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Role of miRNA-210, miRNA-21 and miRNA-126 as diagnostic biomarkers in colorectal carcinoma: impact of HIF-1α-VEGF signaling pathway

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Abstract

Colorectal cancer (CRC) is a major cause of death worldwide. Novel non-invasive, high diagnostic value screening test is urgently needed to improve survival rate, treatment and prognosis. Stable, small, circulating microRNA (miRNA) offers unique opportunities for the early diagnosis of several diseases. It acts as tumor oncogenes or suppressors and involve in cell death, survival, and metastasis. Communication between miRNA and carcinogenesis is critical but it still not clear and needs further investigation. The aim of our study is to evaluate the role of miR-210, miR-21, miR-126, as non-invasive diagnostic biomarkers for screening, early detection of CRC, studying their correlation with prognostic variables, and clarifying the roles of miRNAs on HIF-1α-VEGF signaling pathway. The expression of miR-210, miR-21 and miR-126 was performed using qRT-PCR in adenocarcinoma (no = 35), adenomas (no = 51), and neoplasm free controls (no = 101). Serum levels of VEGF and HIF-1α was determined by ELISA Kit. The results show that the expression of miR-210, miR-21, VEGF, HIF-1α was significantly up-regulated while that miRNA-126 was down-regulated in both adenocarcinoma and adenomas compared with controls ($p < 0.001$ for each). No significant difference was noted comparing patients with adenocarcinoma and adenomas. The three miRNAs correlated with VEGF, HIF-α. The miR-210 and miR-21 associated with TNM classification and clinical staging of adenocarcinoma ($p < 0.001$) and they show high diagnostic value with sensitivity and specificity 88.6%, 90.1% and 91.4%, 95.0% respectively. Our study revealed that circulating miR-210, miR-21 were up-regulated while miR-126 was down-regulated in CRC and adenomas patients, they all correlated with TNM staging and they had high diagnostic value. HIF-1α VEGF signaling pathways regulated by miRNAs played a role in colon cancer initiation. To the best of our knowledge, this is the first study of this miRNAs panel in CRC in our community. These data suggested that these biomarkers could be a potential novel, non-invasive marker for early diagnosis, screening and predicting prognosis of CRC. Understanding the molecular functions by which miRNAs affect cancer and understanding its roles in modulating the signaling output of VEGF might be fruitful in reducing the incidence and slowing the progression of this dark malignancy.

Keywords miR-210 · miR-21 · miR-126 · VEGF · HIF-1α · CRC

Abbreviations

CRC	Colorectal carcinoma
miR	microRNA
qRT-PCR	Quantitative real time polymerase chain reaction
VEGF	Vascular endothelial growth factor

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HIF-α	Hypoxia inducible factor alpha
NF-kB	Nuclear transcription factor
STAT3	Signal transducer and activator of transcription 3
TNM stages	Tumor-nodal-metastasis stages
PPV	Positive predictive value
NPV	Negative predictive value

Introduction

Colorectal cancer (CRC) poses a significant threat to the health of global populations; it is the second most commonly diagnosed cancer in females and the third in males with more than 1.3 million diagnoses and almost 700.000 deaths per year [1]. It is accounting for 8% of all cancer deaths, making it the fourth most common cause of death from cancer [2]. In Egypt, CRC contributes 6.5% of all cancers. Further, the National Cancer Institute Registry, Cairo University cleared that CRC was among the most common cancers registered (6th) and it was 4.2% in males, 3.8% in females and diagnosed in 14% of all patients who undergoes colonoscopy [2, 3].

It is a heterogeneous disease with various patient-related factors such as the anatomic location of the tumor, race of the patient, genetic and dietary factors that the interactions between them influencing the development of the disease [4].

The tumor stage at diagnosis is the most important prognostic factor in CRC, making early detection of crucial importance in reducing mortality and improving compliance rate. Also, up to 30% of stage II patients relapse after surgery and many of them die due to metastatic disease [5]. Advanced adenomas clearly link the chain from benign adenomas to advanced CRC. The advanced adenomas having high grade dysplasia is associated with a high risk of progression to an invasive lesion, and represent the optimal target lesion for strategies to prevent CRC [6]. It has been estimated that over 95% of cases of CRC would benefit from curative surgery if diagnosis was made at an early or premalignant polyp stage [5].

A large number of blood markers have been evaluated, but diagnostic performance has been insufficient for application as a primary tool in screening [5]. Colonoscopic screening for CRC and adenomas is now the most reliable diagnostic tool, but its cost and invasive nature limit its use. Thus, there is a great need for new, non-invasive, accurate biomarkers to improve the detection of this dark malignancy and understanding its initiation [6].

microRNAs (miRNAs) are short (20–22 nucleotides) non-coding RNAs that negatively regulate gene expression through either miRNAs degradation or translational repression. Its expression show a lot of variations in cancerous tissue compared to normal tissue and different miRNAs have been attributed oncogenic and tumor suppressor qualities. The finding that they play an important role in oncogenesis has led to thinking to use miRNAs as potential biomarkers in early diagnosis of cancer, predicting prognosis, treatment response, or even as targets in cancer treatment strategies. The stability, abundance, and easy detectability make circulating miRNAs an ideal candidate to serve as a biomarker for cancer detection and screening [7].

Since then, approximately 1400 human miRNAs have been discovered, amongst them almost 400 to be deregulated in CRC [8]. microRNA-210 (miR-210) is frequently up-regulated in carcinogenesis and its overexpression promotes the migration and invasion of cancer cells. Furthermore, it can be induced by hypoxia and mediate the hypoxia-induced metastasis [8, 9].

Also, (miR-21) overexpression could increase cell proliferation, migration, invasion, and survival in a variety of cancer cell lines and found to be elevated in many cancers [10]. It inhibits the expression of tumor suppressive genes and has been linked to more advanced stages, lower cellular differentiation, decreased overall and disease-free survival [11].

On the other hand, miR-126 appears to inhibit tumor development by targeting certain genes and down-regulation of miR-126 has an important role in tumorigenesis, tumor metastasis and invasion [12, 13]. It may act as tumor suppressor and low expression have been related to poor prognosis in CRC patients [11].

Vascular endothelial growth factor (VEGF) is a signal protein produced by cells that stimulates angiogenesis. It is part of the system that restores the oxygen supply to tissues in case of hypoxia. Cancers that can express VEGF are able to grow and metastasize [14]. Hypoxia-inducible factor-α (HIF-1α) are transcription factors that respond to hypoxia which stimulates the release of VEGF that binds to VEGF receptors on endothelial cells triggering a Tyrosine Kinase Pathway leading to angiogenesis [15].

