases.

Colonoscopic examination of the studied cases revealed that the main lesion site of CRC was colon (24/35 = 68.6%), followed by rectum (9/35 = 25.7%) and lastly rectosigmoid (2/35 = 5.7%). Small lesions (<2.5 cm) were seen in 4 cases (11.4%), medium sized ones (2–5 cm) were 37.1% (13 cases), while large lesions were seen in 18 cases (51.4%). Mass was the main lesion seen (94.2%) with only one case showed ulcer and another one showed a stricture. The main histo-pathological CRC types in the studied cases were adenocarcinoma (24/35 = 68.6%) followed by mucinous adenocarcinoma (9/35 = 25.7%) and signet ring cell adenocarcinoma (2/35 = 5.7%).

The majority of the studied cases were Dukes B (88.6%). According to TNM staging, cases with T1IB were (54.3%) followed by T1IA (34.3%). Three cases only were T1s (8.5%). None of the studied cases had nodal involvement or metastases (all were N0M0).

Table (1): Circulating miR-21 and miR-92a levels in the studied groups.

Variable	Cases (N°=35)		Controls (N°=15)		Z of MWU test	P
	Mean \pm SD	Range	Mean \pm SD	Range		
MiR-21 level	5.53 \pm 0.17	4.93 - 5.67	4.82 \pm 0.20	4.62 - 5.41	5.55	<0.001**
MiR-92a level	7.01 \pm 0.234	6.46 – 7.33	6.56 \pm 0.20	6.05 – 6.9	4.56	<0.001**

**Highly significant P value

Table (2): Mean values of serum miR-21& miR-92a in relation to colonoscopy findings.

Lesion	N° (T= 35)	Serum miR-21 (log RU)		KWT	P	Sig pairs	microRNA-92		KWT	P	Sig pairs	
		Mean± SD	Range				Mean± SD	Range				
Site	Colon	24	5.51 ± 0.19	4.93 - 5.67	0.28	0.87	-----	6.98±0.25	6.94-7.32	4.02	0.13	-----
	Rectum	9	5.56 ± 0.09	5.41 - 5.66				7.14±0.13	6.94-7.32			
	Recto-sigmoid	2	5.57± 0.05	5.54 - 5.61				6.82±0.02	6.8-6.84			
Size	Small	4	5.22 ± 0.31	4.93 - 5.54	14.5	0.001**	Small ≠ medium Small ≠ large	6.75±02	6.46-6.94	11.9	0.003*	.Small ≠ medium
	Medium	13	5.51 ± 0.09	5.41 - 5.65				6.93±024	6.48-7.28			.Medium ≠ large
	Large	18	5.61 ± 0.07	5.44 - 5.67				7.13±015	6.8-7.33			
Morphology	Mass	33	5.54 ± 0.14	4.93 - 5.67	2.93	0.23	-----	7.02±023	6.46-7.33	2.19	0.33	-----
	Ulcer	1	5.63 ± ----	5.64 - 5.64				6.79±--	6.8-6.8			
	Stricture	1	4.98 ± ----	4.98 - 4.98				6.94±--	6.94-6.94			
Pathological Grade	Grade I	3	5.27±0.296	4.93-5.45	8.06	0.018 *	I≠III	5.21±0.286	4.83-5.45	8.02	0.017 *	I≠III
	Grade II	27	5.51±0.176	4.98-5.65				5.41±0.186	4.78-5.55			
	Grade III	5	5.64±0.028	5.61-5.67				5.60±0.029	5.51-4.67			

*Significant P vaule

**Highly significant P value

RU:relative uncertainty RU

A statistically significant relationship was found between serum- miR-21& -miR-92a and the tumor size (P = 0.001 & 0.003 respectively) as well as the tumor pathological grade (P = 0.018 & 0.017 respectively), while tumour site and morphology showed non-significant relationships with serum miR-21 and miR-92a expression.

Table (3): Correlation between serum miR-21& miR-92a and assessed variables in the cases group.

