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ORIGINAL ARTICLE

Serum overexpression of miR-301a and miR-23a in patients with colorectal cancer

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Abstract

Background: Extracellular vesicles (EVs) are a heterogeneous group of membrane-bound vesicles with complex cargoes including proteins, lipids, and nucleic acids. EVs have received significant attention due to their specific features including stability under harsh conditions and involvement in cell-to-cell communication. Circulating EVs and the molecules associated with them are important in the diagnosis and prognosis of cancers. MicroRNAs (miRNAs) are a group of small noncoding RNAs that have a role in regulating gene expression. Current literature shows that circulating miRNAs can be used as noninvasive biomarkers for early detection of cancers. The present study was set to investigate the potential role of serum exosomal miRNA expression levels in colorectal cancer (CRC) patients and evaluate their correlation with clinicopathologic features.

Methods: Exosome-enriched fractions were isolated from the serum of 25 CRC patients and 13 age- and sex-matched healthy controls using a polymer-based precipitation method. During the pilot phase, real-time polymerase chain reaction (RT-PCR) was carried out on 12 CRC patients and eight healthy participants to evaluate the expression difference of 11 candidate miRNAs between CRC patients and tumor free subjects. Finally, the results were validated in a separate group, which was similar in size to the pilot group. The clinicopathologic data were also collected and the relationship between aberrant miRNA expression and clinicopathological parameters were investigated.

Results: There were high expressions of exosomal miR-23a and miR-301a in serum samples of CRC patients compared to normal controls in training and validation phases; these differences were not significantly correlated with clinicopathologic features. Receiver operating characteristic curve analysis showed that miR-301a and miR-23a were able to discriminate CRC patients from normal subjects.

Conclusion: The findings provide evidence on the roles of miR-301a and miR-23a in CRC development and their potential roles as noninvasive biomarkers for early detection of CRC.

Keywords: Biomarkers; Colorectal cancer; Serum

1. INTRODUCTION

Colorectal cancer (CRC) is the second most common cancer type among females and the third among males; there were 1.4 million new cases and 639 000 deaths in 2012 according to reports. High alcohol consumption, obesity, smoking, physical inactivity, age, and family history are some of the risk factors for CRC, leading to the increase in the rate of CRC in recent years.[1,2] Unfortunately, due to the late manifestation of CRC symptoms, patients develop metastasis; that is why CRC is considered as one of the deadliest cancer types. Thus, screening programs have important roles in reducing the incidence and mortality of CRC. Several potential screening tests are currently used to detect CRC such as colonoscopy, flexible sigmoidoscopies, guaiac-based fecal occult blood tests, and fecal immunochemical tests. Colonoscopy is a golden test, but its invasive manner and high cost limit its application. On the other hand, these methods suffer from different problems including invasive, expensive, low sensitivity, and low specificity nature.[3–5] In 2008, the American Cancer Society declared a guideline that emphasized the importance of screening tests that facilitate prevention of cancer by early detection of the disease. The attempt of the scientific investigation has thus changed to finding noninvasive markers from biofluids such as blood, urine, and saliva.[6]

MicroRNAs (miRNAs) are short noncoding RNAs that regulate gene expression by either mRNA degradation or translational repression. In fact, 30% of human genes are regulated by miRNAs; a miRNA can regulate hundreds of different protein coding genes.[7] miRNAs have important roles in biological processes involved in carcinogenesis including apoptosis, proliferation, organ morphology, and developmental transitions. The miRNA expression has been shown to aberrantly change in different types of cancers including lung, pancreas, thyroid, breast, and colon.[8] New findings on miRNAs have recently shed light over cancer-related researches, especially the field of biomarkers.[9] Reports have successfully

