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Research Article

Serum and Stool miR-135b Levels as a Potential Diagnostic Biomarker for Colorectal Cancer

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ARTICLE INFO

Article history:

Received: 6 January, 2020

Accepted: 3 February, 2020

Published: 7 February, 2020

Keywords:

Serum

stool

miR-135b

biomarker

colorectal neoplasm

ABSTRACT

Background: By considering the high incidence and mortality rate of colorectal cancer (CRC), finding the noninvasive biomarker for detection of patients with cancer is the main purpose of more and more cancer studies.

Methods: In this research, the expression level of miR-135b in serum and stool of patients with colorectal cancer was investigated as a diagnostic marker. Using the real-time PCR, the relative expression level of miR-135b in serum and stool in 40 patients with colorectal cancer, paired with 40 healthy controls, was determined. Then its sensitivity and specificity were rated, via ROC curves analysis.

Results: Expression levels of miR-135b in serum and stool of CRC patients were 32.4 and 15.7 times higher in serum and stool, compared to that of healthy control respectively ($P < 0.05$). ROC curves analysis exhibited that serum miR-135b levels were powerful in detecting CRC patients from control subjects, with a sensitivity of 92.7% and a specificity of 89.7% (AUC: 0.929). In addition, stool miR-135b levels strongly distinguished CRC patients from control subjects with a sensitivity of 92.7% and a specificity of 87.2% (AUC: 0.919).

Conclusions: The results of the current study indicate that serum and stool miR-135b expression levels seem to be used as a potential diagnostic biomarker for CRC patients. However further studies with large sample size are needed for approving the miR-135b as a noninvasive diagnostic biomarker of CRC.

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Introduction

Colorectal cancer is one of the most commonly diagnosed malignancies in human and is the second leading cause of cancer death [1]. TNM staging is considered as a standard method in routine use to determine CRC prognosis, in patients with high risk of mortality who could also respond to treatment [2]. Recently, molecular techniques, especially gene expression profiling, have been used increasingly in order to improve CRC classification [3, 4]. CRC is a multistep process

characterized by genetic and epigenetic alterations that affect the main cellular pathways involved in growth and development [5]. Moreover, a better understanding of the molecular mechanisms involved in CRC development may contribute to exploit potential helpful prognostic biomarker and therapeutic target for CRC [6]. Early detection of cancer improves survival and therefore, the search for early detection biomarkers is highly warranted [7, 8]. In recent years, looking for prognostic biomarkers in CRC has increased and as a result, different miRNA expression analysis in tumor tissue studies also soared [9-11].

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There are several interesting candidates, but the different finding makes it difficult to draw undoubted conclusions [12]. In addition to being found in tissue, miRNAs have also stably been detected in feces and serum making them potential good biomarkers [13-16].

MicroRNAs (miRNAs) are a class of short, non-coding single stranded RNA molecules (20-24 nucleotides) that regulate gene expression by post-transcriptional silencing of target mRNAs (mRNAs) through complementary binding and they also lead to translation of some target mRNAs [17-20]. In humans, miRNAs play a substantial role in the regulation of biologic processes such as development, differentiation, cell proliferation and apoptosis [17, 18, 21]. Recent studies indicated that dysregulation of miRNA is the hallmark of tumorigenesis so, miRNAs can be used as a diagnostic biomarker of cancer [19, 21, 22].

MiR-135b is overexpressed in adenomas and adenocarcinomas and one of its targets is APC, implying that the upregulation of miR-135b is an early event in CRC and might be used as an early detection biomarker [23, 24]. Adenomatous Polyposis Coli (APC) is a tumor suppressor gene that regulates beta-catenin concentrations and its protein interacts with E-cadherin [1, 10]. Mutations in the APC gene, leading to reduced expression, are defined as an early event in CRC [25, 26]. The aim of this research was to evaluate miR-135b expression through a quantitative reverse transcription PCR (qPCR) in CRC, to investigate its clinical significance and to evaluate the potential usefulness of miR-135b as a diagnostic biomarker.

