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Decreased expression of fecal miR-4478 and miR-1295b-3p in early-stage colorectal cancer

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Abstract.
BACKGROUND: Colorectal cancer (CRC) is a major cause of cancer-related deaths world-wide. Detection of molecular markers in stool samples is a promising strategy for CRC screening. MicroRNAs (miRNAs) are short, non-coding RNA molecules that are commonly dysregulated in neoplasia.
OBJECTIVE: The objective of this study was to evaluate the fecal miRNAs differentiation between early-stage CRC patients and healthy subjects.
METHODS: Stool samples were collected from 40 patients with early stage (I, II) CRC and 16 healthy controls. RNA was extracted from all samples using miRNAeasy Mini Kits. MiRNA microarray expression profiling was performed with Agilent's miRNA Microarray system on 12 CRC and 8 normal stool samples. The expression levels of miR-4478 and miR-1295b-3p were determined by the SYBR Green miScript PCR system.
RESULTS: In profiling study, we found 215 down-regulated miRNAs in CRC group. Furthermore, in validation study we found that the expression levels of fecal miR-4487 and miR-1295b-3p were significantly decreased in CRC patients compared to healthy controls.
CONCLUSIONS: The expression of miR-4478 and miR-1295b-3p were significantly diminished in stool samples of CRC patients with early stage (I, II) in comparison with normal group. These miRNAs may be used as potential non-invasive molecular markers for CRC diagnosis, but further studies are needed.

Keywords: Profiling, microarray, diagnosis

1. Introduction

Colorectal cancer (CRC) is the third most common cancer in men and the second in women worldwide [1] with an estimated 1.4 million new cases and with more than half million deaths annually [2,3]. The survival rate of CRC patients is significantly dependent on the

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stage of disease at the time of diagnosis. Grave consequences will follow for patients who have been diagnosed in the terminal phases of the disease [4]. Since great proportion of CRC cases develop from precancerous lesions, accurate screening could greatly improve the chance of treatment and survival rate [5]. Although colonoscopy is currently the most reliable method for CRC screening, it is not generally accepted due to its invasive nature and high cost [6]. FOBT (Fecal Occult Blood Test) is a non-invasive procedure for CRC screening, but its sensitivity and specificity is low [5]. Therefore, to develop new non-invasive and early diagnostic methods are highly needed..

MicroRNAs (miRNAs) are short (18–25 nt) noncoding RNAs that regulate gene translation and can be used as markers for various disease [7]. Nowadays, utilization of miRNA provides a promising opportunity for early detection of cancers. Changes in miRNAs expression have been observed in several types of cancer, including CRC [8–11]. MiRNAs have been shown to be stably present in a wide range of clinical specimens [12–14] such as stool samples [15,16].

Stool specimens are in direct contact with the colonic luminal surface and may contain colonocytes and tumoral cells in carcinoma cases [17,18]. These cells carry important genetic and epigenetic information that can be used as diagnostic biomarkers in CRC [19–21]. In the present study, we have investigated the feasibility of using fecal miRNAs in early diagnosis of CRC patients.

2. Materials and methods

2.1. Patients

A total of 40 stool samples from primary CRC patients (Stage I, II) were collected from August 2011 to March 2013 at Digestive Disease Research Institute (DDRI), Shariati Hospital, Tehran University of Medical Science. The histology was confirmed and staged according to the tumor-node-metastasis (TNM) staging system of the International Union Against Cancer. In the control group, 16 stool samples were collected from individuals proven to be free of colonic disease with colonoscopy. The control group was matched to the CRC patient group in terms of age and gender. No significant differences were observed between the CRC patients and controls in age ($p = 0.101$) or gender distribution ($p = 0.414$). Clinical and demographical data of the patients and the controls are presented in Table 1. The samples were quickly frozen at $-70°C$ until subsequent analysis. Written informed consent was obtained from each participant.

Table 1
Patients' demographics: characteristics of CRC patient and control

Characteristic	CRC patient	Control	p value
Age	63 ± 9.03	59.5 ± 8.64	0.101[a]
Gender	Male: 23	8	0.414[a]
	Female: 17	8	
TNM stage	I 11	–	
	II 29	–	
Tumor location	Colon 35	–	
	Rectum 5	–	

[a]p value for age was calculated by independent sample t-test; p values for gender was calculated by Fisher's exact test.