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shown that miRNAs can act as reliable diagnosis biomarkers in tissue samples from different types of cancers; however, there are some problems about collecting tissue samples. On the other hand, most researchers have focused on finding noninvasive tools for detecting different cancers. In this context, circulating nucleic acids have received considerable attention. A primary study was conducted by Chen et al (2008), which demonstrated that miRNAs are stable in serum in harsh conditions such as low or high pH, freezing and thawing cycles, long-time storage, boiling, and RNase digestion.[10] Although there are some reports on their protective mechanisms, the exact mechanism is still unknown. There are some models for stability including formation of Argo-miRNA complex and/or encapsulation in exosomes. Several reports have distinctly demonstrated that cells actively secrete miRNAs to the extracellular environment by exosomes and function as bio messengers in the communication among cells.[11–13] Malignant cells also release exosomes containing miRNAs, which may play important roles in tumor progression.[14,15] In this regard, the scientific community is recently interested in finding noninvasive biomarkers in extracellular vesicle (EV) cargoes for early detection of cancers, predicting the responses to different types of treatments, and better understanding the tumor prognosis.

The current knowledge suggests that expression profile of miRNAs in serum may reflect the miRNA profile of tumor. Despite the results of previous reports suggesting that several miRNAs are aberrantly expressed in CRC, there are still not enough data on the description of serum exosomal miRNAs in CRC and the correlation between serum exosomal miRNAs and clinicopathologic features of CRC. Accordingly, in this study, 11 top deregulated miRNAs were examined in terms of their potential roles as a reliable, cost-effective, and efficient screening method for CRC.

2. METHODS

2.1. Patient and sample processing

Cases with positive colonoscopy results for CRC, confirmed by histology as colon, rectum, or sigmoid, were referred to Amiralmomenin Hospital of Tabriz, Iran. Patients with a history of chemotherapy and radiotherapy, familial adenoma, and malignant tumors were excluded from the study. All patient samples were histologically confirmed, and the stage of the tumor was determined according to surgery findings using TNM (tumour, node, metastases) staging system. Control participants were selected from individuals undergoing colonoscopy with negative results for polyp, malignancy, and other gastrointestinal complaints, matching to the patients' age, gender, and ethnicity. Detailed questionnaires including informed consent and questions on clinical and family history were obtained from the participants. The study was approved by the Research Ethics Committee of Tabriz University of Medical Sciences. Blood samples were collected from the patients and healthy participants before the surgery and 1 hour after colonoscopy, respectively. After filling out the consent forms, 10 mL of the blood samples was transferred into clot activator serum tubes, which were immediately placed on ice. The tubes were centrifuged at 2000*g* for 10 minutes. The supernatant was transferred into new tubes and centrifuged at 2500*g* for another 10 minutes. Serum samples were collected, were aliquoted into Eppendorf tubes, and were stored at −80°C until use. The time span from sample collection to storage at −80°C was <2 hours. Each sample underwent not more than two freeze-thaw cycles before the analysis.

2.2. Exosome isolation and characterization

Exosome-enriched fractions were isolated from 500 µL serum by ExoQuick reagent (System Biosciences Inc., Mountain View, CA, USA), and exoRNA purification was performed using the SeraMir Exosome RNA Amplification kit (System Biosciences Inc., Mountain View, CA, USA) according to the manufacturer's instruction. The obtained exoRNA was quantified by spectrophotometry at 260 nm. After isolation, the exosome pellet was resuspended in Phosphate buffered saline (PBS) and submitted to several analyses to control the purity and yield, and they were then stored at −80°C until further use.

During the initial step of exosome characterization, an ExoELISA kit (System Biosciences Inc., Mountain View, CA, USA) was used to detect exosomal markers, including CD9 and CD63 in an Enzyme-Linked ImmunoSorbent Assay (ELISA) assay. Subsequently, the western blot was used to characterize exosomal markers. Briefly, the protein concentration of the precipitated exosomes was measured by BCA Protein Assay Kit with BSA as standard according to manufacturer's instruction (Pierce, Thermo Scientific, Rockford, IL, USA). Equal amounts of protein were used in western blot analysis. The samples were loaded on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Polyvinylidene difluoride (PVDF) membrane. The following primary antibodies were used: CD81 (Santa Cruz Biotechnology, Santa Cruz, CA) and TSG-101 (Abcam, Cambridge, UK). Afterward, proteins were visualized using enhanced chemiluminescence System. Finally, exosome-enriched fractions were submitted to nanoparticle tracking analysis (NTA). NTA provides information on particle size distribution of samples in liquid suspension using the properties of Brownian motion and light scattering. The concentration and size distribution of particles were evaluated by NTA according to manufacturer's instruction.

