Original Article

Expression Analysis of Previously Verified Fecal and Plasma Down-regulated MicroRNAs (miR-4478, 1295-3p, 142-3p and 26a-5p), in FFPE Tissue Samples of CRC Patients

Reza Ghanbari PhD[1], Sama Rezasoltani PhD[2], Javad Hashemi PhD[3], Ashraf Mohamadkhani PhD[1], Arash Tahmasebifar PhD[4], Ehsan Arefian PhD[5], Naser Mobarra PhD[6], Jahanbakhsh Asadi PhD•[6], Ehsan Nazemalhosseini Mojarad PhD[7], Yaghoub Yazdani MD[8], Sakari Knuutila PhD[9], Reza Malekzadeh MD[1]

Abstract

Background: Colorectal cancer (CRC) is one of the most common causes of cancer-related mortality worldwide. Early diagnosis of this neoplasm is critical and may reduce patients' mortality. MicroRNAs are small non-coding RNA molecules whose expression pattern can be altered in various diseases such as CRC.

Methods: In this study, we evaluated the expression levels of miR-142-3p, miR-26a-5p (their reduced expression in plasma samples of CRC patients was previously confirmed), miR-4478 and miR-1295-3p (their reduced expression in stool samples of CRC patients was previously confirmed) in tissue samples of CRC patients in comparison to healthy subjects.

To achieve this purpose, total RNA including small RNA was extracted from 53 CRC and 35 normal subjects' Formalin-fixed, Paraffin-embedded (FFPE) tissue samples using the miRNeasy FFPE Mini Kit. The expression levels of these four selected miRNAs were measured using quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR).

Results: We found that the expression levels of miR-4478 and miR-1295b-3p (two previously down-regulated fecal miRNAs) were significantly decreased in FFPE samples of CRC patients compared to healthy controls. On the other hand, no significant differences were seen in expression levels of miR-142-3p and miR-26a-5p (two previously down-regulated circulating miRNAs) in FFPE samples between these two groups.

Conclusion: Regarding current findings, it may be concluded that to diagnose CRC patients based on the miRNAs approach, stool samples are more likely preferable to plasma samples; nevertheless, additional studies with more samples are needed to confirm the results.

Keywords: Biomarker, colorectal cancer, early detection, tissue microRNA

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Introduction

Colorectal cancer (CRC) is one of the most common malignancies with approximately 1.36 million new cases annually in the world. In men, CRC is the third most common cancer worldwide after lung and prostate cancer and is also the second most common malignancy in women after breast cancer.[1] During the past two decades, despite all advances in chemotherapy and cancer control strategies, the survival rates of CRC patients have not changed, especially in patients with metastatic disease.[2]

Overall, prognosis, response to therapy and survival in patients with CRC appear to depend on the stage of the tumor at the time of diagnosis and disease progression.[3] Most patients are usually diagnosed when cancer is in its advanced and uncontrollable stages[4]; therefore, there is now an urgent need to identify and explore new biomarkers and reliable CRC diagnostic techniques.

In recent years, there have been numerous studies on microRNAs (miRNAs) and their role in cellular and molecular processes of live organisms.[5–7] miRNAs are small RNA molecules with 18 to 22 nucleotides which play an important role in regulating gene expression.[8] Previous studies been shown that the expression of these small RNA molecules is closely associated with the formation, progression and prognosis of CRC, by affecting oncogenes and tumor suppressor genes.[9,10]

Considering the fact that a specific miRNA can simultaneously regulate the expression of more than 100 mRNA,[11] to identify diagnostic biomarkers, miRNA dysregulation can be more efficient compared to the expression of their target mRNAs. Small size and hairpin structure are other advantages of miRNAs that

Authors' affiliations: [1]Digestive Oncology Research Center, Digestive Diseases Research Institute, Tehran University of Medical Sciences, Tehran, Iran, [2]Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran, [3]Department of Clinical Biochemistry, Tehran University of Medical Sciences, Tehran, Iran, [4]Department of Molecular Medicine and Nanomedicine, Faculty of Advanced Medical Technologies, Golestan University of Medical Sciences (GOUMS), Iran, [5]Department of Microbiology, School of Biology, College of Science, University of Tehran, 14155-6455, Tehran, Iran, [6]Metabolic Disorders Research Center, Golestan University of Medical Sciences, Gorgan, Iran, [7]Gastroenterology and Liver Disease Research Center, Research Institute for Gastroenterology and Liver Dseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [8]Infectious Disease Research Center, Golestan University of Medical Sciences, Gorgan, Iran, [9]Department of Pathology, Haartman Institute, Helsinki University, Helsinki, Finland.