Variable	miR-21		miR-92a	
	rho	P	rho	P
Age	0.231	0.18 (NS)	0.076	0.66 (NS)
Hb%	-0.138	0.43 (NS)	0.115	0.51 (NS)
RBCs	0.083	0.063 (NS)	0.232	0.18 (NS)
WBCs	-0.041	0.81 (NS)	-0.149	0.39 (NS)
PLTs	0.477	0.008 (S)	-0.177	0.31 (NS)
RBS	0.062	0.72 (NS)	0.234	0.17 (NS)
ALT	0.058	0.74 (NS)	0.016	0.92 (NS)
AST	0.055	0.75 (NS)	0.162	0.35 (NS)
ALP	-0.158	0.36 (NS)	0.199	0.25 (NS)
S.albumin	-0.1	0.57 (NS)	-0.161	0.35 (NS)
T. Bilirubin	0.126	0.47 (NS)	0.292	0.09 (NS)
Creat.	0.246	0.15 (NS)	0.249	0.15 (NS)
BUN	0.023	0.89 (NS)	0.052	0.77 (NS)
CEA	0.131	0.45 (NS)	0.022	0.9 (NS)
CA19-9	0.161	0.35 (NS)	0.034	0.84 (NS)

There was a statistically significant positive correlation between serum miR- 21and platelets count (P=0.008), while there were non- significant correlations between serum miR-21 and miR-92a and the other assessed variables.

Table (4) : Sensitivity and specificity of miRNA-21 and microRNA-92a for CRC diagnosis.

Variable	Sens %	Spec %	PPV%	NPV%	AUC	95%CI	P
miR -21 $\geq 5.25 \log RU$	94.3%	93.3%	97%	87.5%	0.99	0.97-1.0	<0.001**
miR -92a $\geq 6.75 \log RU$	91.4%	80%	91.4%	80%	0.91	0.83-0.99	<0.001**
Combined miR -21 & miR -92a	97.1%	93.3%	97.1%	93.3%			

**Highly significant P value

At a cut off value $\geq 5.25 \log RU$, serum miR-21 was 94.3% sensitive and 93.3% specific for detection of CRC, with AUC = 0.99. At a cut off value $\geq 6.75 \log RU$, serum miR-92a was 91.4% sensitive and 80% specific for detection of CRC with AUC = 0.91. When both markers were combined, the sensitivity increased to 97.1% and specificity was 93.3%.

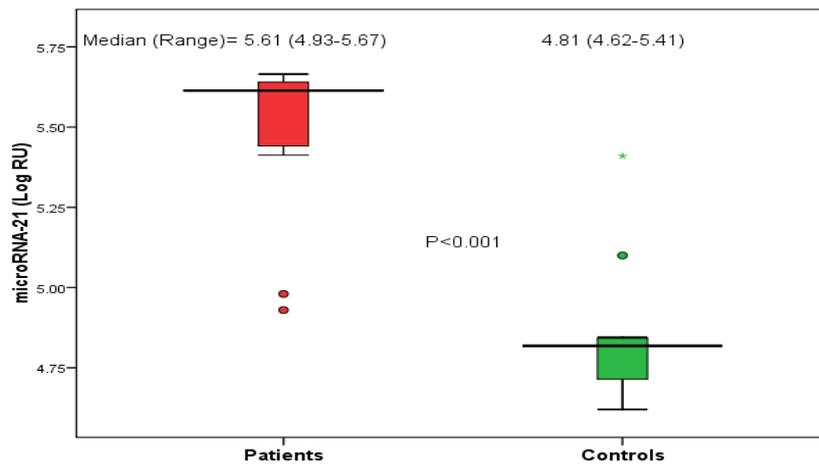


Fig. (1): Box plot showing the median and range of serum miR-21 among cases and control group

Serum miR-21 expression was significantly higher in the cases group compared to the control ($5.53 \pm 0.17 \neq 4.82 \pm 0.20 \log RU$) with ($P < 0.001$).

