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ORIGINAL ARTICLE

Serum microRNA-18a, microRNA-21 and microRNA-92a as diagnostic markers in colorectal cancer patients

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Summary

Purpose: *The present study was done to measure the serum relative expression levels of microRNA-18a, microRNA-21, and microRNA-92a in colorectal cancer patients compared to healthy volunteers to evaluate their use as diagnostic markers in colorectal cancer patients.*

Methods: *The relative serum quantification of each of microRNA-18a, microRNA-21, and microRNA-92a normalized to microRNA-16 was studied in 50 patients diagnosed with colorectal cancer and 50 age- and sex-matched healthy volunteers using real-time polymerase chain reaction (RT-PR).*

Results: *The expression levels of microRNA-18a, microRNA-21, and microRNA-92a were found to be significantly up-regulated in serum of colorectal cancer patients compared to the healthy control group. MicroRNA-18a demonstrated an area under the receiver operating characteristics curve of 0.906; microRNA-21 yielded an AUC of 0.918, while microRNA-92a demonstrated an area under the receiver operating characteristics curve of 0.672 when discriminating colorectal cancer patients from healthy controls.*

Conclusions: *Serum microRNA-18a and/or microRNA-21 might serve as non-invasive diagnostic markers in colorectal cancer, while serum microRNA-92a is better to be combined with either microRNA-18a or microRNA-21 as it has limited usefulness when used as sole diagnostic marker.*

Key words: *colorectal cancer, marker, microRNAs, microRNA-18a, microRNA-21 and microRNA-92a.*

Introduction

Worldwide, colorectal cancer (CRC) accounted for 10% of all cancers in men and 9.2% in women in 2012, making it the third and the second most common cancer in men and women respectively [1]. In Egypt, results from the year 2008 to 2011 of the National Population-Based Cancer Registry Program showed that of all cancers, colon cancer accounted for 2.63% in males and 2.28% in females. Also, rectal cancer accounted for 0.84 and 0.72% in males and females, respectively [2,3].

The mainstay for CRC screening and detection remains colonoscopy, while in contrast to endoscopy or radiology-based tests, biomarker assays are more cost-effective and less invasive [3].

MicroRNAs (miRNAs) are RNA molecules that consist of approximately 18–25 nucleotides, acting epigenetically either by preventing the translation of messenger RNA (mRNA) or causing mRNA degradation, and that could be more conveniently used than routine colonoscopy in CRC screening and diagnosis. However, their use might never surpass the successful performance of routine colonoscopy [4].

The purpose of this work was to study the serum expression levels of miRNA-18a, miRNA-21 and miRNA-92a in CRC patients compared to healthy volunteers and to assess their performance when used as diagnostic tests in CRC.

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Methods

Subjects

The subjects enrolled in the present cross-sectional study were recruited between June 2016 and January 2018 and consisted of two groups: CRC patients' group included 50 Egyptian subjects who were scheduled to undergo surgery in the gastrointestinal tract surgical department at the National Cancer Institute (NCI), Cairo University; Control group included 50 healthy subjects attending the Chemical Pathology Department's Outpatients' Section for a routine check-up.

Inclusion criteria for the patients' group were diagnosed CRC patients confirmed by pathological examination and blood sample taken before any surgical intervention for the disease. Exclusion criteria were chemotherapy or radiotherapy preceding the blood sampling and blood transfusion received in the 3 months preceding the blood sampling.

The study was approved by the local Ethics Committee of the Clinical and Chemical Pathology Department (August 2015) and written consent was obtained from all the participants before the commencement of the study.