•Corresponding author and reprints: Jahanbakhsh Asadi PhD, Metabolic Disorders Research Center, Golestan University of Medical Sciences, Gorgan, Iran. Tel: +98-17-3242 1651, E-mail: ja_asadi52@yahoo.com.

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make them relatively stable, so that during the storage of various samples as well as samples processing, values of miRNAs remain almost unchanged.[12,13]

Given the necessity of new non-invasive molecular diagnostic biomarkers for colorectal carcinoma, the present study was designed to determine which sample (fecal or plasma) is better matched with dysregulation of miRNAs in CRC tissues. For this purpose, the expression levels of four specific miRNAs were analyzed in FFPE samples of CRC patients and neoplasm-free individuals as controls. These four specific miRNAs were as follows: "miR-142-3p, miR-26a-5p" and "miR-4478, miR-1295-3p" whose reduced expression was confirmed by two separate previous studies,[14,15] in plasma and stool samples of CRC patients compared to normal subjects, respectively.

Material and Methods

Subjects and FFPE samples

At first, a total of 53 patients at different stages of colorectal carcinoma and 35 healthy individuals as the control group, who had colonoscopy and had no gastrointestinal disorders, were selected for this study. After approval of the Digestive Disease Research Institute (DDRI), Shariati Hospital, Tehran, Iran and collecting the relevant clinical information, FFPE samples were obtained from all these CRC patients and normal subjects that had been preserved between 2012 and 2013. Tumors were staged after surgery, according to the criteria of Tumor-Node-Metastasis system (TNM) classification.[16]

Patients with a history of gastrointestinal cancer or other malignancies in themselves or their close relatives were excluded from the study. Written informed consent was obtained from all participants for using their FFPE samples in this study.

RNA isolation from FFPE samples

Extraction of total RNA, including miRNAs, from all CRC and normal subjects' Formalin-fixed, Paraffin-embedded (FFPE) tissue samples was carried out using the miRNeasy FFPE Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

The concentration and purity of RNA were determined using NanoDrop-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE).

Moreover, Agilent's Bioanalyzer (Agilent Technologies, Palo Alto, CA) was used for checking the quality of extracted total RNA and miRNA with the RNA 6000 chip and small RNA chip, respectively.

MiRNA quantification by qRT-PCR and statistical analysis

A set of 53 CRC and 35 healthy individual FFPE samples were used for miRNA quantification by SYBR-Green based qRT-PCR. Then, 100 ng of extracted total RNA was reverse transcribed with the miScript Reverse Transcription Kit (Qiagen, Valencia, CA) according to the manufacturer's guidelines.

miRNA expression levels were assessed in duplicate for each sample by Real-time PCR using the SYBR Green miScript PCR system (Qiagen, Valencia, CA) according to the manufacturer's instructions on Roche Light-cycler, software v.3.5 (Roche Applied Science, Mannheim, Germany). miRNA amplification was performed using miRNA-specific and universal primers (Qiagen, Valencia, CA). A human U6 small nuclear RNA miScript Primer Assay (Qiagen) was used for normalization of the data and threshold cycle (Ct) values of 40 or greater were defined as undetectable.

The relative expression of FFPE tissue miRNA levels was calculated and compared between patient and control groups using the $2^{-\Delta\Delta Ct}$ method and Relative Expression Software Tool (REST, version 2009).[17] Statistical analysis and the graphs were achieved using the GraphPad Prism (V.6) software.

Results

Patients and normal subjects

In the present study, we assessed the expression levels of four selected microRNAs in tissue samples of 53 CRC patients (stage I, n = 13; stage II, n = 26; stage III, n = 10; stage IV, n = 4) in comparison to 35 healthy subjects.

No significant differences were found in terms of age (*P*-value = 0.741, independent samples *t*-test) or gender (*P*-value = 0.534, chi-square test) between the CRC patients and the control group. Demographic data of participants enrolled in this study are summarized in Table 1.

miRNAs expression analysis

An adequate amount of total RNA, including miRNA in FFPE

Table 1. Demographic characteristics of CRC patients and healthy individuals.

