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Plasma Level of *miRNA-7*, *miRNA-409* and *miRNA-93* as Potential Biomarkers for Colorectal Cancer

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ABSTRACT

Colorectal cancer (CRC) is among deadliest cancers all over the world. Regarding its high mortality, previous researches have focused to discover new applicable diagnosis methods. In this regard, circulatory miRNAs have been received huge consideration as promising biomarkers for early detection of CRC. The study aimed to evaluate the expression level of *miRNA-7*, *miRNA-93*, and *miRNA-409* in plasma of CRC patients compared to healthy controls. According to the results, *miRNA-7* and *miRNA-409* were significantly upregulated in plasma of CRC patients ($p< 0.05$) while *miRNA-93* was insignificantly elevated in CRC patients ($p> 0.05$). Therefore, it seems that upregulation of *miRNA-7*, *miRNA-93*, and *miRNA-409* is involved in CRC carcinogenesis. Additionally, *miRNA-7* and *miRNA-409* could be considered as novel potential biomarkers for CRC.

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Introduction

Colorectal cancer (CRC) is one of the worst lethal cancer in the world. It ranks as the fourth most prevalent cancer and the fourth leading cause of cancer deaths worldwide, in which more than 1.23 million new cases with CRC and about 608,700 CRC deaths occurrence are reported every year (Ferlay *et al.*, 2015). Family history, age, sex, inflammatory bowel disease, lynch syndrome, a diet with low fiber and high red meat, low physical activity, smoking, obesity, and alcohol consumption are described as the well-known risk factors for CRC (Ren *et al.*, 2015). CRC is mostly originated from dysplastic adenomatous polyps and progressed slowly in a stepwise route via activation and inactivation of key oncogenes and tumor suppressor genes, respectively (Stiegelbauer *et al.*, 2014). CRC diagnosis commonly occurs at the late metastasis stages and its survival rate is less than 30 months (Scartozzi *et al.*, 2014). Early detection at premalignant polyp stages, with no symptoms, would lead to effective treatment and a higher survival rate of patients (Pawa *et al.*, 2011; Ren *et al.*, 2015). So, it seems that the availability of high sensitive screening methods could be beneficial for early diagnosis and advance treatment.

There are some routine methods for CRC diagnosis such as flexible sigmoidoscopies (Ng and Wong, 2013; Ren *et al.*, 2015), colonoscopies, fecal occult blood tests (FOBTs) (Ng and Wong, 2013; Sovich *et al.*, 2015) and FITs test (Sung *et al.*, 2014). However, they have several disadvantages and limitations such as invasiveness, low sensitivity and high cost (Altobelli *et al.*, 2015; Sovich *et al.*, 2015). Therefore, introducing a reliable, cost-effective and simple procedure for early detection of CRC is highly demanded.

In this regard, the application of miRNAs as novel biomarkers and therapeutic targets has been more focused in recent researches (Moles, 2017). miRNAs are tiny RNA molecules that belong to small non-coding RNAs family and regulate gene expression. Their attachment to 3' untranslated (3'-UTR) region of mRNA leads to mRNA degradation or translation inhibition (Jonas and Izaurralde, 2015; Tafrihi and Hasheminasab, 2019). It was shown that the functions of miRNAs, both as oncogenes or tumor suppressor genes, is deregulated in

several human cancers (Damandan and Moradpour Hesari, 2016; Reddy, 2015; Ren *et al.*, 2015; Zare *et al.*, 2018). Considering the important role of miRNAs and their cancer cell-specific alteration pattern (Aminisepehr *et al.*, 2018; Reddy, 2015), they could represent as a novel accurate and predictive biomarker for cancer diagnosis. Additionally, miRNAs are simply detectable and measurable in different sample sources such as tissue, blood, and stool that make them as a desirable noninvasive tool for CRC diagnosis. Consistently, various studies have shown the altered expression of miRNAs in different sample sources of CRC (Ahmed *et al.*, 2013; Dong *et al.*, 2014; Hunter *et al.*, 2008; Luo *et al.*, 2013). In the current study, the expression level of *miRNA-93* (*hsa-miR-93-5p*), *miRNA-7* (*hsa-miR-7-5p*) and *miRNA-409* (*hsa-miR-409-5p*) were evaluated in plasma of CRC patients compared with healthy controls, to identify their potential application as CRC diagnostic biomarker.

Materials and Methods

Clinical specimens

In the present study, a total of 30 patients including 15 men and 15 women with CRC diagnosis besides 30 healthy donors including 15 men and 15 women participated. The blood samples from patients were collected before receiving any treatment or colorectal resection surgery. Patients who had received preoperative radiotherapy or chemotherapy and the ones with the history of cancers in other organs or inflammatory bowel disease were excluded. Asymptomatic and healthy volunteers at the same age range with patients and without any previous history of cancer were selected as healthy control group. Volunteers with any systemic infection were excluded as well. All patients and healthy donors provided their informed consent to take part in this study.

Plasma preparation and RNA extraction

For plasma preparation, 3 ml blood sample in EDTA was collected from patients and healthy cases. The whole blood was centrifuged at 2000 g for 5 min and separated into 2 phases. The supernatant was further centrifuged at 12000 g for 15 min and then the plasma was collected from upper layer and stored at -80 °C until the later use. Total RNA was extracted from plasma samples applying an RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer instruction. Finally, total RNA was eluted from spin column of RNeasy Mini Kit in 20 µl of RNase-free water. Extracted RNA was stored at -80°C.