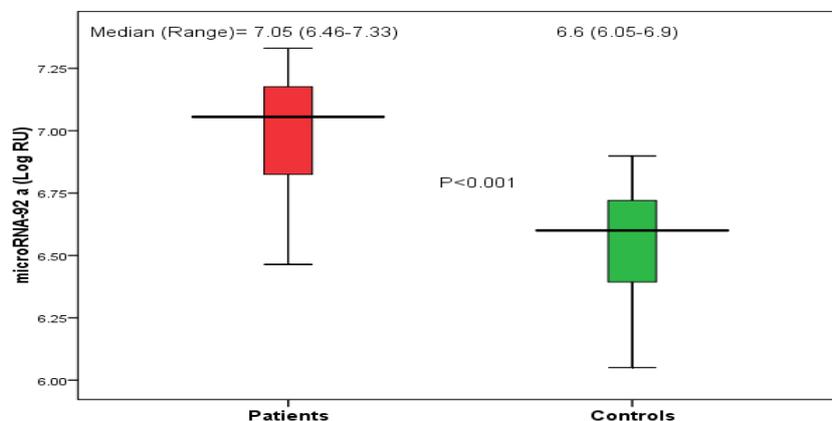


Fig. (2): Box plot showing the median and range of serum miR-92a in cases and control groups

Serum miR-92a expression was significantly higher in the cases group compared to the control ($7.01 \pm 0.234 \neq 6.56 \pm 0.20 \log RU$) with ($P < 0.001$).

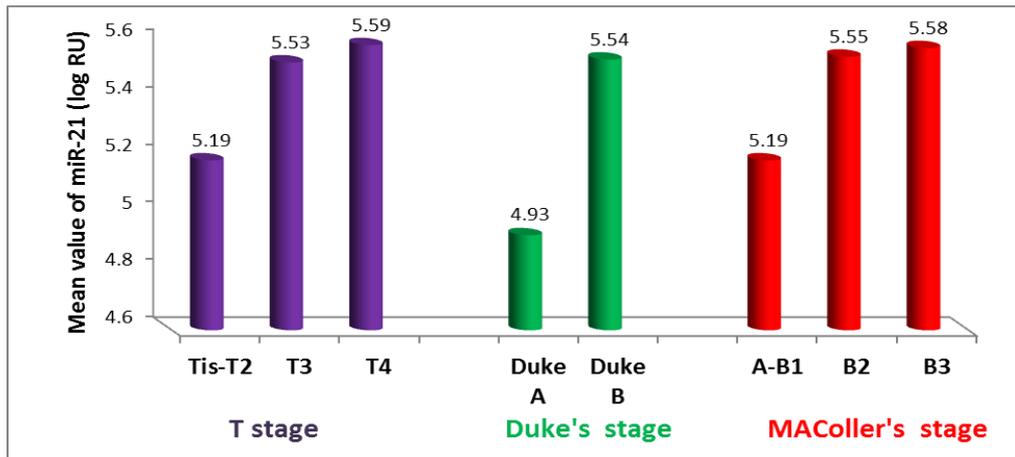


Fig. (3): Mean values of serum miR-21 in different CRC stages

A statistically significant positive relationship was found between T stage ($P = 0.01$) as well as MAC Coller stage ($P = 0.008$) and serum miR-21 expression, while a statistically non-significant relationship was found between Duke's Stage and serum miR-21 expression.