	CRC patients	Healthy subjects
Age	65.42 ± 9.320	62.09 ± 9.360
Gender		
Male	28	19
Female	25	16
TNM[a] stage		
I	13	- - -
II	26	- - -
III	10	- - -
IV	4	- - -
Tumor location		
Colon	45	- - -
Rectum	8	- - -

[a] TNM: tumor-node-metastasis staging system

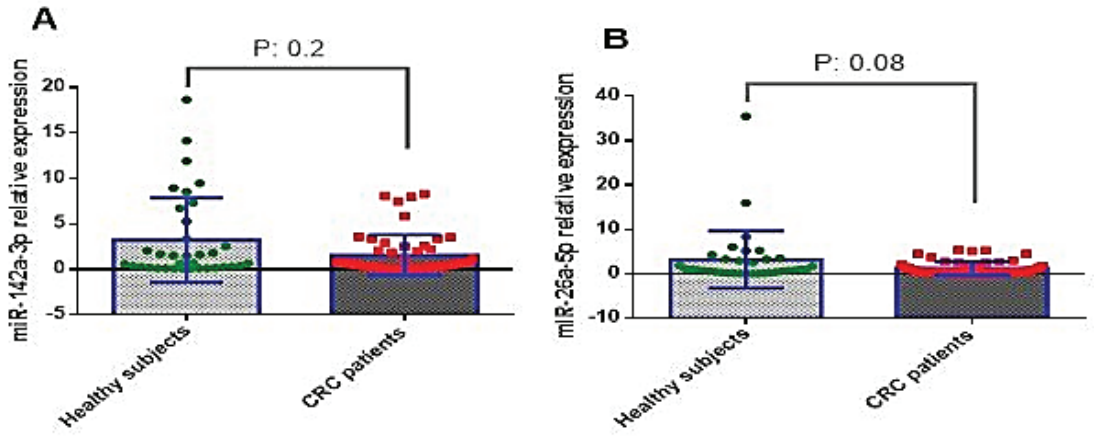

Figure 1. The expression levels of miR-142-3p and miR-26a-5p in 53 patients with CRC compared to 35 healthy individuals. Expression levels of the miRNAs were normalized to RNU6B. Data were analyzed using non-parametric Mann-Whitney test; **A)** miR-142-3p was not different in the patient group compared to control group (*P*-value = 0.2); **B)** miR-26a-5p was not different in the patient group compared to the control group (*P*-value = 0.08).

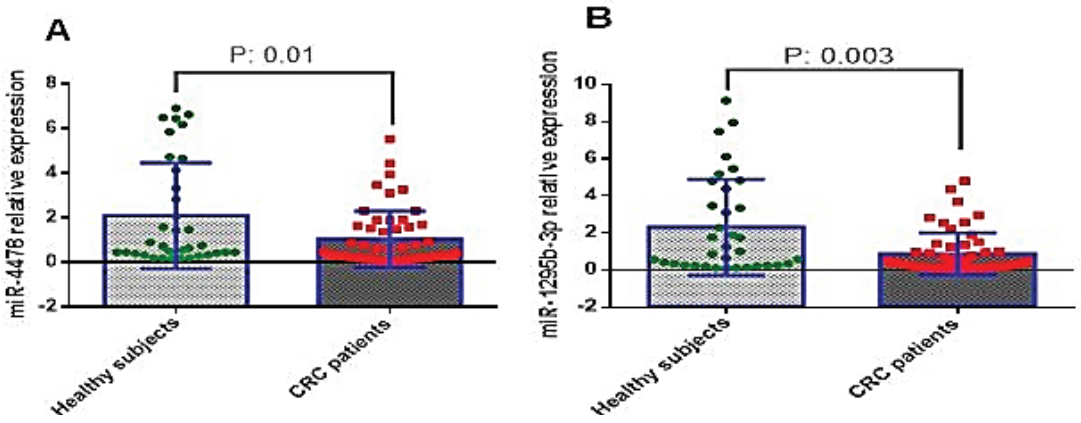

Figure 2. The expression levels of miR-4478 and miR-1295b-3p in 53 patients with CRC compared to 35 healthy individuals. Expression levels of the miRNAs were normalized to RNU6B. Data were analyzed using non-parametric Mann-Whitney test; **A)** miR-4478 was significantly downregulated in the patient group compared to the control group (*P*-value = 0.01); **B)** miR-1295b-3p was significantly downregulated in the patient group compared to the control group (*P*-value = 0.003).

tissue samples of all CRC patients and healthy individuals was isolated, following the commercial protocols. Subsequently, after reverse transcription of RNA to cDNA, the expression levels of previously verified fecal and circulating downregulated microRNAs (miR-4478, miR-1295-3p, miR-142-3p and miR-26a-5p) were analyzed in tissue samples of 53 CRC patients and 35 healthy subjects.