2.3. Screening of candidate miRNAs by quantitative real-time polymerase chain reaction

In this study, a total of 11 candidate miRNAs (miR-23a, miR-92a, miR-221, miR-301a, miR-31, miR-143, miR-142, miR-223, miR-18a, miR-135b, and miR-18b) were selected on the basis of the most reported deregulated miRNAs.[16] Each 500 µL of serum yielded 50 µL of total RNA, which was kept at a temperature of −80°C. Reverse transcription and quantitative polymerase chain reaction (qPCR) were performed on equal RNA volumes of each sample using SeraMir Exosome RNA Amplification kit (System Biosciences Inc., Mountain View, CA, USA) according to the manufacturer's protocol. Briefly, 5 µL of the total RNA was polyadenylated and reverse transcribed to cDNA using the kit; 10 µL of this product was used in adaptor anneal reaction. Finally, 5 µL of this product was used for further polymerase chain reaction (PCR) analysis. PCR was performed by SYBR® Green Suppermix kit (Takara, Dalian, China) in a Roche PCR system. The primers used in the study were manufacturer-provided universal primers and miRNA-specific forward primers (System Biosciences Inc., Mountain View, CA, USA). Each reaction was performed in a total of 12 µL volume, and 45 PCR cycles were used as follows: denaturation at 95°C for 30 seconds, denaturation for 15 seconds at 95°C, and extension at 60°C for 1 minute. Melting curve analysis was also used to explore the specificity of the expected PCR. Each reaction was performed in triplicate, and one reaction was used without template as the negative control. Cycle threshold (C_t) value of ≥38 was considered as a low expression. Relative quantification of miRNAs was performed using the comparative C_t method software provided by the manufacturer.

2.4. Validation of differentially expressed miRNAs

The separate group of cases and controls (13 CRC patients and five tumor-free subjects) were used to validate differentially expressed miRNAs from the screening step.

2.5. Statistical analysis

All calculations were performed using GraphPad Prism 7.01 (GraphPad Software, Inc., La Jolla, CA, USA). Student's *t*-test, one and two-way analysis of variance, and chi-squared test were used for data analysis. *p*-value of <0.05 was considered to be statistically significant. Receiver operating characteristic (ROC) curve and the area under the ROC curve (AUC) were used to assess the sensitivity and specificity of miRNA biomarkers for the diagnosis of CRC.

3. RESULTS

3.1. Patient population

The present study utilized the sera derived from the following: who were diagnosed with CRC (n = 25), matching age and gender to participants with no evidence of CRC (n = 13). All 25 patients were pathologically and clinically diagnosed with CRC (Table). No significant difference was observed on the distribution of age (p = 0.51), gender (p = 0.76), alcohol consumption (p = 0.82), smoking (p = 0.93), and other diseases (p = 0.62) between the CRC patients and tumor-free subjects.

3.2. Characterization of exosome-enriched fraction

According to NTA, more particles were observed in exosome-enriched fraction isolated from serum and cell-derived exosomes in comparison with PBS and exosome-free medium. The levels of tested vesicle markers (CD63 and CD9) by ELISA were also higher in cell-derived exosome and the exosome-enriched fractions isolated from serum compared to exosome-free medium, which is consistent with western blotting results, confirming the presence of EVs in the isolated samples (Fig. 1A–C). Electrophoresis of the protein content of the exosomes isolated by Exo-Quick also revealed that the tested samples shared a similar mixture of proteins. Moreover, RNAs were separated by size, representing the presence of a population of small RNAs.