Table 1: Patient's clinical characteristics.

		Patients	Healthy Controls
Age	≤ 50	16	18
	> 50	24	22
Genus	<i>Male</i>	21	21
	<i>Female</i>	19	19
TNM staging	<i>I</i>	11	
	<i>II</i>	16	
	<i>III</i>	6	
	<i>IV</i>	7	
Tumor location	<i>Right side Colon</i>	32	
	<i>Left side colon</i>	18	

These Patient *Demographics* information were extracted from patients Files in Shariati hospital.

Methods

I Participants and Sample Collection

This study was based on 40 patients and 40 control samples collected from Shariati hospital, Tehran, Iran, from 2014 to 2015 (Table 1). All processes were carried out with advised consent and approved by the ethical committee for clinical research at the hospital. Exclusion and inclusion criteria for the patients and controls were regarded throughout the study (Table 2).

Table 2: Inclusion and Exclusion Criteria to undergoing the study.

	<i>Patient</i>	<i>Healthy control</i>
Inclusion Criteria	A person that Was confirmed as malignant or precancerous by colonoscopy and histopathologic examination and was agreed to undergo the study.	- No history of colorectal cancer or adenoma - No positive fecal occult blood test or FIT within the previous six months. - No prior colorectal resection for any reason other than sigmoid diverticular disease. - No Inflammatory bowel disease (IBD) including chronic ulcerative colitis (CUC) and Crohn's disease - Consent to undergo the study
Exclusion Criteria	A person who: - Has no hospital file documentation. - Disagreed to undergo the study.	A person who: - has a history of colorectal cancer or adenoma - Has positive fecal occult blood test or FIT within the previous six months. - Has prior colorectal resection for any reason other than sigmoid diverticular disease. - Has Inflammatory bowel disease (IBD) including chronic ulcerative colitis (CUC) and Crohn's disease - Was disagreed to undergo the study.

Patients and healthy control group based on these criteria Were enrolled and the most of case histories were extracted from patient records.

II Serum and Stool Collection and RNA Extraction

Whole blood and stool samples of Patients and control group were collected after colonoscopy, and before surgery. Whole blood samples were collected in 5ml tubes (RNase-free) and were left to clot at room temperature for 30 minutes then they were centrifuged at 2000 rpm at 4°C for 10 minutes. The serum was then separated, aliquoted and stored at -80°C until needed. Stool samples were collected freshly and immediately flash frozen in liquid nitrogen and stored at -80 °C until

used. Demographic and clinical information documented at the time of sampling included the stage, type, and grade of the tumor. Tumors were staged in accordance with American Joint Committee on Cancer (AJCC) TNM staging [13, 14]. One of the very important impacts on the results is the quality of total RNA, which was extracted from prototype [27]. Therefore, in this study, miRNeasy serum/plasma kit (Cat number: Q217184; Qiagen, Germany) for serum samples and miRNeasy mini kit (Cat number: Q217004; Qiagen, Germany) for stool were used, that are most qualified methods for miRNAs extraction [28]. Total miRNAs

were extracted in accordance with the manufacturer's instructions, and the concentration and purity of separated miRNA were assayed by Bio photometer system (Eppendorf AG, Hamburg, Germany).