2.2. RNA isolation

Total RNA (including small RNA) was extracted from stool samples using miRNAeasy Mini Kits (Qiagen) according to the manufacturer's instructions with some modifications. Briefly, approximately 100 mg of stool was homogenized with 1000 μL RNase free water and 200 μL of this homogenate was lysed in a proportion of 1:6 with QIAzol lysis reagent (Qiagen, Valencia, CA). After homogenization, RNA was precipitated with chloroform. The aqueous phase was mixed with 1.5 volumes of 100% ethanol. The concentration of extracted RNA was measured using the NanoDrop-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE), and Agilent's Bioanalyzer (Agilent Technologies, Palo Alto, CA) was used for checking the quality of total RNA with the RNA 6000 chip and miRNA with the small RNA chip.

2.3. MiRNA microarray expression profiling and data analysis

Stool samples from 12 CRC patients (including 3 cases of stage I and 9 cases of stage II) and 8 healthy controls were used for miRNA profiling study. The miRNA expression profiles were generated by Agilent's miRNA Microarray system (Release 19.0), containing 2006 human miRNAs (Agilent Technologies, Santa Clara, CA). Labeling and hybridization of RNA samples were performed according to the Agilent's protocol. Microarray data processing was performed with Agilent's Feature Extraction Software with default parameters. The statistical analyses of microarray data were performed with the GeneSpring GX version 12.0 Analysis Software (Agilent). Data were preprocessed by log 2 transformation and normalization

between all arrays was done by the 75th percentile method. Differences between the two groups were analyzed using t-test for those miRNAs with at least a 2-fold change in expression level between the two groups. Only those miRNAs with P value < 0.05, q value < 0.3 were considered as significantly changed.

2.4. MiRNA quantification by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

A total of 40 stool samples from CRC patients (stage I, $n = 11$; stage II, $n = 29$) and 16 stool samples from healthy controls were used for qRT-PCR in the validation study. The miRNA microarray results were validated using a SYBR Green method. Reverse transcription of RNA was performed with the miScript Reverse Transcription Kit (Qiagen, Valencia, CA) according to the manufacturer's guidelines. Real-time PCR was performed on a Roche Light-cycler, software v.3.5 (Roche Applied Science, Mannheim, Germany) by the SYBR Green miScript PCR system (Qiagen, Valencia, CA) according to the manufacturer's instructions. Each reaction was performed in a 20 μL volume with 10 ng template cDNA. All reactions were run in duplicate. The most significant differentially expressed miRNAs, miR-4478 and miR-1295b-3p, were selected for validation study. The RNU6B (Qiagen) was used as an endogenous control for normalization of data. The relative quantification (RQ) for each miRNA compared with RNU6B and evaluation of statistically significant differences in miRNA expression between the two groups were calculated using $\Delta\Delta$Cq method and GraphPad Prism (V.6) software respectively. P values less than 0.05 were considered as statistically significant.

3. Results

3.1. MiRNA profiling

We isolated an adequate amount of total RNA, including miRNA in stool samples of all CRC patients and normal subjects, following optimization of the commercial RNA extraction protocols.

Microarray analysis was performed to reveal the miRNAs differentially expressed in stool samples of 12 CRC patients and 8 normal subjects. We found 215 significantly altered miRNAs which were down-regulated in CRC group compared to the control group. The complete list of differentially expressed miRNAs between primary CRC patients and healthy subjects is provided in Supplementary Table S1.

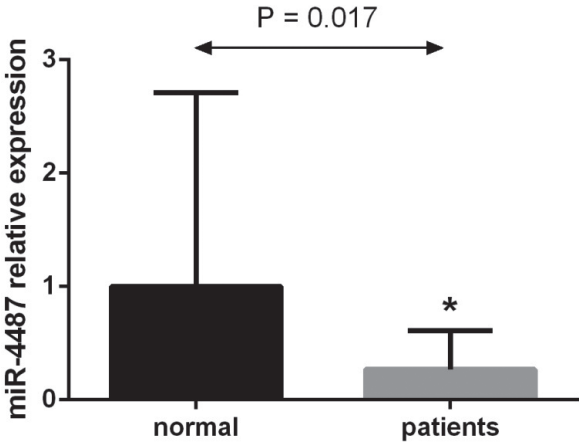

Fig. 1. MiR-4487 down-regulation in the fecal samples from CRC patients compared to the normal group (P value 0.017). Expression levels of the miR-4487 are normalized to RNU6B (Non-parametric Mann-Whitney t-test).