Table

Clinicopathological features of CRC patients

Characteristics	CRC patients, n (%)
Age, y (mean)	58.7
Gender	
Female	10 (40)
Male	15 (60)
Location	
Cecum	5 (20)
Terminal ileum	9 (36)
Sigmoid colon	5 (20)
Rectum	6 (24)
Size, cm	
≥4	10 (40)
<4	15 (60)
TNM stage	
II	12 (48)
III	13 (52)
Family history of CRC	No
Surgery	
Yes	25
No	0
Chemotherapy	
Yes	0
No	25

CRC = colorectal cancer.

The 18S and 28S ribosomal RNAs were hardly observed, generally detected with cell-derived RNAs. The examination of miRNA concentrations in the serum samples from the tested samples showed that miRNAs are the important components of small RNAs in serum.

3.3. Circulating miRNA screening

Newly diagnosed CRC patients from Amiralmomenin Hospital of Tabriz, Iran, were included in the study. A two phase case-control study was designed to identify serum miRNAs as blood-based biomarkers for CRC diagnosis. We selected 11 candidate miRNAs reported in previous studies and used quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays to investigate their expression in the 12 CRC cases and age- and gender-matched eight tumor-free samples in the training set. miR-301a and miR-23a presented a detection rate of >90% in both CRC and tumor-free samples, and were selected for further analysis. Statistical analysis revealed miR-301a (3.1 times upregulated) and miR-23a (3.8 times upregulated) that have significantly different levels in CRC patients compared to healthy controls (p < 0.0001) (Fig. 2). ROC curve analysis was also used to examine the diagnostic potential of differentially expressed miRNAs to distinguish between the cancer and normal groups. AUC values for miR-23a (p < 0.05; 95% CI: 0.74-1.0) and miR-301a (p < 0.05; 95% CI: 0.65-1.0) in discriminating CRC patients from healthy controls were 0.89 and 0.84, respectively, which showed that serum levels of miR-23a and miR-301a were sensitive biomarkers for discriminating CRC patients from healthy samples (Fig. 3). In addition, miR-92a showed upregulation in patients, although it was not statistically significant; it tended to be higher in CRC patients. The levels of other tested miRNAs in serum were too low to be quantified accurately. qRT-PCR is the most sensitive and reproducible method to quantify gene expression, but the accuracy is limited provided that the expression of miRNAs is low. As a result, some miRNAs with a low expression cannot be tested or compared between the patients and healthy controls. Subsequently, we tried to correlate expression levels of analyzed miRNAs to clinical-pathological characteristics of CRC patients. No significant difference was observed in the expression levels of miR-301a and miR-23a in the serum of CRC patients according to age and gender as well as tumor type, size, and location. Taken together, it was hypothesized that miR-301a and miR-23a contribute to CRC pathogenesis. Hence, it was investigated whether these two miRNAs would show altered expression levels in different sets of samples. Validation of differentially expressed miRNAs (miR-23a and miR301a) detected by the pilot study performed on a separate group (13 cases and five controls) confirmed the significant overexpression of miR-23a (>3-fold times) and miR-301a (>3-fold times) in the case subjects compared to the controls. miR-92a and miR-21 were also included in this phase because they have been reported as biomarkers for CRC. Additionally, a high expression of miR-92a and miR-21 has been identified in other cancer types, which proposed that further studies are necessary to reveal their potential roles as cancer biomarkers.[17–19] Surprisingly, the detection rate of