Certain miRNAs are involved in regulating the HIF-1α pathway by targeting both upstream and downstream signaling molecules that function as oncogenes and/or tumor suppressors. The most responsive and influential miRNA that trigger HIF-1α pathway is miR-210 [16].

However, subsequent studies have proved that miRNAs can serve as potential biomarkers for various diseases including cancer [17], the early diagnostic value of circulating miRNAs as screening biomarkers for early detection of CRC still under intensive investigation. Nevertheless, the diagnostic performance of the identified miRNAs was not sufficient to compete with other non-invasive testes. Further research on multi-marker blood based test, potentially including the panel of miRNAs might be promising approach to enhance the repertoire for non-invasive cancer

screening and to identify specific miRNAs that can act as prognostic tools in CRC. It is of great interest to detect CRC by simple test before development of symptoms and find reliable markers indicating which individuals have an increased risk of developing CRC later in life.

The aim of our study is to: (1) Evaluate the role of miR-210, miR-21, miR-126, as non-invasive diagnostic biomarkers for screening and early detection of CRC, (2) Throw the light on the correlation of these biomarkers with the prognostic variables which may monitor cancer progression and provide useful tools for cancer prevention and therapy, and (3) Insight into the involvement of HIF-1α-VEGF signaling pathway.

Patients and methods

Patients

The patients were enrolled in this study, who consecutively referred to Tropical Medicine Department of Assiut, and Cairo University Hospital for colonoscopic examination at a period between 2013 and 2016. An informed consent was obtained from each patient before being recruited in the study. The study was approved by ethical committee of Faculty of Medicine, Cairo University which was conducted according to *Good Clinical Practice (GCP)* guidelines developed by the *International Conference on Harmonization (ICH)* and the principles contained in the World Medical Association Declaration of Helsinki on the "*Ethical Principles for Medical Research Involving Human subjects*" (2004).

A sample size of 187 subjects was included. They divided into two groups according to colonoscopic finding: Group I (no = 86), with mass and Group 2 (no = 101) without any mass in the colon, considered as neoplasm free control. According to the pathological finding the first group subdivided into two subgroups: adenocarcinoma (no = 35) and adenomas (no = 51).

Inclusion criteria All patients who were well prepared for colonoscopy as part of their clinical check up with complete aseptic endoscopic conditions (up to the caecum).

Exclusion criteria Difficult colonoscopic examination due to technical cause such as patient intolerance, poor colonic preparation, and obscuring mucosal examination. Patients who have received any treatment for CRC and/or adenomas and patients with any acute and chronic inflammation, other known malignancy elsewhere were excluded.

Sample collection

Colonoscopic examinations were applied and tissue biopsy samples were preserved in formalin for further histopathological examination. Histopathological information including site, size, grades of tumor will be recorded. Tumor staging will be estimated according to AJCC Cancer Staging Handbook of the American Joint Committee on Cancer, 2010.

Ten milliliter of venous blood were collected and divided into two parts. 5 ml of whole blood for further RNA extraction processing, and the other 5 ml were left in room temperature for serum separation which preserved at − 70 °C till the assay of biochemical markers.

Biochemical analysis

The serum VEGF (pg/ml) was determined by using an ELISA kit, SinoGeneClon Biotech Co., Ltd, Cat. No.: SG-10402, Hangzhou, China. Serum HIF-1α (ng/L) was determined by using an ELISA kit, SinoGeneClon Biotech Co., Ltd, Cat. No.: SG-10669, Hangzhou, China.

RNA extraction

A total RNA was extracted from 300 µL whole blood by miRNeasy extraction kit (Qiagen, Valencia, CA, USA); available online at (http://asmlab.org/public/files/miRNeasy-Mini-Kit-EN.pdf). The blood samples were processed within 2 h. Further analysis for quantity and quality purified RNA assessment was performed with Beckman dual spectrophotometer (USA).

mRNA and miRNA quantitative genes expression by real-time PCR

Five µg of the total RNA from each sample for mRNA expression were used for reverse transcription and PCR amplification by the kit provided from Bioline, a median life science company, UK (SensiFAST™ SYBR® Hi-ROX One-Step Kit, catalog no. PI-50217 V) according to the manufacturer's protocol. Conversion of individual miRNAs into the corresponding cDNA was done with a TaqMan MicroRNA real-time RT-PCR kit (Applied Biosystems, assay ID 000397, catalog no 4427975) according to the manufacturer's protocol. A SYBR Green PCR Master Mix kit (Quanta Biosciences, Amsterdam, The Netherlands) was used for relative quantification of mRNA and micRNAs by qRT-PCR. RT-PCR was performed on the Real-time PCR instrument (Applied Biosystems; StepOne System. SDS software v2.1 and RQ Manager 1.2). Negative controls were included with every real-time RT-PCR assay to exclude any contamination. Changes in the expression of each target gene were normalized relative to the mean critical threshold (CT) values of endogenous reference control genes B-actin and RNU6B for mRNA and miRNA respectively by the $\Delta\Delta C_t$ method. We used 20 nM of both primers specific for each

target gene. Primers sequence and annealing temperature specific for target genes demonstrated in Table 1.

Statistical analysis

Data were coded and entered using the statistical package SPSS (Statistical Package for Social Sciences) version 23. Data were summarized using mean and standard deviation for quantitative data and using frequency (count) and relative frequency (percentage) for categorical data. Comparisons between quantitative variables were done using the non-parametric Kruskal–Wallis and Mann–Whitney test. For comparing categorical data, Chi square (χ^2) test was performed. Exact test was used instead when the expected frequency is less than 5. Correlations between quantitative variables were done using Spearman correlation coefficient. Standard diagnostic indices including sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and diagnostic efficacy were calculated. Receiver operating characteristic (ROC) curve was constructed and area under curve (AUC) was calculated to detect best cut-off value. p values less than 0.05 were considered as statistically significant.

Results

Study population

A total of 187 participant including 35 adenocarcinoma patients, 51 adenomas patients and 101 neoplastic free controls were recruited into the study. The demographic and clinico-pathological characteristic of the subjects under the study are depicted in Table 2. No significant difference was found as regards to age, sex, family history, and clinical symptoms including diarrhea. A significant difference was found between adenocarcinoma, adenomas patients, and controls as regards to abdominal pain and constipation ($p < 0.05$ for each). Also, adenomas patients show significant difference compared to controls as regards to bleeding ($p < 0.001$). One subject (1.0%) of control group show inflammatory bowel disease and two subjects (3.9%) of adenomas group show familial polyposis.