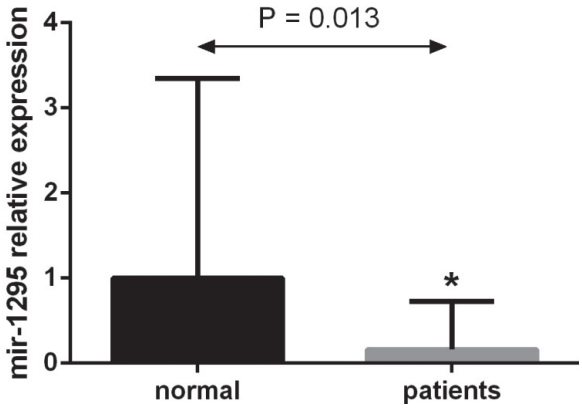

Fig. 2. Fecal miR-1295b-3p expression levels were down-regulated in CRC patients compared to normal subjects (P value 0.013). Expression levels of miR-1295b-3p were normalized to RNU6B (Non–parametric Mann-Whitney t-test).

3.2. Validation of miRNA expressions by qRT-PCR

To confirm the accuracy of the data obtained from microarray, two miRNAs, miR-4478 and miR-1295b-3p, the most significant miRNAs, were selected for further validation by qRT-PCR. A significant alteration was noticed in the levels of these miRNAs: miR-4478 ($P = 0.017$) (Fig. 1) and miR-1295b-3p ($P = 0.013$) (Fig. 2) were significantly down-regulated in the CRC group.

Receiver operating characteristic (ROC) curve analysis for the differential expression of miR-4487 and miR-1295b-3p between CRC patients and normal individuals revealed that these miRNAs are potentially valuable to be used as diagnostic biomarkers. The area

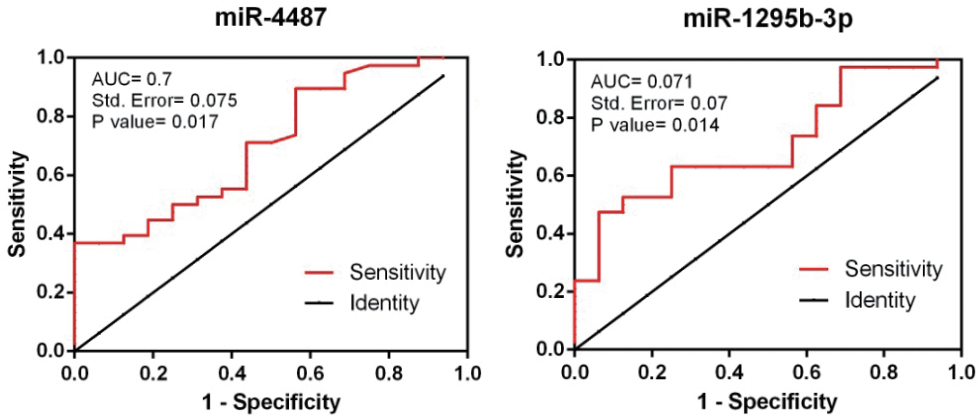

Fig. 3. Area under curve (AUC) of receiver operating characteristic (ROC) for miR-4487 and miR-1295b-3p. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/CBM-140453)

under the curve (AUC value) for miR-4487 was 70% with the standard error of 0.075 (P value = 0.017) while the AUC value for miR-1295b-3p was 71% with the standard error of 0.072 (P value = 0.014) (Fig. 3).

4. Discussion

MiRNAs have been shown to exist stably more than other nucleic acids and proteins in a broad range of clinical specimens [12,13] including stool samples [15, 16]. Moreover, their expression levels are generally representative of the tumorous tissues. Therefore, determining the expression level of miRNAs in stool may serve as a suitable non-invasive diagnostic, prognostic and biomarkers in CRC [22]. Over the past few years, a large number of differentially expressed miRNAs have been identified in colorectal cancer. The first study on detection of fecal miRNA in CRC patients was reported by Ahmed et al in 2009, who identified 7 over-expressed and 7 under-expressed miRNAs [16]. MiR-320 was one of the miRNAs with decreased expression, which consistently confirmed in our profiling study. In separate subsequent studies, up-regulation of miR-92a [23], miR-21 [23,24], miR-17-92 cluster and miR-135 [25], as well as miR-106a [24] and miR-144 [15] have been reported in stools of the CRC patients but not in the controls. Li et al. reported recently down-regulation of fecal miR-143 and miR-145 in CRC patients [26].