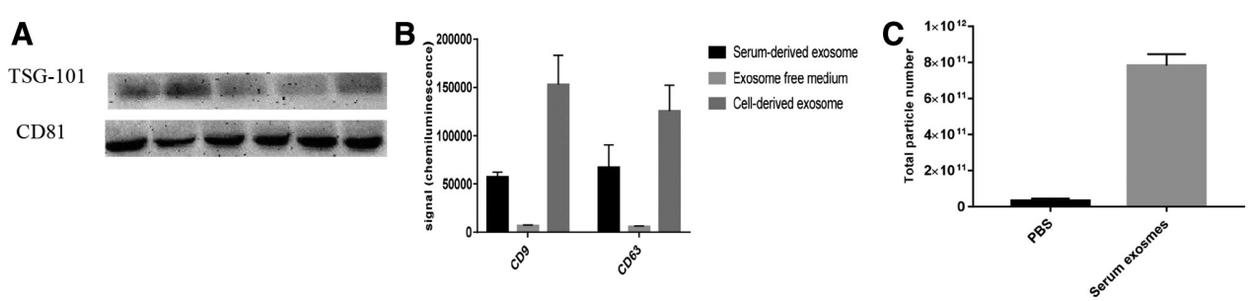

Fig. 1 Characterization of isolated extracellular vesicles (EVs). A, Isolated EVs were evaluated for the expression of EV markers TSG-101 and CD 81 by western blotting. B, Analysis of EV markers by ELISA. C, Evaluation of the EV concentration by nanoparticle tracking analysis (NTA).

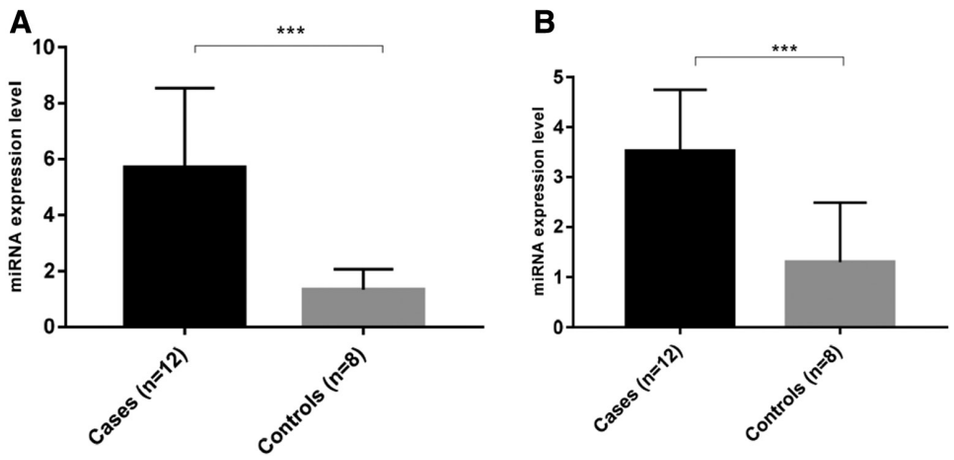

Fig. 2 Serum levels of candidate microRNA (miRNA) biomarkers in the screening phase. Levels of serum miR-23a (A) and miR-301a (B) were compared between tumor-free controls and colorectal cancer (CRC) patients.

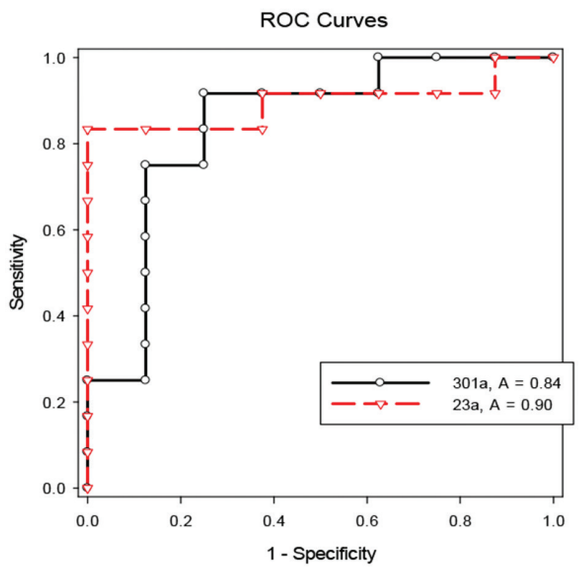

Fig. 3 Receiver operator characteristic (ROC) curves of miR-30a1 and miR-23a for discriminating colorectal cancer (CRC) patients from normal subjects.

miR-21 and miR-92a was <60% in either CRC or healthy group; they were excluded from the analysis. Our results indicated that in CRC, the primary tumor releases high concentrations of miRNAs into the blood circulation, and some of them were found in tumor-derived exosomes.