Pathological feature including mass size, mass site for both adenocarcinoma and adenomas patients and also, TNM staging, clinical staging, grades for adenocarcinoma patients was demonstrated in Table 2. Stage I represented (no = 4, 11.4%), stage II (no = 14, 40.0%), stage III (no = 12, 34.3%), and stage IV (no = 5, 14.3%).

The expression value of studied biomarkers

The expression of miR-210, miR-21, VEGF and HIF-1α was significantly higher in both adenocarcinoma and adenomas compared with controls ($p < 0.001$ for each). On the other hand miR-126 was significantly lower in the both groups compared with controls ($p < 0.01$). No significant difference was noted comparing patients with adenocarcinoma and adenomas, Table 3 and Fig. 1.

Correlation between these biomarkers was studied and demonstrated in Table 4. miR-210 show significant positive correlation with miR-21, VEGF and HIF-1α ($r = 0.5$, $p < 0.001$; $r = 0.306$, $p < 0.001$ and $r = 0.509$, $p < 0.001$ respectively) and significant negative correlation with miR-126 ($r = -0.160$, $p = 0.028$). In addition, miR-21 show significant positive correlation with VEGF, HIF-α ($r = 0.337$, $p < 0.001$ and $r = 0.125$, $p = 0.038$ respectively) and significant negative correlation with miR-126 ($r = -274$, $p < 0.001$). Also, mir-126 show significant negative correlation with HIF-1α ($r = -0.209$, $p = 0.004$).

Table 1 Showing primer sequence of target studied genes

Gene symbol	Primer sequence from 5′–3′	Gene bank accession number
miR-210	F: ACACTCCAGCTGGGTAGCTTATCAGACTGA R: GTGTCGTGGAGTCGGCAATTC	NG_051107.1
miR-21	F: CGTCTGATCTCGGAAGCTAAGC R: GGCGGTCTCCCATCCAA	NG043905.1
miR-126	F: GGAATGTAAGGAAGTGTG R: GAGCAGGCTGGAGAA	NR_029713.1
VEGF	F: CGGGAACCAGATCTCTCACC R: AAAATGGCGAATCCAATTCC	NM_003376.5
HIF-α	F: AGCTGCTGGAGACACAATCA R: TGGGGCATGGTAAAAGAAAG	NM_181054.2
B-actin	F: GGC GGCACCACCATGTACCCT R: AGG GGCCGGACTCGTCATACT	NM_001101.3
RNU6B	F: GCTTCGGCAGCACATATACTAAAAT R: CGCTTCACGAATTTGCGTGTCAT	NG_044085.2

Table 2 Demographic and clinico-pathological characteristic of studied groups

Demographic data (No/%)	Adenocarcinoma No = 35	Adenoma No = 51	Controls No = 101	*p* value[a]	*p* value[b]	*p* value[c]
Age	51.97 ± 12.18	49.59 ± 13.99	48.47 ± 15.16	0.167	0.158	0.156
Sex						
Male	19 (54.3%)	34 (66.7%)	52 (51.5%)	0.246	0.775	0.075
Female	16 (45.7%)	17 (33.3%)	49 (48.5%)			
Family history of CRC						
Yes	1 (2.9%)	2 (3.9%)	0 (0%)	1	0.257	0.111
No	34 (97.1%)	49 (96.1%)	101 (100%)			
Abdominal pain						
Yes	20 (57.1%)	4 (7.8%)	24 (23.8%)	0.013*	0.052*	0.017*
No	15 (42.9%)	47 (92.2%)	77 (76.2%)			
Constipation						
Yes	19 (54.3%)	15 (29.4%)	38 (37.6%)	0.020*	0.054*	0.016*
No	16 (45.7%)	36 (70.6%)	63 (62.4%)			
Diarrhea						
Yes	1 (2.9%)	5 (9.8%)	12 (11.9%)	0.349	0.183	0.701
No	34 (97.1%)	46 (90.2%)	89 (88.1%)			
Bleeding						
Yes	13 (37.1%)	27 (52.9%)	25 (24.8%)	0.149	0.159	0.001*
No	22 (62.9%)	24 (47.1%)	76 (75.2%)			
Mass size						
< 1 cm	0 (0.00%)	8 (15.7%)		0.019*		
> 1 cm	35 (100%)	43 (84.3%)				
Site						
Ascending colon						
Yes	11 (31.4%)	14 (27.5%)		0.690		
No	24 (68.6%)	37 (72.5%)				
Transverse colon						
Yes	3 (8.6%)	10 (19.6%)		0.160		
No	32 (91.4%)	41 (80.4%)				
Descending colon						
Yes	22 (62.9%)	36 (70.6%)		0.452		
No	13 (37.1%)	15 (29.4%)				
TNM staging						
T category						
T1	4 (11.4%)					
T2	12 (34.3%)					
T3	16 (45.7%)					
T4	3 (8.6%)					
N category						
N0	18 (51.4%)					
N1	17 (48.6%)					
M category						
M0	30 (85.7%)					
M1	5 (14.3%)					
Clinical stage						
Stage I	4 (11.4%)					
Stage II	14 (40.0%)					
Stage III	12 (34.3%)					
Stage IV	5 (14.3%)					
Clinical stage (grouped)						

Table 2 (continued)

Demographic data (No/%)	Adenocarcinoma No = 35	Adenoma No = 51	Controls No = 101	*p* value[a]	*p* value[b]	*p* value[c]
Stage I + II	18 (51.4%)					
Stage III + IV	17 (48.6%)					

A significant difference was found between adenocarcinoma, adenoma patients and controls as regards to abdominal pain and constipation ($p < 0.05$ for each), adenoma patients showed significant difference compared to controls as regards to bleeding ($p < 0.001$)

[a]Comparison between adenocarcinoma and adenoma

[b]Comparison between adenocarcinoma and controls

[c]Comparison between adenoma and controls

*Statistically significant ($p < 0.05$)