Methodology

a) Sample collection

From each CRC patient, 6 mL venous blood was withdrawn and divided into two serum vacutainer tubes, then both tubes were left to clot and then centrifuged. The separated serum from one tube was used for determination of serum carcinoembryonic antigen (CEA) for CRC patients and the supernatant serum of the other tube was stored at –80°C until RNA purification for determination of the expression level of mature miRNAs. From healthy subjects, 3 mL venous blood was withdrawn for mature miRNAs expression level determination.

b) RNA purification

Purification of total RNA, including miRNA, from serum and plasma was done using Qiagen® miRNeasy Serum/Plasma Kit (Qiagen, cat no 217004) according to the manufacturer's instructions. The concentration of RNA was determined by measuring the absorbance at 260nm (A260) using the NanoDrop 1000A Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

c) MicroRNAs assay

For microRNA based reverse transcription-polymerase chain reaction (RT-PCR) assays, 5 µL of small RNAs from plasma samples were reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, cat no 4366596) according to manufacturer's instructions in a total reaction volume of 15 µL. The quantitative PCR reaction (qPCR) was done using TaqMan® MicroRNA Assays (Applied Biosystems, cat no 4427975) specific to the corresponding mature sequence, the RT-PCR reaction product (complementary DNA), TOPreal™ qPCR 2× PreMIX (TaqMan probe) (Enzynomics, cat no RT600S) and nuclease-free water in a 20 µL final reaction volume. MicroRNA-16 was used as an endogenous control for normalization [5,6]. The qPCR reactions were run on StepOne Real-time PCR system (Applied Biosystems, Foster City, CA, USA).

d) MicroRNAs relative expression

Relative expression (fold change) for each candidate miRNA within each group was then calculated using the comparative cycle threshold (CT) method with the fold change (FC) equation: $2^{-\Delta\Delta Ct}$ [7].

Statistics

Analysis and statistical evaluation of the results were performed using Microsoft® Excel® and IBM© SPSS© Statistics version 22 (IBM© Corp., Armonk, NY, USA). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test or Fisher's exact test was used to examine the relation between qualitative variables. For quantitative data, a comparison between two groups was made using either Student t-test for normally distributed (parametric) or Mann-Whitney U test (non-parametric t-test) for not normally distributed data. The Receiver Operating Characteristic (ROC) curve was used for determining the area under the curve (AUC) for the different markers and prediction of diagnostic cut-off values. A new variable predicted probability was created by using the binary logistic regression to assess if the use of combined markers was better than single marker use. Evaluation of diagnostic accuracy of the markers was done by calculating sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and total accuracy. All tests were two-tailed. A p value <0.05 was considered significant, $p<0.01$ was moderately significant and $p<0.001$ was highly significant.

Results

The demographic data of the studied groups are summarized in Table 1. Compared to the healthy control group, the relative serum expression levels of the three analyzed miRNAs were significantly up-regulated in CRC patients (Table 2 and Figure 1).

ROC curve analysis demonstrated that the serum levels of miRNA-18a and miRNA-21 were beneficial biomarkers for distinguishing between

Table 1. Demographic data of the studied groups

	CRC (n=50)	*Controls (n=50)*
Age, years†	50.2 ± 16.7	44.5 ± 14.1
Gender‡		
Male	27 (54)	28 (56)
Female	23 (46)	22 (44)

† Data are presented as mean ± SD, ‡ Data are presented as numbers (%)

Table 2. Median values of FC of miRNAs among the studied groups

	miRNAs FC of the studied groups		
	CRC (N = 50)	*Control (N = 50)*	*p value*
miRNA-18a†	7.36 (0.44 - 71.55)	1.18 (0.09 - 4.66)	<0.001
miRNA-21†	6.05 (0.64 - 141.16)	0.89 (0.17 - 6.27)	<0.001
miRNA-92a†	1.86 (0.15 - 63.58)	0.99 (0.08 - 6.10)	0.003

† Data are presented as median (minimum-maximum)