In the present study, we investigated the fecal miRNAs in patients with early stage (I, II) CRC. We found 215 differentially expressed miRNAs in the patient group. Moreover, in a validation study by qRT-PCR, we demonstrated that the stool levels of miR-4478 and miR-1295b-3p have been significantly decreased in the CRC patients compared to the healthy subjects.

MiR MiR-4478 has the ability to bind to 3'-UTR of the class II Histone deacetylase (HDAC4) [27], which is a regulator of colon cells proliferation [28]. HDAC4 frequently overexpressed in several malignancies including CRC [29] and promotes growth of colon cancer cells via repression of p21 [28,30]. Moreover, decreased expression of miR-1295 has been already reported in CRC tissue samples [31].

All of our patients were in early and curable stages (I, II) of CRC. Worth of mentioning is that in these early-stage tumors miR-4478 and miR-1295b-3p were down-regulated and thus may serve as a novel non-invasive biomarker for diagnosis of this neoplasia. Nevertheless, this finding requires further validation studies on larger set of samples of different histopathology and stages (hyperplasia, adenoma, carcinoma and metastasing carcinoma). The current study serves as a basis for subsequent investigations on identification of target genes of these detected miRNAs and determine the expression rate of these target genes and their corresponding proteins to identify their potential effects on the CRC tumorigenicity.

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Supplemental material

Supplement Table 1
Differentially expressed miRNAs in fecal samples of CRC patients