Because there is no consensus on the use of internal normalization control in the serum for miRNA qPCR, the expression levels of miR-93 and miR-16 in the serum were examined to be used in data normalization. Both miRNAs were proposed as suitable reference genes for miRNA quantification in the serum.[20] Our results revealed that both miR-93 and miR-16 were not readily detected in the exosome-enriched fractions of all controls and CRC patients. They did not display enough expression levels, high stability, and low variability. Therefore, they were not used for data normalization.

4. DISCUSSION

Despite recent progresses in the diagnosis and treatment of CRC patients, CRC patients die because of distance metastasis. The incidence and mortality of CRC in Asia have rapidly increased in recent decades. Early detection and surgical removal of primary lesions could be helpful in reducing CRC-related deaths and management of CRC. Carcinogenesis of CRC has been extensively studied at the molecular level, but there is a minimal knowledge about descriptions of serum exosomal miRNAs in CRC. The aim of this study therefore was to investigate the potential of 11 candidate miRNAs as biomarkers of CRC and the relationship between the expression levels of selected miRNAs and clinicopathological characteristics of disease. Interestingly, the serum levels of miR-301a and miR-23a were found to be upregulated in CRC patients compared to normal controls, which were capable of distinguishing the patients group from the normal group. To our knowledge, the present study provides the first evidence about the high expression of miR-301a and miR-23 in exosome-enriched fraction from the serum in CRC. On the other hand, with a significant ROC score, miR-23a and miR-301a expression levels were able to discriminate the CRC patients from tumor-free samples. The study could not validate other candidate miRNAs because they could not be measured due to their low sera levels.

One of the most prevalent causes of colorectal cancer is mutation in transforming growth factor beta receptor type II (TGFBR2). It has been evaluated that approximately 30% of colorectal cancers are as a result of mutations in TGFBR2. Multiple miRNAs have been approved to implicate in transforming growth factor (TGF)-ß signaling pathway, miR-301a has been confirmed to regulate TGFBR2. [21] miR-301a increases in various types of cancers and functions as an oncogene-like miRNA. miR-301a has been found to increase CRC cell line proliferation, migration, and invasion in vitro and tumor growth in vivo. It plays an oncogenic role by reducing the expression of cytokine signaling 6 (SOCS6).[22] Liu et al. approved upregulation of miR-301a in colon cancer tissues compared to matched adjacent normal tissues; they reported oncogenic role of miR-301a in colon tumorigenesis by regulating transforming growth factor TGF-β/smad signaling.[23] However, the difference in the expression level of miR-301a between CRC and healthy groups in blood-based approach has not been elucidated. It is questionable whether miR-301a can act as an important messenger mediating CRC progression; however, on the basis of previous reports on miR-301a in tumor biology, it is supposed that miR-301 has a role in poor prognosis and invasion and metastasis in CRC. Differential expression of miR-23a was also found in our tested CRC patients. miR-23a is one of the top 50 deregulated miRNAs in CRC.[24,25] A report has shown that miR-23a increases colon cancer cell growth, invasion, and metastasis via inhibiting metastasis suppressor gene expression. Furthermore, the upregulation of miR-23a expression was correlated with an advanced stage and the depth of invasion as well as lymph node metastasis,

demonstrating that miR-23a could be a biomarker for CRC.[26] Similarly, inhibition of miR-23a enhanced 5- fluorouracil (5-FU) induced apoptosis by the apoptosis-activating factor-1 (APAF-1)/caspase-9 apoptotic pathway.[27] On the basis of our results, it can be suggested that miR-301a and miR-23a have important roles in the regulation of CRC invasion and proliferation.