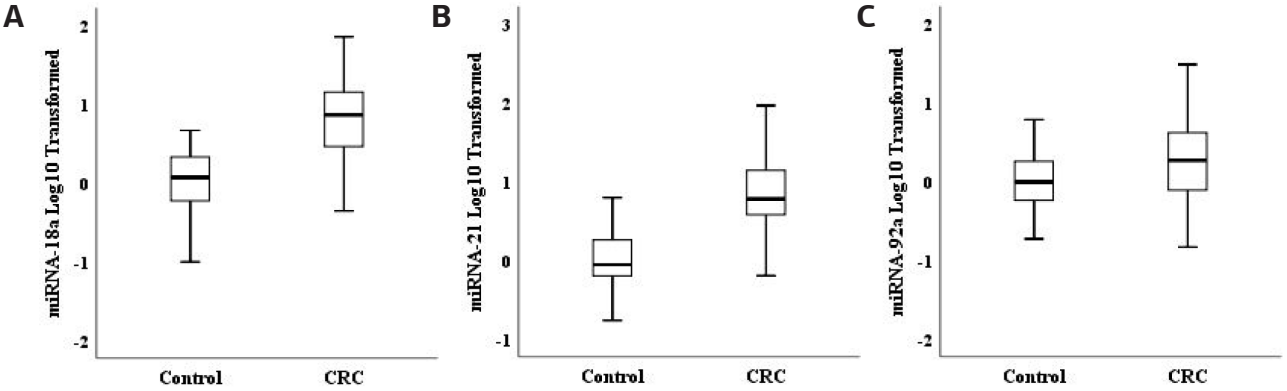

Figure 1. Box plots of median fold change of serum **(A)** miRNA-18a, and **(B)** miRNA-21, and **(C)** miRNA-92a of the studied groups.

Table 3. Results of receiver operating characteristic (ROC) for serum miRNA-18a, miRNA-21 and miRNA-92a and their combinations

	Cutoff	*Sensitivity (%)*	*Specificity (%)*	*PPV† (%)*	*NPV‡ (%)*	*Accuracy (%)*
miRNA-18a	2.379	84	84	84	84	84
miRNA-21	2.806	84	90	89.4	84.9	87
miRNA-92a	1.469	66	68	67.3	66.3	67
miRNA-18a + miRNA-21		88	92	91.7	88.5	90
miRNA-18a + miRNA-92a		80	92	90.9	82.1	86
miRNA-21 + miRNA-92a		84	90	89.4	84.9	87
miRNA-18a + miRNA-21 + miRNA-92a		86	90	89.6	86.5	88

PPV: Positive predictive value, NPV: Negative predictive value

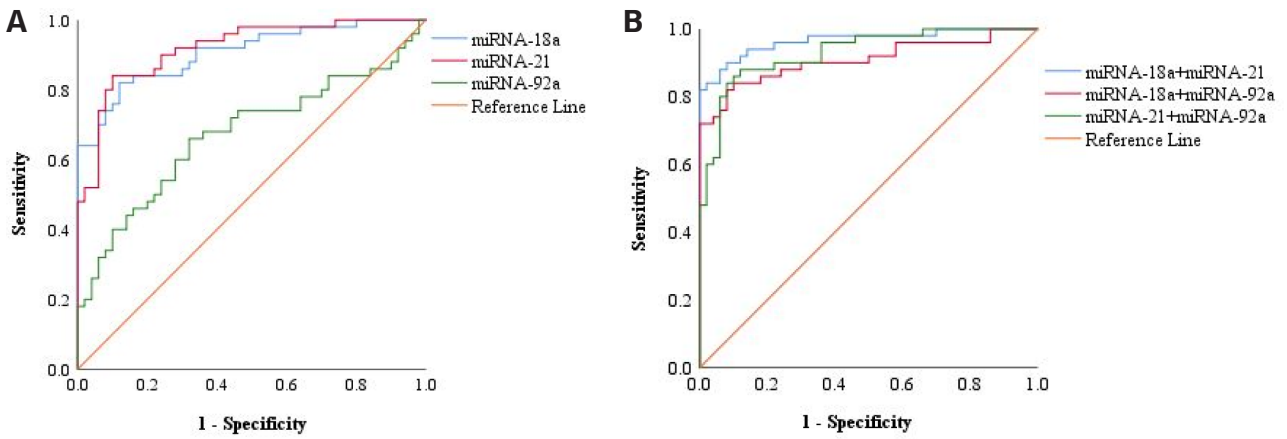

Figure 2. The receiver operating characteristic curves showing **(A)** the AUC of the studied miRNAs and **(B)** the AUC of the combined miRNA-18a, miRNA-21 and miRNA-92a in discriminating CRC patients.