III cDNA Synthesis and Real-Time PCR

For the cDNA synthesis and real-time PCR, the LNA kits and primers of Exiqon company were used that are totally exclusive to miRNA amplification [29]. CDNA synthesis was done by miRCURY LNA Universal cDNA synthesis kit II (Cat number: 203301; Exiqon, Vedbaek, Denmark). Real-time PCR was done by Exilent Sybergreen master mix (Cat number: 203403; Exiqon, Germany) and miR-16, RNU6B and miR-135b specific LNATM PCR primer sets (Exiqon) according to manufacturer's instructions. The real-time PCR condition was initial denaturation at 95 °C for 10 min, followed by 48 cycles of denaturation at 95 °C for 10 s (40 cycles for manufacturer), annealing and extension at 60 °C for 1 min and ramp-rate 1.6°C/s, in CFX96 real-time PCR system (Bio-Rad, Milan, Italy). The specificity of the PCR products were verified by melting curve analysis after last amplification procedure. To assure reproducibility and uniformity of the results, for all of the samples, real-time PCR reaction was done duplicated. In a pilot study, before normalization step, U6 small nuclear RNA (RNU6B) and miR-16 expression were examined as a reference gene, because a faultless internal control for stool-based research of miRNA, has not yet been approved. Due to the unchanging expression level, miR-16 was preferred as reference housekeeping gene for data normalization in serum and stool examinations.

IV Statistical Analysis

The efficiency of real-time PCR for both miR-135b and miR-16 was about 2; so, the rate of upregulation was measured using $\Delta\Delta CT$ methods, by REST (Relative Expression Software Tool) 2009 (Qiagen, Hilden, Germany) based on the relative expression of a miR-135b versus miR-16 as a reference gene [30]. The computing of $2^{-\Delta\Delta CT}$ was accomplished

via transform menu of IBM SPSS statistics® version 20 (Chicago, IL, USA). For the analyses of variance, Kruskal-Wallis and Mann-Whitney statistics test were used to evaluate differences in serum or stool miRNA expression. Receiver operating characteristic (ROC) analysis was performed to determine the sensitivity and specificity of miR-135b expression in serum and stool of CRC patients as a diagnostic test. Data analysis was done with MedCalc® version 13.1.2.0-64bit (Acaciaaan 22, 8400 Ostend, Belgium).

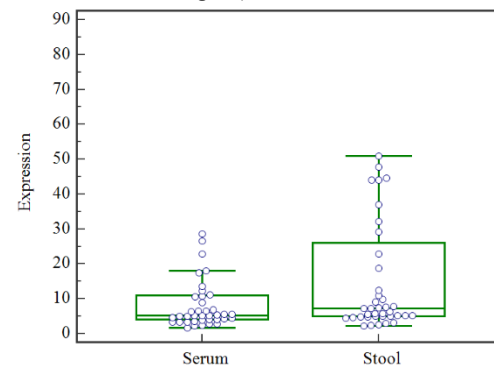


Figure 1: The expression levels of miR-135b in serum and stool. The results of Mann-Whitney test show that, In the CRC patients, the expression levels of miR-135b in serum compared to stool was not different significantly ($P < 0.05$).

Results

As a primary step, we determined whether miR-135b in stool and serum of colorectal cancer patient in comparison with normal control is upregulated. MiR-135b in serum and stool were upregulated in CRC patients by 32.4 times in serum and 15.7 times, in the stool, compared to the normal serum and stool ($P < 0.05$). The results of Mann-Whitney test show that the expression levels of miR-135b in serum and stool was significantly associated ($P < 0.05$) (Figure 1).