Table 3 Levels of biomarkers in studied groups

Biomarkers	Adenocarcinoma No = 35	Adenoma No = 51	Control No = 101	*p* value[a]	*p* value[b]	*p* value[c]
miR-210	1.18 ± 0.68	1.15 ± 0.63	0.29 ± 0.14	0.954	< 0.001*	< 0.001*
miR-21	0.82 ± 0.44	0.66 ± 0.32	0.21 ± 0.08	0.342	< 0.001*	< 0.001*
miR-126	0.22 ± 0.11	0.23 ± 0.16	0.33 ± 0.19	0.823	< 0.004*	< 0.001*
VEGF (pg/ml)	1.75 ± 1.13	1.62 ± 0.68	0.78 ± 0.36	0.128	< 0.001*	< 0.001*
HIF-1α (ng/l)	1.26 ± 0.58	1.13 ± 0.51	0.23 ± 0.11	0.487	< 0.001*	< 0.001*

The expression levels of miR-210, miR-21, VEGF, HIF-α were significantly elevated in adenocarcinoma and adenoma patients compared to controls but the expression levels of miR-126 was significantly decreased

[a]Comparison between adenocarcinoma an adenoma

[b]Comparison between adenocarcinoma and controls

[c]Comparison between adenoma and controls

*Statistically significant ($p < 0.05$)

Association between biomarkers and clinico-pathological finding of adenocarcinoma group

The association between the expression of biomarkers and clinical parameters was demonstrated in Table 5. No significant association was found between miR-210, miR-21, miR-126, VEGF, and HIF-1α with demographic and clinical findings including age, sex, family history, and clinical manifestation. As regard, TNM staging, the present study demonstrated significant higher expression of miR-210, miR-21 and VEGF with advanced T classification, positive nodal and metastatic status ($p = 0.001$ each for miR-210; $p = 0.06$, $p = 0.001$, $p = 0.003$ respectively for miR-21; $p = 0.029$, $p = 0.001$, 0.001 respectively for VEGF). HIF-1α showed significant higher expression in case of positive nodal status ($p = 0.012$). On the other hand miR-126 showed significant down-regulation with positive metastatic status ($p = 0.001$). All these biomarkers, with the exception of miR-126, were significantly elevated in early stage (stage I + II) in comparison with late stage (stage III + IV) of adenocarcinoma ($p < 0.05$ for each) Table 5.

The value of studied biomarkers for early detection of CRC

The difference between miRNA expression in various stage and neoplasm free control was demonstrated at Table 6. There was significant up-regulation of miR-210, miR-21 in early stage (stage I + II) and late stage (stage III + IV) in comparison to controls (p‹0.001 for each). On the other hand, there was significant down-regulation of miR-126 in early and late stage in comparison with controls ($p = 0.052$ and $p = 0.005$ respectively) Table 6. When adenocarcinomas patients separated into 4 groups and compared with neoplasm free controls, there was significant up-regulation of miR-210, miR-21in all stages ($p < 0.05$, p‹0.00, $p < 0.001$, p‹0.001 respectively) and significant down-regulation of miR-126 in stage II, III, and VI compared with controls ($p = 0.024$, $p = 0.009$ and $p = 0.003$ respectively) (Fig. 2, 3).

The diagnostic value of studied biomarkers

To determine whether the levels of mir-210, mir-21 and mir-126 had diagnostic value for adenocarcinoma and adenomas, the ROC curve was applied to analyze

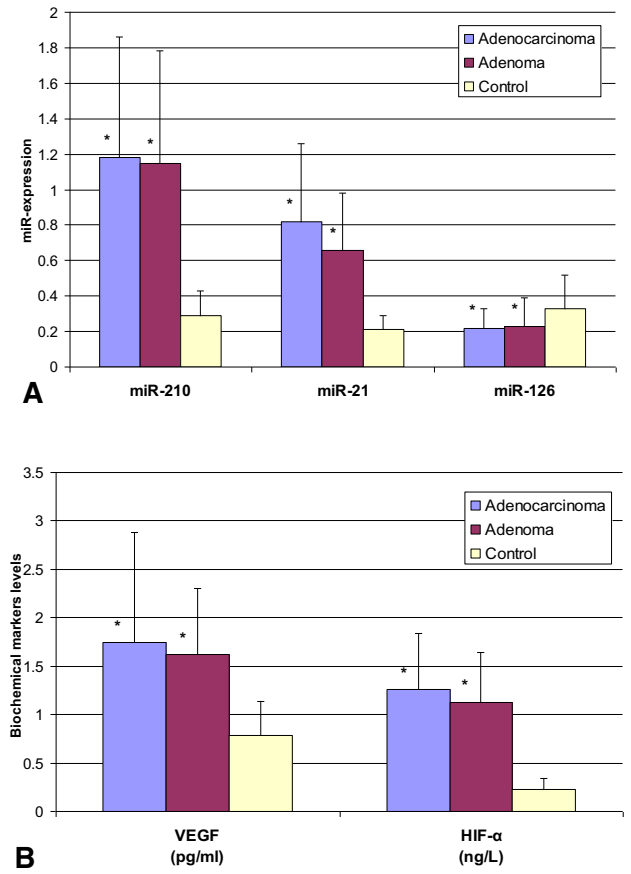

Fig. 1 The expression levels of Biochemical markers in studied groups. **a** The expression of miR-210, miR-21, was significantly higher in both adenocarcinoma and adenomas compared with controls ($p < 0.001$ for each). On the other hand miR-126 was significantly lower in the both groups compared with controls ($p < 0.01$). **b** VEGF, HIF-α was significantly higher in both adenocarcinoma and adenomas compared with controls ($p < 0.001$ for each). No significant difference was noted comparing patients with adenocarcinoma and adenomas

their diagnostic sensitivity and specificity (Table 7, Figs. 4, 5). ROC curve analysis showed that miR-210, miR-21 and miR-126 could differentiate adenocarcinoma and adenoma from controls with AUC of 0.934 for miR-210 (95% CI 0.873–0.995, p), 0.973 for miR-21 (95% CI 0.946–1.000) and 0.665 for miR-126 (95% CI 0.571–0.759). At the cut-off value of 0.512, the sensitivity and specificity of miR-210 was 88.6 and 90.1%. At the cut of value 0.333 for miR-21, the sensitivity and specificity was 91.4 and 95.0%. In addition, at the cut-off value of 0.294 for miR-126, the sensitivity and specificity was 88.6 and 50.5%. PPV, NPV, and area under the ROC curve were also demonstrated in Table 7 and Figs. 4 and 5.