According to the results of REST 2009 and GraphPad Prism software using the Mann–Whitney test, there was no significant difference for miR-142-3p ($P = 0.2$) and miR-26a-5p ($P = 0.08$) between CRC patient and control group (Figure 1); however, miR-4478 ($P = 0.01$) and miR-1295b-3p ($P = 0.003$) were downregulated in tissue samples of patients with CRC compared to the control group samples (Figure 2).

Discussion

Although colorectal cancer mortality rates have decreased due to advances in diagnosis and treatment during the past three decades,[2,18] identifying diagnostic and prognostic biomarkers, as well as new targeted therapies for colorectal cancer, seem vital.

Numerous studies have shown that miRNAs are differentially expressed in cancers and the expression patterns of miRNAs can be used as a diagnostic or prognostic biomarker for several types of cancer.[19,20]

In the case of colorectal carcinoma, it might be possible to use miRNAs dysregulation as an effective diagnostic and prognostic biomarker. Furthermore, miRNAs have the potential to be used as a tool to treat this malignancy in the future. However, further studies are needed to identify the miRNAs with high specificity and sensitivity in tissue and other samples such as plasma and stool.

To our knowledge, similar to our previous study on simultaneous evaluation of miRNA expression in both CRC plasma and stool samples,[21] no study has been conducted to date on expression analysis of verified fecal and plasma dysregulated microRNAs, in tissue samples of CRC patients. In the present investigation, the expression of miR-4478 and 1295-3p, as well as miR-142-3p and miR-26a-5p (previously shown reduced expression of miRNAs in stool and plasma samples of CRC patients, respectively)[14,15] were

explored in FFPE samples of CRC patients compared to healthy subjects. Finally, in the expression analysis study using qRT-PCR, we demonstrated that the FFPE tissue levels of miR-4478 and miR-1295b-3p were significantly decreased in the patients compared to the controls; however, there was no significant difference in the expression levels of miR-142-3p and miR-26a-5p between these two groups.

Studies have revealed that miR-4478 is able to bind to the 3'-UTR of class II histone deacetylases (HDACs),[22] which can act as a regulator of cell growth in the colon.[23] In addition, overexpression of class II HDACs has been observed in a number of malignancies such as colorectal carcinoma.[24] Furthermore, in a study on expression pattern of miRNAs in CRC conducted by Slattery *et al.* (2011), decreased expression of miR-1295 was reported in tissue samples of patients with colorectal cancer.[25]

Presence of miRNAs in the stool sample may be probably due to exfoliation of gastrointestinal tract cells,[26] and their presence in blood[27] might be due to exiting exosomes containing miRNAs from the cells of the digestive tract. Although working with blood samples is more practical and easier, regarding the high leakage of colonic epithelial cells (colonocytes) into the colon,[28] there is the possibility of more accurate CRC diagnosis by investigating stool samples.

According to the current findings, it might be hypothesized that to implement non-invasive laboratory techniques for identifying colorectal malignancies based on evaluating miRNAs expression, stool samples appear to have a higher priority than blood samples due to the similar miRNAs expression profile with the cancerous tissue. However, a definitive viewpoint in terms of this finding requires further complementary studies with more samples and other identified fecal and circulating dysregulated miRNAs in patients with colorectal carcinoma.