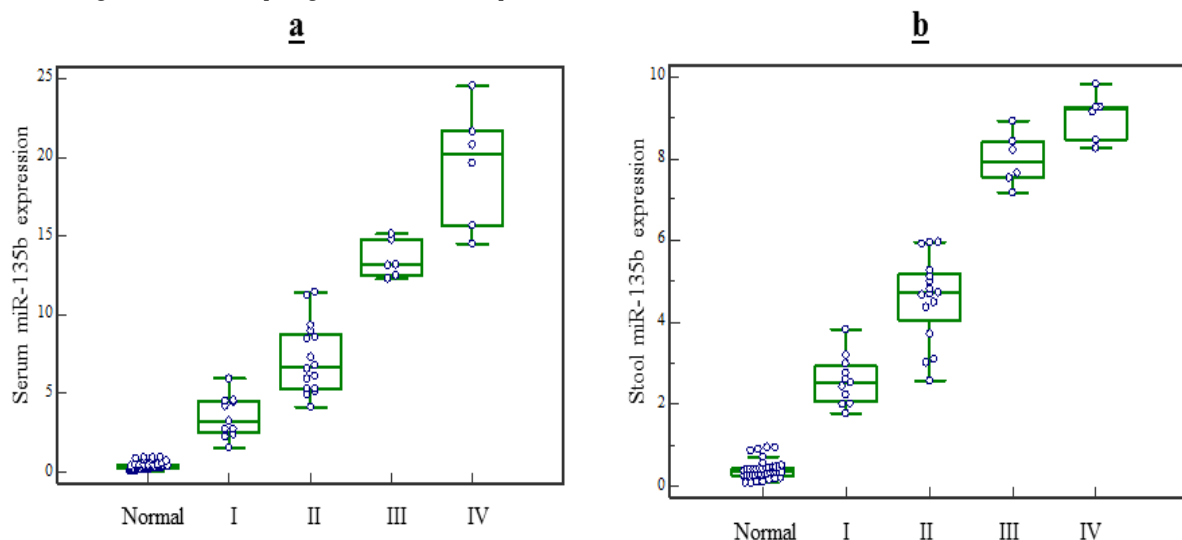


Figure 2: MiR-135b expression level in serum and stool based on patients clinical TNM stages. The result of Kruskal-Wallis test showed: miR-135b expression level in serum and stool based on patients clinical TNM stages. **a)** The expression level of miR-135b in serum can be hold as diagnostic marker to detect the TNM stage of CRC patients ($P < 0.05$). **b)** The expression level of miR-135b in stool can be hold as marker to detect the TNM stage III, IV from I or II ($P < 0.05$) but is unable to distinguish stage III from IV ($P > 0.05$).

I MiR-135b Expression Level in Serum and Stool, Based on Patients Clinical Characteristics

Based on TNM staging, the expression level of miR-135b in serum raised respectively with stages, and expression levels in stage IV patients significantly elevated compared to stage I, II or III patients ($p < 0.05$ for

all stages). Nevertheless, stool expression levels in stage IV patients did not significantly elevate compared to stage III patients ($p > 0.05$), but it raised compared to stages I or II ($p < 0.05$) (Figure 2). on the other hand, the expression level of miR-135b in serum and stool of CRC patients did not vary with age, genus, tumor type, tumor size and tumor location ($p > 0.05$) (Table 3).

Table 3: MiR-21 expression level in serum and stool, based on patients' clinical characteristics.

		<i>MiR-21 in serum</i>		<i>MiR-21 in stool</i>	
		<i>Average rank</i>	<i>P value</i>	<i>Average rank</i>	<i>P value</i>
<i>Age</i>	≤ 50	20.37	0.688(>0.05)	17.25	0.151(>0.05)
	> 50	19.90		22.67	
<i>Genus</i>	<i>Male</i>	19.81	0.694(>0.05)	20.52	0.989(>0.05)
	<i>Female</i>	21.26		20.47	
<i>Tumor type</i>	<i>Colon</i>	21.09	0.52(>0.05)	20.33	0.852(>0.05)
	<i>Rectum</i>	18.12		20.19	
<i>Tumor size</i>	$\leq 5\text{cm}$	18.88	0.38 (>0.05)	18.52	0.250(>0.05)
	$> 5\text{ cm}$	22.23		22.96	
<i>Tumor location</i>	<i>Right side</i>	21.14	0.478(>0.05)	22.00	0.212(>0.05)
	<i>Left side</i>	18.53		17.41	

There are no significant association between Stool and Serum MiR-21 expression level and clinical characteristics (age, genus, tumor type, tumor size and tumor location) of colorectal patients (P value > 0.05).