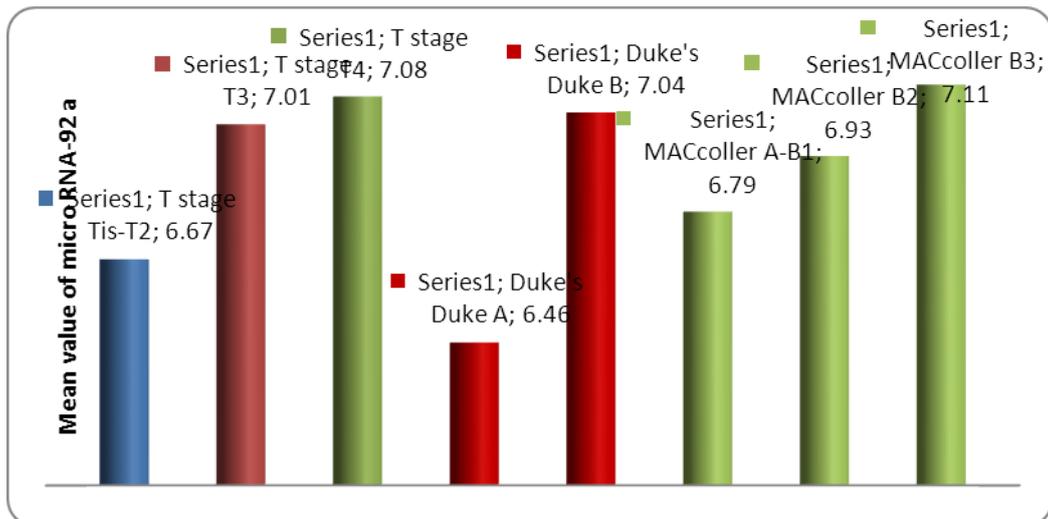


Fig. (4): Mean values of serum miR-92a in different CRC stages

A statistically significant positive relationship was found between T stage ($P = 0.03$) as well as MAC Coller stage ($P = 0.031$) and serum miR-92a expression, while a statistically non-significant relation was found between Duke's Stage and serum miR-92a expression.

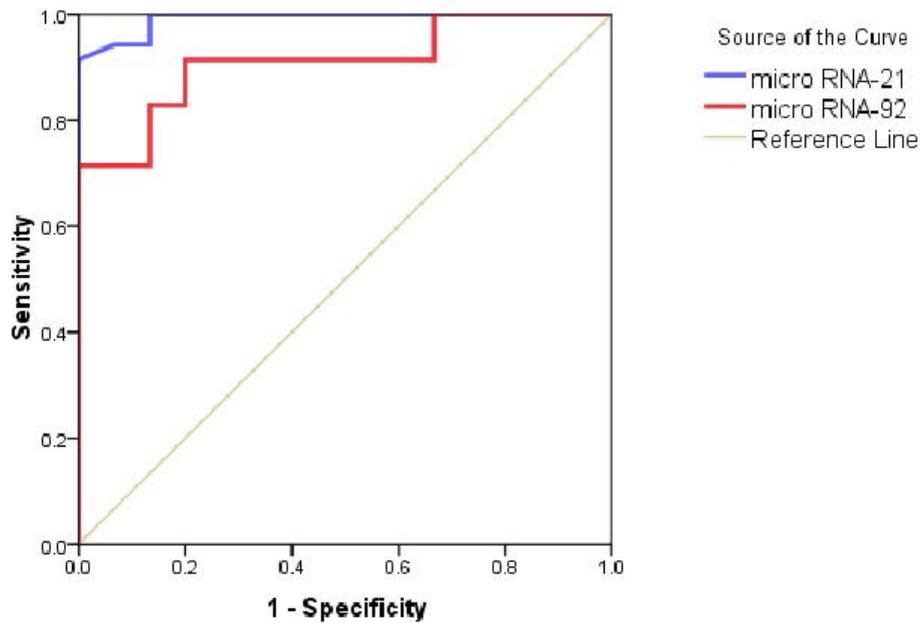


Fig. (5): ROC curve for the performance of miR-21 and miR-92 in CRC detection.

DISCUSSION

CRC is the third most prevalent cancer worldwide and is a leading cause of cancer-related mortality for both men and women [19]. In Egypt, CRC is one of the most common malignant neoplasms. It ranks the sixth most common cancer in both males and females [20,21]. The median age of CRC cases in Egypt is 48 and 51.5y, for males and females, respectively [22].

In the present study, the age of the studied cases ranged between 19-74ys, with a mean of 50.6 ± 15.1 ys. More than 60% were >50ys and 26% (more than 1/4 of patients) were ≤ 40 ys old. This comes in agreement with many Egyptian studies Gado et al. [22] found that the mean age of their assessed patients was 51ys and 44.8ys respectively and El Attar [23] and Sakr et al. [21] told that the mean age of their studied patients was 48ys and 51.2 ys respectively.