Table 4 Correlation between biomarkers in studied groups

Biomarkers	miR-21	miR-126	VEGF	HIF-1α
miR-210				
Correlation coefficient	0.500	−0.160	0.306	0.509
Sig (*p* value)	< 0.001*	0.028*	< 0.001*	< 0.001*
miR-21				
Correlation coefficient		−0.274	0.337	0.152
Sig (*p* value)		< 0.001*	< 0.001*	0.038*
miR-126				
Correlation coefficient			−0.118	−0.209
Sig (*p* value)			0.107	0.004*
VEGF				
Correlation coefficient				0.394
Sig (*p* value)				< 0.001*

*Statistically significant ($p < 0.05$). miR-210 showed significant positive correlation with miR-21, VEGF and HIF-1α and significant negative correlation with miR-126. In addition, miR-21 showed significant positive correlation with VEGF, HIF-α and significant negative correlation with miR-126. Also, mir-126 showed significant negative correlation with HIF-1α

Discussion

About 52.5% miRNAs coding sequences are located at fragile sites and genomic regions and more vulnerable to mutation or environmental influences. This miRNA contributed to cancer initiation, progression and regulated expression of many oncogens and tumor suppressor genes in cancer pathogenesis [8]. The full understanding of miRNAs role in carcinogenesis is a laborious task since their function may vary according to the target tissue. Its estimation promise non-invasive, stable biomarkers that can be detected in the blood of cancer patients [11]. However, the molecular mechanisms by which miRNAs modulate cellular processes still need to be further elucidated and studying the specific function of miRNAs in human carcinogenesis may offer a foundation for evaluating its therapeutic potential as diagnostic, prognostic, and screening biomarkers [18].

To the best of our knowledge, the published reports of circulating miRNA in CRC in our community are limited so, the present study aimed to evaluate the role of miR-210, miR-21, miR-126, as a non-invasive diagnostic biomarkers for screening and early detection of CRC and throw the light on the correlation of these biomarkers with the prognostic variables which may monitor cancer progression and provide useful tools for cancer prevention and therapy.

In this study, we found that the levels of miR-210 and miR-21 were significantly higher in adenocarcinoma and adenoma than neoplasm free controls. On the other hands, levels of miR-126 were significantly lower in the same groups of patients than controls. All these biomarkers showed variation in levels between adenocarcinomas and

Table 5 Association between biomarkers and clinico-pathological finding of adenocarcinoma group

Clinico-pathological finding	miR-210		miR-21		miR-126		VEGF		HIF-1α	
	Mean ± SD	*p* value	Mean ± SD	*p* value	Mean ± SD	*p* value	Mean ± SD	*p* value	Mean ± SD	*p* value
Abdominal pain										
Yes	1.43 ± 0.63	0.377	0.49 ± 0.21	0.185	0.19 ± 0.08	0.702	2.41 ± 2.16	0.444	1.86 ± 0.88	0.068
No	1.13 ± 0.69		0.74 ± 0.33		0.22 ± 0.11		1.47 ± 1.01		0.99 ± 0.50	
Constipation										
Yes	1.11 ± 0.62	0.947	0.65 ± 0.28	0.329	0.22 ± 0.08	0.337	1.52 ± 0.74	0.305	1.09 ± 0.49	0.573
No	1.20 ± 0.76		0.80 ± 0.36		0.25 ± 0.13		1.59 ± 1.50		1.03 ± 0.68	
Bleeding										
Yes	1.19 ± 0.86	0.759	0.88 ± 0.35	0.054*	0.21 ± 0.14	0.188	1.47 ± 1.31	0.384	0.89 ± 0.47	0.246
No	1.13 ± 0.57		0.63 ± 0.27		0.24 ± 0.09		1.59 ± 1.05		1.17 ± 0.62	
Diarrhea										
Yes	1.91 ± 0.68	0.235	0.72 ± 0.33	0.921	0.34 ± 0.11	0.165	1.24 ± 1.65	0.092	1.32 ± 0.56	0.137
No	1.13 ± 0.62		0.69 ± 0.30		0.21 ± 0.09		1.59 ± 1.13		1.09 ± 0.57	
Mass										
< 1 cm	1.15 ± 0.57	0.212	0.63 ± 0.23	0.53	0.22 ± 0.12	0.327	1.55 ± 1.12	0.098	1.06 ± 0.58	0.134
> 1 cm	1.45 ± 0.68		0.73 ± 0.32		0.24 ± 0.13		1.87 ± 1.13		1.28 ± 0.56	
Grade										
LG	0.95 ± 0.53	0.289	0.67 ± 0.28	0.195	0.21 ± 0.09	0.709	1.48 ± 1.10	0.694	1.04 ± 0.61	0.455
HG	1.21 ± 0.70		0.88 ± 0.43		0.23 ± 0.17		1.78 ± 1.55		1.15 ± 0.49	
T stage										
T1 + T2	0.65 ± 0.48	0.001*	0.83 ± 0.48	0.052*	0.26 ± 0.12	0.072	1.38 ± 0.55	0.029*	1.02 ± 0.62	0.246
T3 + T4	1.47 ± 0.79		1.12 ± 0.42		0.17 ± 0.06		1.93 ± 0.84		1.24 ± 0.48	
N stage										
N0	0.51 ± 0.28	0.001*	0.74 ± 0.36	0.001*	0.24 ± 0.12	0.241	1.17 ± 0.41	0.001*	0.90 ± 0.59	0.012*
N1	1.71 ± 0.66		1.26 ± 0.41		0.18 ± 0.07		2.18 ± 0.72		1.39 ± 0.39	
M stage										
M0	0.84 ± 0.49	0.001*	0.80 ± 0.40	0.003*	0.23 ± 0.10	0.001*	1.47 ± 0.58	0.001*	1.03 ± 0.03	0.528
M1	1.60 ± 0.32		1.26 ± 0.47		0.10 ± 0.05		2.52 ± 0.55		1.59 ± 0.13	
Clinical stage										
Stage I + II	0.51 ± 0.28	˂0.001	0.74 ± 0.36	˂0.001	0.24 ± 0.12	0.241	1.19 ± 0.42	˂0.001*	0.90 ± 0.59	0.012*
Stage III + IV	1.72 ± 0.66		1.27 ± 0.41		0.18 ± 0.07		2.19 ± 0.71		1.39 ± 0.39	

*Statistically significant ($p < 0.05$), There was significant higher expression of miR-210, miR-21 and VEGF with advanced T classification, positive nodal and metastatic status, HIF-1α showed significant higher expression in case of positive nodal status, miR-126 showed significant down-regulation with positive metastatic status, The biomarkers were significantly different in early stage in comparison with late stage of adenocarcinoma