Several studies have attempted to examine the potential roles of circulating miRNAs as invasive biomarkers in CRC. Some reports have shown the potential roles of miR-17-3p, miR-29a, miR-92a, and miR-135b as biomarkers for CRC.[12,25,27,28] In this regard, Faltejskova et al. used qPCR to evaluate the expression levels of these four miRNAs in 100 CRC patients and 30 healthy controls; they did not find any significant change in tested miRNAs between the healthy and patient groups.[30] Different groups reported that miR-21, miR-141, miR-29a, miR-17-3p, miR-92, miR-601, and miR-760 were significantly expressed in a group of patients compared to healthy individuals.[31,32] Aherne et al. examined the expression of 677 miRNAs in CRC and healthy samples; they did not find biomarkers found by other groups. They identified two circulating miRNAs capable of discriminating groups with different diseases of the colon from each other and patients with advanced cancer from benign disease groups.[29] Therefore, some disparities exist among findings of different reports with regard to miRNA biomarkers; the present findings of upregulation of miR-23a and miR-301a in the serum of tested CRC patients were in accordance with previous studies.[32–35] In contrast, no significance difference was observed in the expression of miR-92a, miR-21, and other studied miRNAs between the two studied groups. Ng et al. demonstrated that miR-92a can distinguish CRC patients from healthy controls with 89% and 70% sensitivity and specificity, respectively.[25] Huang et al. also demonstrated similar findings (sensitivity: 84, specificity: 71.4).[28] In contradiction with these findings, Schee et al. showed miR-92a levels to be low in several CRC tissue samples and concluded that there was no significant association between miR-92a levels and CRC.[36] In addition, an association has been reported between miR-92a expression and other diseases such as breast cancer, hepatocellular carcinoma, and cardiovascular diseases.[37] It can be thus suggested that miR-92a does not have enough specificity to be a biomarker for CRC. Controversial results are also found about expression levels of miR-21 and CRC.[3,36,38,39] Surprisingly, a study revealed that red blood cell (RBC) can contribute significant levels of reported cancer biomarkers into blood, having significant applications for interpretation of biomarkers. A highest tendency for RBC hemolysis in patients diagnosed with colon cancer could especially explain the relatively high level of miR-92a in blood samples reported as a biomarker in patients with colon cancer. Indeed, several miRNAs proposed as circulating cancer biomarkers or used as reference genes including miR-21- 5p, miR-16, miR-29a-5p, and 92a-5p may generally be driven from blood cells, and their blood levels have been shown to be strongly affected by hemolysis.[40] There are several reasons for the contradictory results reported in the literature; for example, studies have evaluated different kinds of samples such as colon, rectum, and sigmoid and different sample types including serum, plasma, and tissue. Furthermore, the genetic background of tumor, ethnic background of patient, sample processing, data acquisition, normalization, and analysis may also affect the results. In an effort to control variable factors in this study, our tested samples were homogenized in terms of age and the stage of disease; the time of sample collection and sample processing was the same as well. In summary, according to the cycle threshold (C_t) values of the tested samples, miR-301a is not a low abundance biomarker in sera. The present findings, if validated with a large group of patients, could be useful in confirming the role of miRNAs as noninvasive biomarkers for CRC screening. Accordingly, further research studies may contribute to the indication of the most accurate miRNAs as biomarkers and better understanding of their roles in CRC.

In conclusion, 11 miRNAs were investigated in the exosome-enriched fractions isolated from serum of CRC patients. The upregulation of miR-301a in the CRC patients' serum was not surprising; however, no previous study has shown upregulation of miR-301a in the CRC serum sample. New potential biomarkers, miR-23a and miR-301a, were identified for the diagnosis of CRC, which are beneficial for the development of blood-based biomarkers for CRC.

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