Males represented 56% of the studied cases in the present study. Rim et al. [24] and Murphy et al. [25] reported that men have more incidence of CRC than women, while El-Bolkainy et al. [26], Abotchie et al. [27] and Sakr et al. [21] told that CRC affects men and women almost equally

The percentage of the studied cases with CRC with a positive family history was 17%. This comes in agreement with chan et al. [28] and Hagggar & Boushey [29] who told that the percentage of

patients with CRC with a positive family history was up to 20%. Fatemi et al. [30] reported a higher figure (31.3%) in their studied Iranian patients. These data make adherence to CRC screening programs is mandatory in subjects with family history of CRC. Lieberman and his colleagues [31] told that screening should be offered to individuals with a family history of CRC earlier than for the average-risk population.

As regards the tumor site, colonic lesions (68.6%) were encountered more than rectal (25.7%) and rectosigmoid (5.7%) ones. Yi et al. [32] reported similar finding of a lower incidence of rectal lesions (25.5-30.5%) compared to colonic lesions. On the other hand, Kenawi et al. [33] and Soliman et al. [34] told that about half of their studied cases were rectal lesions, this disagreement may be attributed to the large number of their studied cases (400 cases) compared to the small number in our study.

Upon histopathological examination, most of the studied cases in the present study were adenocarcinoma (68.6%). Mucinous and signet ring adenocarcinomas constituted around 25.7 and 5.7% respectively. This comes in agreement with Hamilton et al., (2010)⁴⁹, Veruttipong et al. [35], Said et al. [36] and Sakr et al. [21].

Our results exhibited significantly higher mean serum miR-21 and miR-92a levels ($P < 0.001$) in the studied cases compared to the control group

(5.53 ± 0.17 and $7.01 \pm 0.234 \neq 4.82 \pm 0.20$ and 6.56 ± 0.20 log RU, respectively). This was in agreement with, Wang et al. [37] who found that miR-21 & miRNA-92a levels were significantly higher in CRC cases and added that miR92a could distinguish CRC and advanced adenoma from normal controls, with a sensitivity of >62% and specificity of >84%. No statistically significant association was found between serum miR-21 or miR-92a level and either age or sex. This comes in agreement with Guang-Hui and colleagues [38]:

The mean value of serum miR-21 level showed a statistically significant positive relationship with the tumor size ($P < 0.001$) and the mean values in small (<2.5 cm), medium and large (>5cm) CRC lesions were 5.22, 5.51 and 5.61 log RU, respectively. Also serum miR-21 level showed a statistically significant positive relationship with the pathological grade of CRC ($P < 0.05$). The mean value of grade I was (5.27 log RU), grade II (5.51 log RU) and grade III (5.64 log RU). Higher mean values of serum miR-21 were found in higher CRC pathological grades. This comes in agreement with Toiyama et al. [39]. In the present study, the mean value of serum miR-21 level showed a statistically significant difference in different TNM stages ($P > 0.05$) with a statistically significant positive correlation. The mean values in Tis-T2, T3 and T4 were 5.19, 5.53 and 5.59 log RU, respectively. This comes in agreement with Schetter et al. [40] who found that higher expression levels of miR-21 were associated with more advanced clinical stages of CRC. The mean value of serum miR-21 level showed a statistically significant difference between different MAC Coller stages ($P > 0.05$) in the present study with a statistically significant positive correlation. The mean values in A-B1, B2 and B3 were 5.19, 5.55 and 5.58 log RU, respectively.

The mean value of serum miR-92a level showed a statistically significant positive correlation with tumor size ($P < 0.003$). The mean values in small, medium and large CRC lesions were 6.75, 6.93 and 7.13 log RU, respectively This comes in agreement with Zhou et al. [41] who reported that over expression of miR-92a is correlated with TNM stages and poor prognosis in CRC. Nami and his colleagues [42] told that a significant increase in miR-92a expression was more frequently observed in CRCs than in colorectal adenomas, and suggested that miR-92a could be a potential marker for discrimination between

cancers and adenomas and a potential promoter for the phenotypic changes from adenoma into carcinoma. They added that miR-92a expression was related to advanced clinical stages and to the depth of invasion.