Table 6 Biomarkers differently expressed in various stage of adenocarcinoma and controls

Biomarkers	Early stage (I + II)	Late stage (III + IV)	Controls	*p* value[a]	*p* value[b]
miR-210	0.51 ± 0.28	1.72 ± 0.66	0.29 ± 0.14	< 0.001*	< 0.001*
miR-21	0.74 ± 0.36	1.27 ± 0.41	0.21 ± 0.08	< 0.001*	< 0.001*
miR-126	0.23 ± 0.10	0.18 ± 0.07	0.33 ± 0.19	0.051*	0.005*
VEGF (pg/ml)	1.19 ± 0.42	2.19 ± 0.71	0.78 ± 0.36	0.020*	0.003*
HIF-1α (ng/l)	0.90 ± 0.59	1.39 ± 0.39	0.23 ± 0.11	0.053*	0.001*

There were significant up-regulation of miR-210, miR-21 while miR-126 was significantly down-regulated in early stage and late stage in comparison to controls

[a]Comparison between early stage and controls

[b]Comparison between late stage and controls

*Statistically significant ($p < 0.05$)

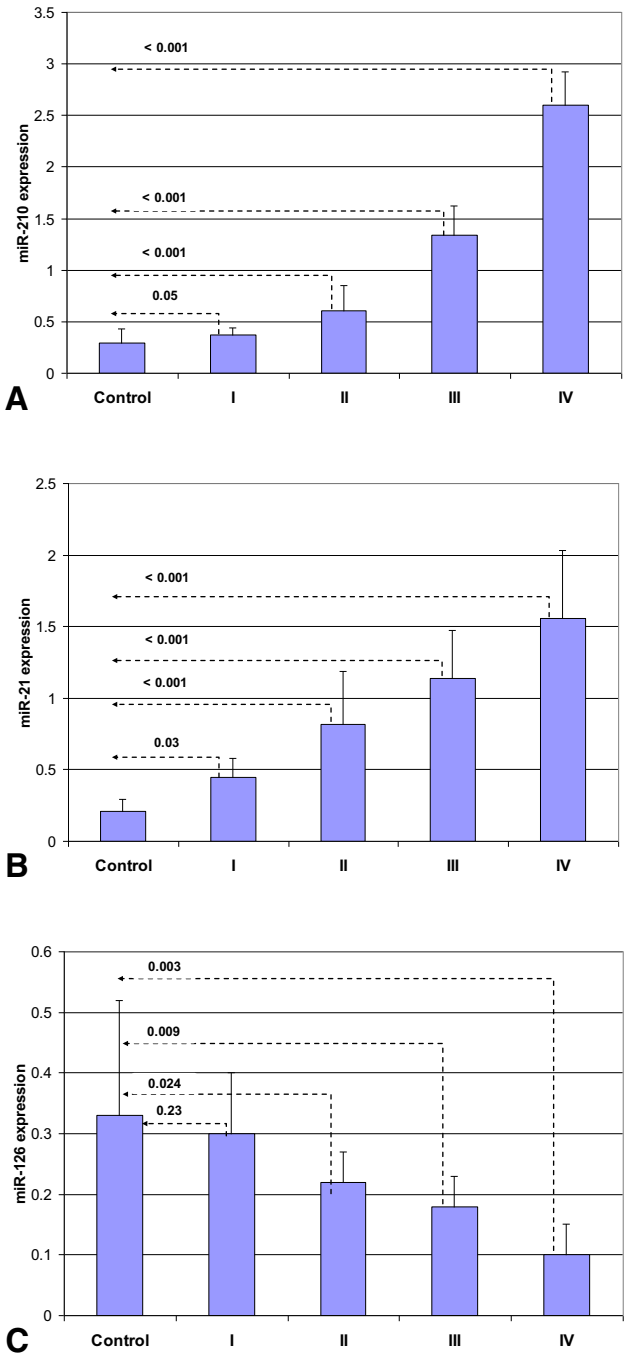

Fig. 2 Association of, miRNAs expression with TNM stages of adenocarcinoma, **a, b** there were significant up-regulation of miR-210, miR-21 in all stages of adenocarcinoma. **c** There was significant down-regulation of miR-126 in stage II, III and VI compared with controls

adenomas but did not reach a significant value. In addition the three miRNAs showed significant correlation with each other.

The miR-210, miR-20 and miR-126 show significant diagnostic value for CRC yielding AUC 0.934, 0.973 and 0.665 respectively with sensitivity and specificity 88.6 and 90.1% for miR-210, 91.4 and 95.0% for miR-21, 88.6 and 50.5% for miR-126. The results of miR-210 and miR-21 were suggesting their potential value for early detection of CRC.

As regards to association between biomarkers and clinico-pathological finding of adenocarcinoma group, the present study showed no significant association between studied biomarkers and demographic and clinical finding. On the other hand, TNM staging demonstrated significant higher expression of miR-210, miR-21 with advanced T classification, positive nodal and metastatic status. Also, there was significant up-regulation of miR-210, miR-21 in early stage (stage I + II) and late stage (stage III + IV) than controls and significant down-regulation of miR-126 in early and late stage than controls.

As regards to miR-210 our results were in agreement with Gee et al. [19] and Tagscherer et al. [9]. They reported that miR-210 up-regulated in CRC compared to controls and associated with poor prognosis. Also another study by Qu et al. [10], showed that miR-210 up-regulated, correlated with aggressive tumor progression and is involved in hypoxia-induced CRC metastasis. Its levels in stage II-IV were significantly higher than in stage I. Wang et al. [20] confirmed that miR-210 were significantly elevated in CRC and it was a useful marker for discriminating CRC from controls with sensitivity 74.6% and specificity 73.5%. They also found that high expression of miR-210 was correlated with clinical TNM stage.

The functional consequences of an increased miR-210 expression in CRC are so far unknown and its role in CRC is still unclear and warrants further investigation. Since hypoxia is one of the main factors contributing to poor prognosis of cancer, elevated miR-210 levels, which are mainly regulated by HIF, may only be a bystander effect instead of directly influencing patients' outcome. miR-210 inhibit cologenicity and proliferation with accumulation of cells in the G2/M phase of cell cycle. Also, there was an increase of ROS generation. Moreover, there was induction of apoptosis associated with up-regulation of pro-apoptotic expression and enhance processing of Caspase 2. Therefore, regulation of apoptosis by miR-210 might be of great biological relevance in CRC [9].