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer. 2015; 136(5): E359 – E86.
2. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA: Cancer J Clin. 2013; 63(1): 11 – 30.
3. Gatta G, Capocaccia R, Sant M, Bell C, Coebergh J, Damhuis R, et al. Understanding variations in survival for colorectal cancer in Europe: A EUROCARE high resolution study. Gut. 2000; 47(4): 533 – 538.
4. Rhodes J. Colorectal cancer screening in the UK: Joint position statement by the British Society of Gastroenterology, the Royal College of Physicians, and the Association of Coloproctology of Great Britain and Ireland. Gut. 2000; 46(6): 746 – 748.
5. Lundstrom K. Micro-RNA in disease and gene therapy. Curr Drug Discov Technol. 2011; 8(2): 76 – 86.
6. Yeo JH, Chong MM. Many routes to a micro RNA. IUBMB Life. 2011; 63(11): 972 – 978.
7. Mirnezami A, Pickard K, Zhang L, Primrose J, Packham G. MicroRNAs: Key players in carcinogenesis and novel therapeutic targets. Eur J SurgOncol. 2009; 35(4): 339 – 347.
8. Bartel DP. MicroRNAs: Target recognition and regulatory functions. Cell. 2009; 136(2): 215 – 233.
9. Srivastava K, Srivastava A. Comprehensive review of genetic association studies and meta–analyses on miRNA polymorphisms and cancer risk. PLoS One. 2012; 7(11): e50966.
10. Tokarz P, Blasiak J. The role of microRNA in metastatic colorectal cancer and its significance in cancer prognosis and treatment. Acta Biochimica Polonica. 2012; 59(4): 467 – 474.
11. Visone R, Croce CM. MiRNAs and cancer. Am J Pathol. 2009; 174(4): 1131 – 1138.
12. MacFarlane LA, Murphy PR. MicroRNA: Biogenesis, function and role in cancer. Curr Genomics. 2010; 11(7): 537 – 561.
13. Krol J, Sobczak K, Wilczynska U, Drath M, Jasinska A, Kaczynska D, et al. Structural features of microRNA (miRNA) precursors and their relevance to miRNA biogenesis and small interfering RNA/short hairpin RNA design. *J Biol Chemistry*. 2004; 279(40): 42230 – 42239.
14. Ghanbari R, Mosakhani N, Asadi J, Nouraee N, Mowla SJ, Yazdani Y, et al. Downregulation of Plasma MiR–142–3p and MiR–26a–5p in patients with colorectal carcinoma. *Iran J Cancer Prevent*. 2015; 8(3): e2329.
15. Ghanbari R, Mosakhani N, Asadi J, Nouraee N, Mowla SJ, Poustchi H, et al. Decreased expression of fecal miR–4478 and miR–1295b–3p in early–stage colorectal cancer. *Cancer Biomark*. 2015; 15(2): 189 – 195.
16. Puppa G, Sonzogni A, Colombari R, Pelosi G. TNM staging system of colorectal carcinoma: A critical appraisal of challenging issues. Arch Pathol Lab Med. 2010; 134(6): 837 – 852.
17. Pfaffl MW. A new mathematical model for relative quantification in real–time RT–PCR. Nucleic Acids Res. 2001; 29(9): e45 – e46.
18. Van Steenbergen L, Elferink M, Krijnen P, Lemmens VEPP, Siesling S, Rutten H, et al. Improved survival of colon cancer due to improved treatment and detection: A nationwide population–based study in The Netherlands 1989–2006. Ann Oncol. 2010; 21(11): 2206 – 2212.
19. Raisch J, Darfeuille-Michaud A, Nguyen H. Role of microRNAs in the immune system, inflammation and cancer. World J Gastroenterol. 2013; 19(20): 2985 – 2996.
20. Lan H, Lu H, Wang X, Jin H. MicroRNAs as potential biomarkers in cancer: opportunities and challenges. BioMed research international. 2015; (2015): 1-17.
21. Ghanbari R, Mosakhani N, Sarhadi VK, Armengol G, Nouraee N, Mohammadkhani A, et al. Simultaneous Underexpression of let–7a–5p and let–7f–5p microRNAs in Plasma and Stool Samples from Early Stage Colorectal Carcinoma. Biomark Cancer. 2015; 7(Suppl 1): 39.
22. Berillo OA, Issabekova AS, Régnier M, Ivashchenko AT. Characteristics of binding sites of intergenic, intronic and exonic miRNAs with mRNAs of oncogenes coding intronic miRNAs. African J Biotechnol. 2016; 12(11): 1016 – 1024.
23. Wilson AJ, Byun DS, Nasser S, Murray LB, Ayyanar K, Arango D, et al. HDAC4 promotes growth of colon cancer cells via repression of p21. Mol Biol Cell. 2008; 19(10): 4062 – 4075.
24. Bolden JE, Peart MJ, Johnstone RW. Anticancer activities of histone deacetylase inhibitors. Nat Rev Drug Discov. 2006; 5(9): 769 – 784.
25. Slattery ML, Wolff E, Hoffman MD, Pellatt DF, Milash B, Wolff RK. MicroRNAs and colon and rectal cancer: differential expression by tumor location and subtype. Genes Chromosomes Cancer. 2011; 50(3): 196 – 206.
26. Link A, Balaguer F, Shen Y, Nagasaka T, Lozano JJ, Boland CR, et al. Fecal MicroRNAs as novel biomarkers for colon cancer screening. Cancer Epidemiol Biomarkers Prev. 2010; 19(7): 1766 – 1774.
27. Kosaka N, Iguchi H, Ochiya T. Circulating microRNA in body fluid: A new potential biomarker for cancer diagnosis and prognosis. Cancer Sci. 2010; 101(10): 2087 – 2092.
28. Koga Y, Yasunaga M, Takahashi A, Kuroda J, Moriya Y, Akasu T, et al. MicroRNA expression profiling of exfoliated colonocytes isolated from feces for colorectal cancer screening. Cancer Prevent Res. 2010; 3(11): 1435 – 1442.