The mean value of serum miR-92a level showed a statistically significant positive correlation and different TNM stages ($P > 0.03$). The mean values in Tis-T2, T3 and T4 were 6.67, 7.01 and 7.08 log RU, respectively. This comes in agreement Zhang et al. [43] who reported that expression of miR-92a was associated with more advanced tumor-lymph node-metastasis (TNM) stage ($P = 0.07$).

When ROC curves were applied; serum miR-21 at a cutoff value of ≥ 5.25 Log RU was 94.3% sensitive and 93.3% specific for diagnosis of CRC with an AUROC = 0.99. Wang et al. [37] reported that serum miR-21 showed a sensitivity and specificity of 93% and 91%, respectively. While, Ahmed et al. [44] and Schetter et al. [40] reported that serum miR-21 could detect CRC with 90% sensitivity and specificity. On the other hand, Guang-Hui et al. [38] reported that the sensitivity of serum miR-21 for detection CRC was only 65% and the specificity was 85%.

In the present study, serum miR-21 level ≥ 5.61 Log RU, could significantly discriminate cases of CRC lesions larger than 5 cm with 83.3% sensitivity and 82.4% specificity, with AUROC = 0.87 as well as cases with high grade adenocarcinoma with 100% sensitivity and 63.3% specificity and AUROC = 0.85. At a level ≥ 5.43 log RU, miR-21 could significantly detect cases of T stage >2 with 90% sensitivity and 100% specificity, with AUROC = 0.95, and at a level ≥ 5.6 log RU, it could significantly detect cases of MaCollerB3 with 68.4% sensitivity and 68.8% specificity, with AUROC = 0.71.

Serum miR-92a at a cut off value of ≥ 6.75 Log RU, was 91.4% sensitive and 80% specific for diagnosis of CRC with an AUROC = 0.91. Other studies reported plasma miR-92a to have a sensitivity and specificity of 89% and 70% respectively at a cut off value of ≥ 6.75 Log RU in distinguishing CRC patients from healthy controls [43,44]. Huang et al. [14] also reported similar results (sensitivity: 84, specificity: 71.4). In addition, many studies have found miR-92a expression to be associated with other diseases such as hepatocellular carcinoma, breast cancer, and even cardiovascular diseases, pointing to the low specificity of miR-92a. MiR-92a which has been studied extensively in plasma has also been

observed to have higher expression levels in the stool of CRC patients [13]. However, the sensitivity and specificity of the miRNA 92a for detecting CRC was significantly lower in stool than in plasma[13].

The present study found that serum miR-92a at a cut off value ≥ 7.05 log RU, could significantly detect cases of CRC lesions larger than 5 cm with 72.2% sensitivity and 70.6% specificity, with AUROC = 0.82. This was in agreement with Guang et al. [38] who reported that, at a cut off ≥ 7.0 , AUROC value of 0.722 (95% CI = 0.633–0.811) with a sensitivity of 70% and specificity of 70%. At level ≥ 7.1 log RU, miR-92a could significantly detect cases of MaC CollerB3 with 63.2% sensitivity and 81.8% specificity, with AUROC= 0.76. This was in agreement with Guang et al. [38] who told that serum levels of miR -92a were potential biomarkers for CRC, and at the cut-off value of ≥ 7.1 , the sensitivity was 65.5% and the specificity was 82.5% with an AUROC = 0.786.

At level ≥ 6.96 log RU, miR-92a could significantly detect cases of T stage >2 with 77.4% sensitivity and 100% specificity, with AUROC= 0.9 This was in agreement with Guang et al. [38].

When serum miR-21 (≥ 5.25 log RU) and miR-92a (≥ 6.75 log RU) were combined together, the sensitivity for detection of CRC was increased to 97.1% and the specificity was 93.3%.

In conclusion, serum miR-21 and miR-92a expression levels represent a sensitive and specific tool for detection of CRC with higher accuracy of miR-21.

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Ethical approval: Approved

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