On the other hand, Ullmann et al. [1] reported that, low oxygen concentration lead to the up-regulation of miR-210 in tissue cultures with increased incidence of tumor, reduced tricarboxylic acid cycle activity and increased lactate levels. This study highlights the importance of hypoxia- induced miR-210 in the regulation of colon cancer initiation.

As regards to miR-21, our results are in accordance with Yan et al. [21], Shibuya et al. [22], Liu et al. [23] and de Almeida et al. [11]. They found that, there was overexpression of miR-21 in CRC which more linked with advanced

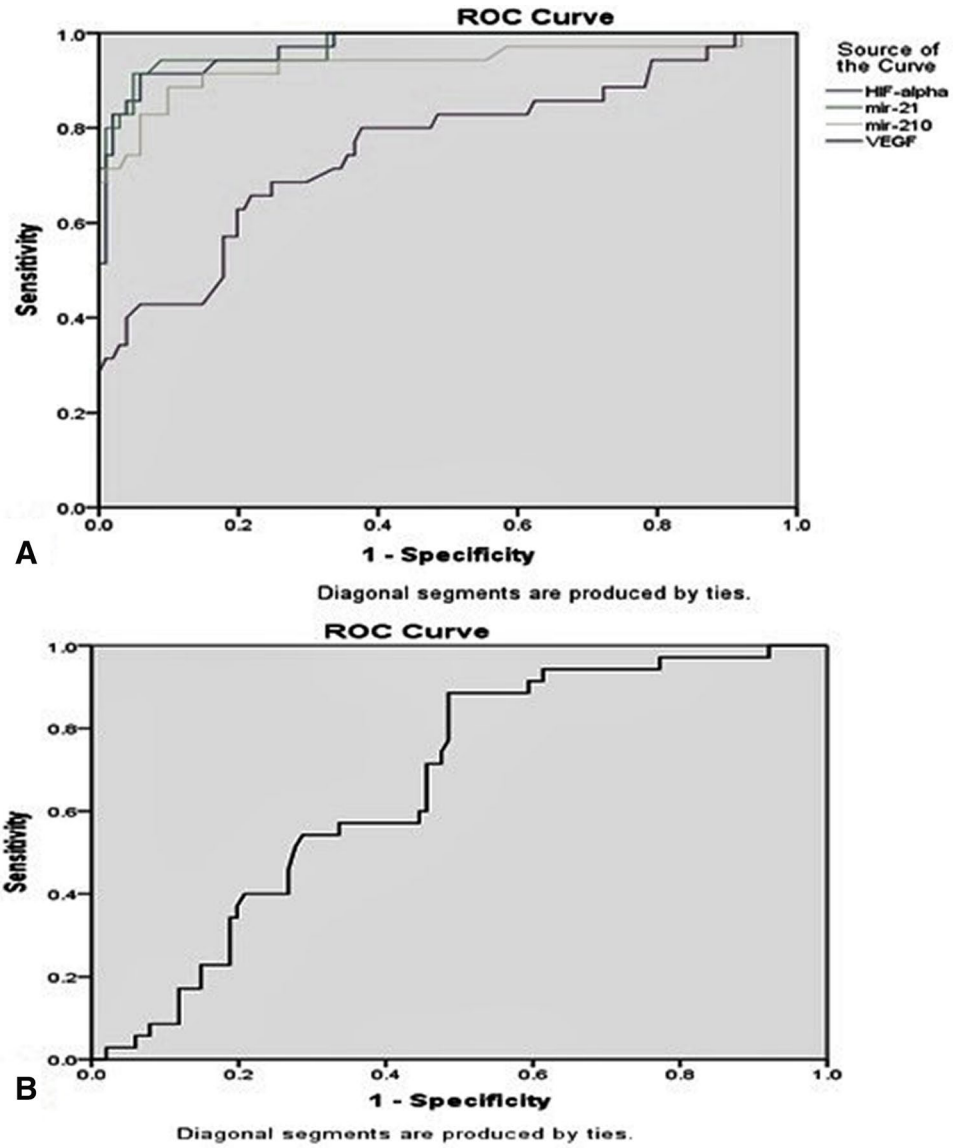

Fig. 3 ROC curve for expression of miR-210, miR-21, VEGV and HIF-α. miR-210, miR-21 and miR-126 could differentiate adenocarcinoma and adenoma from controls. **a** AUC was 0.934 for miR-210, 0.973 for miR-21, 0.758 for VEGF, 0.970 for HIF-α. The sensitivity and specificity were 88.6% and 90.1% for miR-120, 91.4% and 95.0% for miR-21, 65.7% and 78.2 for VEGF, 91.4% and 94.1% for HIF-α. **b** The AUC was 0.665 for miR-126 and the sensitivity and specificity was 88.6% and 50.5%

TNM stage and lower cellular differentiation. The miR-21 inhibits the expression of tumor suppressive genes and induces cell proliferation [11]. The results of Basati et al. [24] and Wu et al. [25] exhibited that miR-21 significantly elevated in adenocarcinoma. Furthermore, there was significant association with clinical staging and clinical TNM classification giving the fact that the majority of miRNA coming from tumor tissue. Also, they found high sensitivity and specificity for miR-21 in discriminating between adenocarcinoma and controls [24]. The same results reported by Tsukamoto et al. [26], Liu et al. [23].

The estimated diagnostic sensitivity and specificity of miR-21 expression about 65 and 85% respectively in plasma and 97 and 91% in saliva indicating that this biomarker was proper for CRC screening [27]. The sensitivity and specificity of miR21 in the diagnosis of CRC were 84.5 and 74.4%, respectively as reported by Wang and Zhang [7]. Similar results were also approved in another study by Kanaan et al. [28].

On the other hand, Kanaan et al. [28] and Wang and Zhang [7] reported no significant association between serum miR-21 levels and clinical staging of CRC.

miR-21 not reliable biomarkers regarding lymph node metastasis but it could support risk assessment in stage T1 tumors. The reason of the controversy deserves further evaluation [29].

The nuclear transcription factor (NF-kB) and signal transducer and activator of transcription 3 (STAT3) maintain constitutive activator of pro-inflammatory pathways as essential components in development of CRC. The miR-21 acted as a negative regulator of NF-kB signaling result in inflammation hyper-responsiveness and tumorigenesis.