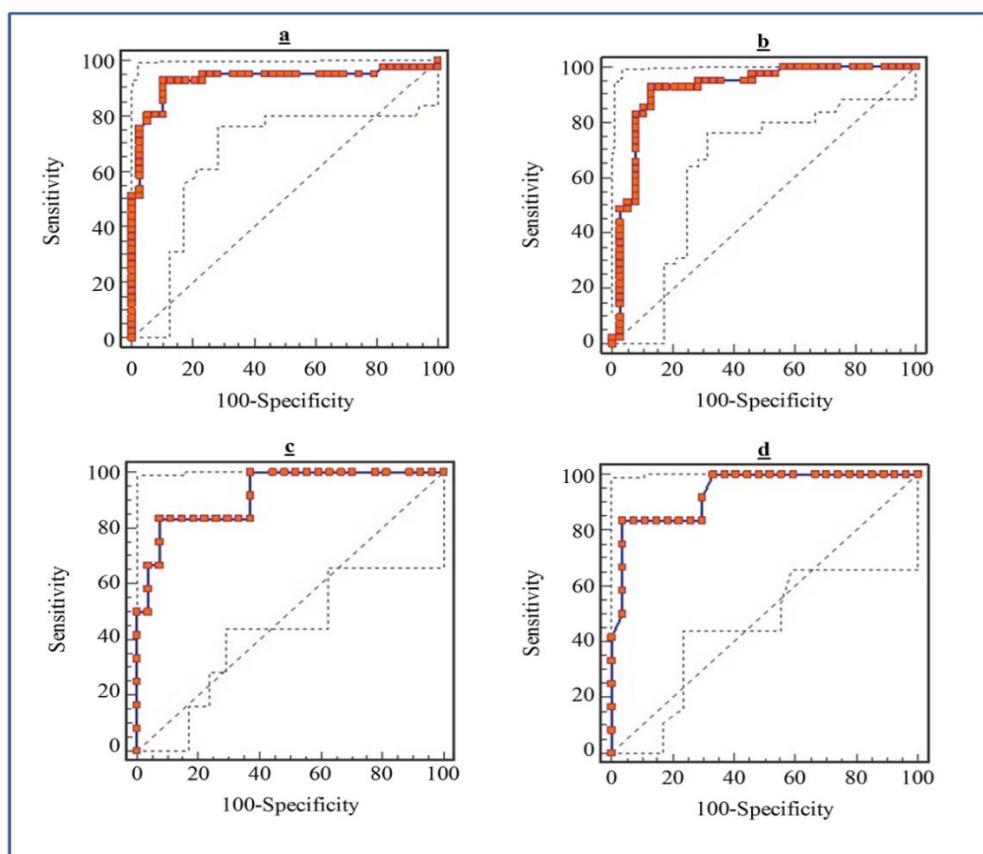


Figure3: ROC curve of serum and stool miR-135b Expression level for detection of CRC patients. **a)** Serum miR-135b expression level for detection of CRC patients, area under the ROC curve (AUC): 0.929, Youden index: 0.8243, sensitivity: 92.7%, specificity: 89.7%. **b)** Stool miR-135b expression level for detection of CRC patients, AUC: 0.919, Youden index: 0.7986, Sensitivity: 92.7%, Specificity: 87.2%. **c)** Serum miR-135b expression level for detection of TNM stages in CRC patients, AUC: 0.920, Youden index: 0.7593, sensitivity: 83.3%, specificity: 92.6%. **d)** Stool miR-135b expression level for detection of TNM stages in CRC patients, AUC: 0.935, Youden index: 0.7963, sensitivity: 83%, specificity: 92.5%.