Table 4. Relationship between miRNA-18a, miRNA-21 and miRNA-92a expression and clinical and pathological features in CRC patients

	N (%)	*miRNA-18a*	*miRNA-21*	*miRNA-92a*
Age, years				
<45	20 (40))	6.46 (0.44-26.37)	5.43 (1.10-92.49)	1.82 (0.17-15.89)
≥45	30 (60)	8.55 (1.02-71.55)	6.64 (0.64-141.16)	1.99 (0.15-63.58)
p value		0.635	0.384	0.593
Gender				
Male	27 (54)	10.93 (0.44-34.55)	5.98 (0.93-78.86)	2.6 (0.17-63.58)
Female	23 (46)	6.28 (1.02-71.55)	6.46 (0.64-141.16)	1.8 (0.15-10.27)
p value		0.126	0.846	0.508
Smoking				
Positive	12 (24)	17.46 (2.91-34.55)	5.71 (1.10-78.86)	3.44 (0.17-30.49)
Negative	38 (76)	6.05 (0.44-71.55)	6.26 (0.64-141.16)	1.82 (0.15-63.58)
p value		<0.001	0.768	0.261
Tumor location				
Colon	20 (40)	7.24 (1.26-34.55)	5.36 (0.93-92.49)	2.57 (0.19-63.58)
Rectum	22 (16)	7.47 (0.44-71.55)	6.44 (0.64-141.16)	1.72 (0.15-28.65)
Colorectal	8 (44)	8.65 (1.58-18.78)	5.32 (1.10-30.94)	1.69 (0.58-15.89)
p value		0.978	0.497	0.338
Pathology				
Conventional adenocarcinoma	43 (86)	6.37 (0.44-71.55)	5.98 (0.64-141.16)	1.80 (0.15-63.58)
p value		0.702	0.213	0.510
Clinical stage				
Early (I-II)	18 (36)	5.04 (1.26-71.55)	6.57 (0.93-141.16)	
Late (III-IV)	32 (64)	8.26 (0.44-30.29)	5.49 (0.64-92.49)	
p value		0.146	0.289	
Preoperative serum CEA level (ng/mL)				
≤5	27 (54)	6.37 (0.75-34.55)	5.55 (0.93-78.86)	
>5	23 (46)	7.84 (0.44-71.55)	6.26 (0.64-141.16)	
p value		0.922	0.915	

Data are presented as median (minimum-maximum)

CRC patients and healthy controls, either each used solely or combined. Also, miRNA-92a used alone had limited usefulness and its performance was markedly improved when combined with either of miRNA-18a or miRNA-21 (Table 3 and Figure 2).

Clinical and pathological data of the CRC patients' group and the association of the relative expression (normalized to miRNA-16) of the studied miRNAs with the clinical and pathological characteristics in CRC patients are shown in Table 4.

Using the diagnostic cutoff for the relative serum expression of microRNA-18a (2.379), it was found that there was no relationship between smoking status and the relative serum expression of microRNA-18a when the smoking status was used to categorize CRC patients' group (p=0.083).

Discussion

The high prevalence of morbidity and mortality from CRC makes the development of sensitive, specific, cost-effective, noninvasive, and technically convenient methods for early detection and diagnosis of an imperative need [8].

Fecal occult blood test (FOBT) and/or fecal immunochemical test (FIT) are generally used to detect microscopic blood in the gastrointestinal tract as the first approach to screen for CRC in suspected individuals. Both are considered noninvasive and inexpensive modalities, but their use doesn't lead to conclusive results [9] and colonoscopy is usually performed to provide definitive diagnosis [10]. However, the use of colonoscopy

has many pre-requisites and has several disadvantages [11].