Table 7 Diagnostic value of studied biomarkers in patients

Biochemical markers	Area under curve	95% confidence interval		*p* value	Cut-off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
		Lower bound	Upper bound							
miR-210	0.934	0.873	0.995	< 0.001*	0.512	88.6	90.1	75.6	95.8	89.7
miR-21	0.973	0.946	1	< 0.001*	0.333	91.4	95	86.5	97	94.1
mirR-126	0.665	0.571	0.759	0.004*	0.294	88.6	50.5	38.3	92.7	60.3
VEGF	0.758	0.658	0.859	< 0.001*	0.992	65.7	78.2	51.1	86.8	75
HIF-1α	0.97	0.942	0.998	< 0.001*	0.349	91.4	94.1	84.2	96.9	93.4

*Statistically significant ($p < 0.05$), *PPV* positive predictive value, *NPV* negative predictive value, miR-210, miR-21 and miR-126 could differentiate adenocarcinoma and adenoma from controls with AUC of 0.934 for miR-210, 0.973 for miR-21 and 0.665 for miR-126. At the cut-off value of 0.512, the sensitivity and specificity of mir-210 was 88.6% and 90.1%. At the cut of value 0.333 for mir-21, the sensitivity and specificity was 91.4 and 95.0%. In addition, at the cut-off value of 0.294 for mir-126, the sensitivity and specificity was 88.6 and 50.5%

STAT3 directly activated miR-21 transcription by binding multiple sites in miRNA promotor resulting in cellular transformation [18]. Also, miR-21 can modulate malignant phenotypes such as proliferation, anti-apoptosis, cell cycle progression and invasion of CRC cells by down-regulating PTEN protein expression [5]. miR-21 promotes proliferation, migration, invasion, tumor growth of CRC by down-regulation of sec23a protein and its mRNA expression [30].

Sideris and Papagrigoridis (2014) [31] and de Almeida et al. [11] showed low expression of miR-126 in CRC patients and associated with poor prognosis. Also, Yuan et al. [32] showed low expression of miR-126 in CRC which inversely correlated with TNM stage and metastasis and low level identified patients with poor prognosis. In addition, miR-126 was down-regulated in CRC which associated with histological subtypes, peri-neural tumor infiltration, microsatellite instability, pathological staging, poorer survival, and advanced nodal and metastatic stage [33]. The same results were reported by Zhou et al. [34], Liu et al. [35] and Faeza et al. [36].

The miR-126 inhibited cell proliferation, migration and invasion [11]. Also, miR-126 coordinate cell behavior signaling cascades via modulating gene expressions of VEGF/PI3K/AKT and MAPK signaling that may be more effective in inhibition of cancer development and metastasis [37]. Yuan et al. [32] added that, miR-126 act as tumor suppressor that inhibit colon cancer proliferation and invasion by inactivating RhoA signaling via (C-X-C motif) receptor 4 and Ras homolog gene family, member A, signaling pathway. It increased apoptosis and decreased accumulation of cells in the G0–G1 phase with reduced BCL-2 and increased P53 protein expression [33].

The results of the present study showed that, the levels VEGF and HIF-1α were significantly higher in adenocarcinoma and adenoma than neoplasm free controls. These two biomarkers showed variation in levels between adenocarcinomas and adenomas but not reach a significant value. In addition the three studied miRNAs showed significant correlation with VEGF and HIF-1α.

Our results were in agreement with Jiang et al. [38], Greijer et al. [39] and Simiantonaki et al. [40]. They found that HIF-1α (mRNA and/or protein) is detected in both adenomas and colorectal adenocarcinomas, and is more frequently expressed in adenocarcinomas compared to adenomas. The levels of HIF-1 were positively correlated with VEGF expression. Additionally Takaaki et al. [41] suggested that HIF-α promoted the growth of colon cancer cells. Angiogenesis in CRC has been shown to be induced by HIF-1α through the activation of expression of the HIF-1 target gene. The activation of the HIF-1α/VEGF pathway in CRC tissue specimens has been shown in a number of studies using immunohistochemistry [42, 43].

HIF-1 mediated VEGF expression increases survival of colon cancer cells in culture under hypoxic conditions, dependent on the expression of a functional VEGFR-2 (KDR receptor). The colon cancer cells differentially express a functional VEGF/KDR/HIF- 1α autocrine loop that mediates survival under hypoxic conditions [44].

Our results can be explained by HIF-1α-VEGF pathway. It was evident that the VEGF signaling was highly regulated at multiple levels. The miRNAs provide an additional layer of regulation by titrating the levels of proteins that involved in the transduction of angiogenic signals. These miRNAs were highly induced in response to HIF-1α that produced in response to hypoxia as in cancer cells [45]. They concluded that in hypoxia different miRNA work consonantly to fine-tune the cellular adaptation; when a master miRNAs alters its expression, dynamic of others vary accordingly which together contribute to aberrant RNA/protein profiles observed in the pathophysiology in cancers. Under normoxia, endogenous levels of miR-210 are at very low levels. Stabilization of miR-210 is achieved by the binding of HIF-1α to the Hypoxia Responsive Element present on its proximal promoter. HIF-1α promotes increased expression of miR-210 which promotes the stabilization of HIF-1α, suggesting presence of a positive feedback loop. Because the level of miR-210 is dependent on the level of HIF-1α, the presence of elevated miR-210 in tissues has become a predictive marker for tumor hypoxia [46]. HIF-1α drives miR-210's overexpression and this result in alteration of cellular processes, including cell cycle regulation, mitochondria function, apoptosis, angiogenesis, and metastasis [47]. Since VEGF plays a broad role in multiple aspects of endothelial biology and has become a target for therapeutic manipulation of pathological blood vessel in cancer, miRNAs that affected VEGF signaling output will undoubtedly be major targets of clinical value and may be useful in clinical settings where precise control of vascular growth is desired [45].

Conclusion

Our study revealed that circulating miR-210, miR-21, and miR-126 expression was varied in CRC and adenomas patients, correlated with TNM staging and they had high diagnostic value. HIF-1α VEGF signaling pathways regulated by miRNAs played a role in colon cancer initiation. To the best of our knowledge, this is the first study of this miRNAs panel in CRC in our community. These data suggested that these biomarkers could be a potential novel, non-invasive marker for early diagnosis, screening and predicting prognosis of CRC. Understanding the molecular functions by which miRNAs affect cancer and understanding its roles in modulating the signaling output of VEGF might be fruitful in reducing the incidence and slowing the progression of disease. Further studies with large numbers and follow up are required to demonstrate whether it can be incorporated into routine clinical practice.

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Compliance with ethical standards

Conflict of interest The authors report no conflict of interest.

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