miRNA	P	FC[1]	miRNA	P	FC	miRNA	P	FC	miRNA	P	FC
4478	0.0009	10.66	1226-5p	0.009	10.05	3147	0.02	8.48	1227-5p	0.03	5.75
1295b-3p	0.001	16.00	5739	0.01	8.05	3177-3p	0.02	8.72	5703	0.03	5.89
124-3p	0.002	10.76	4515	0.01	9.76	193b-5p	0.02	8.12	4497	0.03	6.00
320a	0.002	10.59	3648	0.01	7.61	4507	0.02	8.06	4442	0.03	4.55
3185	0.002	16.00	631	0.01	7.09	3141	0.02	7.57	4800-5p	0.03	5.28
3605-5p	0.002	10.90	629-3p	0.01	10.48	3610	0.02	6.48	4651	0.03	5.25
4688	0.002	11.61	760	0.01	7.76	3620-5p	0.02	8.26	4632-5p	0.03	6.27
3911	0.002	16.00	4634	0.01	7.86	6076	0.02	8.85	3917	0.03	16.00
1247-3p	0.003	9.10	4665-5p	0.01	7.05	3186-3p	0.02	16.00	4538	0.03	6.65
4451	0.003	12.38	4465	0.01	7.00	let-7b-5p	0.02	12.28	1587	0.04	6.22
4640-3p	0.003	16.00	6126	0.01	6.22	1202	0.02	6.06	149-3p	0.04	5.25
1260a	0.003	16.00	4286	0.01	11.81	4738-3p	0.02	6.70	4476	0.04	5.59
494	0.003	13.03	518c-5p	0.01	6.88	150-3p	0.02	6.06	4505	0.04	5.43
6085	0.003	16.00	4793-5p	0.01	6.14	584-5p	0.02	11.15	4459	0.04	5.28
4690-5p	0.003	10.70	6133	0.01	8.63	602	0.02	7.36	320b	0.04	5.95
4454	0.003	16.00	526b-5p	0.01	6.70	3196	0.02	6.75	4257	0.04	6.69
4428	0.004	8.19	622	0.01	8.59	483-5p	0.02	5.40	4788	0.04	4.82
33a-3p	0.004	16.00	550a-5p	0.01	7.84	4327	0.02	6.57	4741	0.04	5.03
1233-1-5p	0.004	8.16	4698	0.01	6.56	5006-5p	0.02	5.18	33b-3p	0.04	7.08
1236-5p	0.004	7.46	4486	0.01	6.36	766-3p	0.02	7.12	5195-3p	0.04	6.47
3200-5p	0.005	10.52	623	0.01	6.86	133b	0.02	14.20	1281	0.04	9.72
4513	0.005	9.26	5088	0.01	5.64	3614-5p	0.02	11.56	933	0.04	8.94
5190	0.005	12.89	4271	0.01	9.96	1229-5p	0.02	4.67	638	0.04	5.21
4259	0.005	9.28	4672	0.01	7.56	3188	0.02	5.35	197-5p	0.04	4.21
4648	0.005	7.08	1273g-3p	0.01	7.90	4710	0.02	5.96	191-3p	0.04	7.07
574-5p	0.005	12.49	302c-5p	0.01	7.48	4743-5p	0.02	6.42	4323	0.04	8.58
3195	0.005	12.24	193a-5p	0.01	12.61	575	0.02	5.72	658	0.04	4.33
483-3p	0.005	16.00	3652	0.01	8.60	3937	0.02	5.94	6724-5p	0.04	4.91
4746-3p	0.005	16.00	3176	0.01	16.00	4484	0.02	5.03	4322	0.04	5.60
4532	0.006	9.02	3682-3p	0.01	8.19	5189	0.02	4.92	1207-5p	0.04	6.35
663a	0.006	14.24	3180-5p	0.01	16.00	4664-3p	0.02	6.20	939-5p	0.04	5.11
4776-5p	0.006	7.67	4669	0.02	7.58	6510-5p	0.02	5.01	671-5p	0.04	5.60
4419a	0.006	8.47	1469	0.02	12.71	4429	0.02	4.91	2276	0.04	5.50
4430	0.006	6.42	1914-3p	0.02	6.87	6127	0.03	5.49	4749-3p	0.04	6.35
3174	0.006	14.33	765	0.02	7.09	211-3p	0.03	6.34	1246	0.04	6.09
3676-5p	0.007	10.18	3173-3p	0.02	14.94	4745-5p	0.03	5.25	6068	0.04	5.29
1287	0.007	12.82	4298	0.02	8.41	345-3p	0.03	4.29	324-3p	0.04	4.71
3934-5p	0.007	16.00	371a-5p	0.02	7.17	4534	0.03	6.02	4767	0.04	7.37
6717-5p	0.007	12.58	652-5p	0.02	6.75	188-5p	0.03	6.28	1234-3p	0.04	6.05
6511b-5p	0.007	7.78	3194-5p	0.02	6.73	6507-5p	0.03	10.48	1225-3p	0.045	6.13
3646	0.007	11.93	1182	0.02	7.30	711	0.03	4.79	498	0.045	5.22
601	0.007	10.34	5001-5p	0.02	6.21	3124-5p	0.03	7.84	1304-3p	0.045	9.42
4485	0.007	10.29	3667-5p	0.02	5.90	5787	0.03	6.91	6165	0.046	4.44
3935	0.008	12.04	4655-5p	0.02	7.19	762	0.03	5.74	3659	0.047	6.28
4496	0.008	7.71	877-5p	0.02	6.55	572	0.03	6.93	937-5p	0.047	5.96
125a-3p	0.008	13.30	4728-5p	0.02	5.46	4499	0.03	7.89	6125	0.047	5.00
874	0.009	7.90	5196-5p	0.02	7.77	3137	0.03	4.69	525-5p	0.048	6.42
4419b	0.009	8.29	6086	0.02	6.04	4758-5p	0.03	7.25	1237-3p	0.048	5.98
514b-5p	0.009	7.14	4721	0.02	6.19	3162-5p	0.03	7.42	4656	0.049	4.28
718	0.009	9.75	642a-3p	0.02	5.73	4443	0.03	6.03	3680-3p	0.049	16.00
4261	0.009	10.43	5100	0.02	14.13	6723-5p	0.03	5.00	4466	0.049	5.42
320d	0.009	8.86	636	0.02	5.64	3138	0.03	4.45	1238-3p	0.049	6.01
4444	0.009	7.55	769-3p	0.02	9.49	4313	0.03	7.32	4665-3p	0.049	6.17
583	0.009	9.76	1185-1-3p	0.02	6.68	3663-3p	0.03	4.85			

[1]FC: Fold change.