MicroRNAs expression patterns differ in the serum and plasma of cancer patients [12], and it has been shown that during CRC progression, the expression levels of several miRNAs are altered [13]. In 2008, Lawrie et al were the first to establish the existence of miRNAs in the circulation [14]. Being packed in exosomes or vesicles, or bound to proteins/lipoproteins [15], miRNAs are protected from enzymes that can rapidly degrade RNA [16]. In addition, a small quantity of blood is required for miRNA measurement in plasma [17].

Based on the considerations mentioned above and to address the drawbacks of routine colonoscopy, circulating miRNAs have been suggested as potential biomarkers for many cancers, including CRC [8].

MicroRNA-18a may function as a potential tumor suppressor through its targeting effect on KRAS [18] and by inducing cancer cell apoptosis [19]. MicroRNA-21 is one of the miRNAs that are frequently overexpressed in CRC and is, therefore, considered an oncogenic miRNA [20]. MicroRNA-92a was found to have a pro-tumorigenic effect, as miRNA-92a inhibitor has significantly increased the incidence of apoptosis in tumor cells [21].

The present study was performed to evaluate the serum expression levels of miRNA-18a, miRNA-21 and miRNA-92a in CRC patients compared to healthy volunteers and to assess their performance as diagnostic tests in CRC.

MiRNA-18a, miRNA-21 and miRNA-92a were found to be significantly up-regulated in the CRC patients' group compared to the healthy control group and demonstrated an AUC of 0.906, 0.918 and 0.672 respectively, with a better performance of both miRNA-18a and miRNA-21 than miRNA-92a. When the three miRNAs were used in different combinations, miRNA-18a with miRNA-21 had the best performance among all combinations with an AUC of 0.966, demonstrating the additive effect in the diagnostic potential of both biomarkers.

Several studies that were done over the years evaluating the same miRNAs studied in the present work in CRC patients but of different ethnic origins as part of different miRNAs panels, were in accordance with the results presented here. These studies have demonstrated that miRNA-21 and miRNA-17~92 cluster members, including miRNA-18a and miRNA-92a, were increased in the serum of CRC patients making them useful biomarkers for discriminating CRC patients from healthy controls [5,22-30].

On the contrary, other studies haven't found a significant difference in serum miRNA-18a and miRNA-92a levels of CRC patients than in healthy controls [31-34]. As per Faltejskova et al, trying to explain their contradictory results with previous studies, no internal consensus control for circulating miRNAs has been determined, the correlation between the levels of miRNAs in plasma and serum was not established, and the specificity of these miRNAs for CRC was uncertain. Moreover, they have reported that the patients that were enrolled in their study were of Caucasian race while the antecedent studies to their work, as they mentioned, were performed on patients of Mongoloid race [31].

Vega et al results concerning serum miRNA-21 levels have revealed its significant down-regulation in CRC patients compared to healthy control group [34]. The reasons for these contradictory results with the present study may be related to the different studied populations. In the same context, Li et al found that miRNA-21 relative serum expression was not significantly altered in CRC patients classified as having stage two and stage three cancer, compared to healthy controls. As per Li et al, the combined use of let-7d/g/i as housekeeping genes for the normalization of serum miRNA levels, was better than others in common use, as miRNA-16 [35].

In conclusion, serum microRNA-18a and/or microRNA-21 might serve as non-invasive diagnostic markers in CRC, CRC while serum microRNA-92a is better to be combined with either microRNA-18a or microRNA-21 as it has limited usefulness when used as a sole diagnostic marker. However, the evaluation of the studied microRNAs in patients with early stage CRC is required to evaluate the role of these markers for early diagnosis, which will contribute to early management of patients and improve the disease outcome.

Ethical approval

This study was approved by the local Ethics Committee of the Clinical and Chemical Pathology Department (August 2015).

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Conflict of interests

The authors declare no conflict of interests.

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