II MiR-135b as a Noninvasive Biomarker for Diagnosis of Colorectal Cancer

To evaluate the diagnostic potential of miR-135b as a noninvasive biomarker, the ROC curves were studied (Figure 3). ROC curve analysis exhibited that serum miR-135b levels can be used as perfect diagnostic biomarker for CRC detection, with a sensitivity: 92.7%, specificity: 89.7%, AUC (area under the ROC curve) 0.929. Moreover, ROC analyses revealed that stool miR-135b levels discriminated CRC patients from control subjects with a sensitivity: 92.7%, specificity: 89.7%, AUC 0.984, with no significant statistic differences with serum miR-135b ($P=0.72$). The ROC analyses also, showed that serum miR-135b levels were robustious in detecting TNM stage III-IV from I-II with a sensitivity: 83.3% and specificity: 92.6%, AUC: 0.920. Furthermore, in patients with CRC, stool miR-135b levels, as well as serum miR-135b ($P=0.7$), were diagnosed TNM stage III-IV from I-II with a sensitivity: 83%, specificity: 92.5%, AUC: 0.935.

Discussion

In recent years, in order to achieve a safe and non-invasive method for the diagnosis of cancer, researchers have tended to consider micro-RNA profiles that are overexpressed in various cancers [11, 19, 28, 29]. Since the recognition of miRNAs in 1993, they have emerged as a favorable new class of biomarkers for cancer diagnosis [19, 31]. However, only a small number of miRNA are detectable in serum and stool, to the same extent that they are in the cancer cells [18, 32]. Hence, more studies should be done to specify how much the miRNAs are released into the bloodstream. Also, many of the cells in the colon cancer, in addition to the blood flow, drop their content into the lumen and sometimes the cells excreted in the stool which makes miRNAs released into the stool [14, 33, 34].

In this study, it was found that miR-135b in serum and stool of patients with colorectal cancer is upregulated, which confirms previous studies [35-39]. Statistical analysis of our study showed that the expression level of the miR-135b in serum and stool did not differ significantly, compared to the results of previous studies, which showed that miRNA expression is increased in tumor tissues [4, 34]. It can be said that the expression level of the miR-135b in serum, stool and tumor tissues of CRC patients is a correspondence of its expression in cancer cells. The other results of our study which examined the relationship between the expression of the miR-135b and some clinical and demographic characteristics showed that overexpression of the miR-135b in serum and stool of CRC patients was not significantly associated with age, gender, tumor size, tumor type and tumor location. Previous studies have shown that high levels of this miRNA had a positive significant relationship with the TNM stages [4, 34].

This study also revealed that miR-135b amount increased from the stage I to the IV, in the serum of CRC patients but in the stool, the expression level in stages III, IV had no significant difference. On the other hand, evaluating of ROC curve indicated that miR-135b expression in serum and stool can be used as an efficient diagnostic test to identify patients with colorectal cancer and determine the TNM stages of CRC patients with high sensitivity and specificity. The study of Wu.CW et al. (2014) showed that the sensitivity of fecal miR-135b for detection of colorectal

cancer was 78% and the specificity was 68%, whereas in our study higher levels of sensitivity and specificity were acquired both in serum and stool. The comparison of ROC curves, using Medical statistical software (2009) indicated that the sensitivity and specificity of the miR-135b in serum and stool were not significantly different for CRC diagnosis and TNM stages determining.

Overall, the results of this study suggest that miR-135b in serum and stool have diagnostic value. Nonetheless, for efficiency analysis, the $2^{-\Delta\Delta Ct}$ equation was used, that is relative and does not indicate, the exact amount of miRNAs, and to get the correct results, it would need data normalization [40, 41]. Determination of appropriate internal control is the most important approach to normalizing the real-time PCR data [41]. Mostly, in previous studies, miR-16 and RNU6B were used as internal control, but until then there was no exact recommended miRNA as reference gene [42]. For this reason, we evaluate both miRNAs and it was found that miR-16 due to the greater stability was more suitable as an internal control. In summary, the results of the current study indicate that Serum and stool miR-135b expression levels seem to be a potential diagnostic biomarker for CRC patients. However, further well-designed studies with larger sample sizes are needed to well approve the role of miR-135b in CRC noninvasive diagnosis.

Acknowledgments

We appreciate the members of the Molecular medicine and Genetics department for their suggestions and assistance. This Study was supported by Hamadan University of Medical Sciences, Hamadan, Iran.

Conflicts of Interest

None.

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