Real-time RT-PCR

The concentration of extracted RNA was measured by NanoDrop (Thermo Scientific, USA) Apparatus. For cDNA synthesis, 500 ng of RNA was initially polyadenylated, using *E. coli* poly (A) polymerase (New England BioLab) in a total reaction volume of 10 µl. The reaction mixture was incubated at 37 °C for 30 min. Subsequently, polyadenylated RNA was reverse transcribed using PrimeScript RT reagent Kit (TAKARA, Japan) in a total volume of 10 µl. The reaction was incubated at 37°C for 30 minutes followed by 85°C for 5 minutes.
Following to cDNA synthesis, Real-time PCR was performed using Rotor-Gene Q system (QIAGEN) and SYBER Green method in a total volume of 25 µl. All reactions were done in duplicate. The amplification condition was as following: 30 sec. at 95ºC for pre incubation, followed by 40 cycles of quantification step containing; 10 sec. at 95ºC for denaturation, 30 sec. at 60ºC for annealing and extension, and ended with 1 cycle of melting curve step containing; 40 sec. at 60ºC and ramping to 95ºC with ramp rate 0.1ºC/sec. For internal control, U6 gene was used.
The designed specific primer sequence was as following: *miRNA-409*: AGGTTACCCGAGCAACTTTGC; *miRNA-93*: CAAAGTGCTGTTCGTGCAGG; *miRNA-7*: GCGCTGGAAGACTAGTGATTTTG; and U6: CGCAAGGATGACACGCAAATTC.
The expression of the miRNAs was normalized to that of U6 and determined by the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

All statistical analysis was performed using SPSS (version 20) software to evaluate the significance of miRNAs levels between patients and control groups. Values are

presented as means ± SE. chi-square test was used for categorical variables and *t*-test was applied for continuous variables between patients and control groups. *p*-value less than 0.05 was considered as statistically significant.

Results

As mentioned above, Real-time RT-PCR was used to evaluate the plasma level of *miRNA-407*, *miRNA-7*, and *miRNA-93* in CRC patients and compare them with healthy controls. Totally, 30 patients with colorectal cancer and 30 healthy donors were included in the present assessment. 15 men and 15 women were involved in both patients and normal control groups. The age of the patients at the time of diagnosis and the healthy donors ranged from 50 to 78 years. There was no significant correlation between the gender and the age of the patients and control groups. For assessment of the specificity of the PCR procedure, the products of amplified miRNAs were analyzed on agarose gel (Fig. 1).

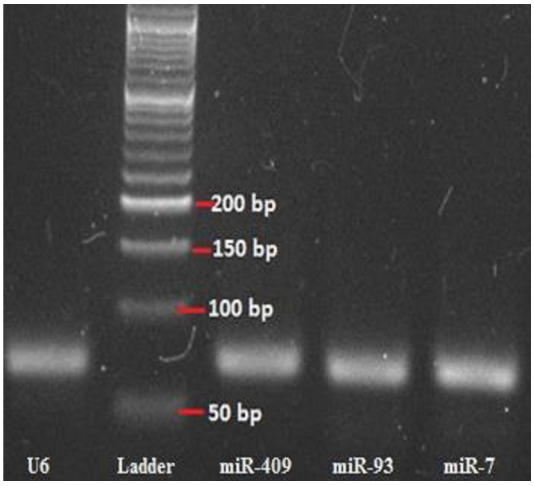

Fig. 1. Assessment of PCR products on agarose gel: From right to left, the specific products of *miRNA-7*, *miRNA-93*, *miRNA-409*, and *U6* were shown on an agarose gel.

According to our results, the plasma level of *miRNA-7* and *miRNA-409* showed significant differences between patients and normal control group. The expression level of *miRNA-7* was significantly higher in plasma of colorectal cancer patients relative to the normal control group ($p< 0.001$). Accordingly, patients with colorectal cancer express *miRNA-7* by 3.4 fold higher than healthy donors (Fig. 2).

Additionally, *miRNA-409* was considerably elevated in colorectal cancer patients in comparison with the normal control group ($p< 0.001$). The plasma level of *miRNA-409* in colorectal cancer patients was increased by 2.4 fold compared with healthy donors (Fig. 3). Similarly, the expression of *miRNA-93* was upregulated in plasma of colorectal cancer patients in comparison with the normal control group, while the difference in expression level was not significant ($p> 0.05$) (Fig. 4).

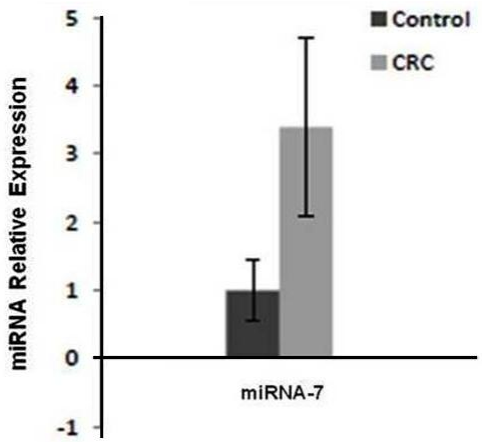

Fig. 2. Expression level of *miRNA-7* in plasma of colorectal cancer patients: Real time RT-PCR was used to analyze the expression level of *miRNA-7* in plasma samples. *miRNA-7* was significantly overexpressed by 3.4 fold in CRC patients relative to normal group ($p<0.05$).

Discussion

CRC represents as a serious deadly malignancy both in men and women in the world. However, early detection could reduce the mortality rate (Siegel et al., 2014).

Nowadays, several approaches are recommended for early diagnosis of CRC in adults which are colonoscopy every 10 years and also flexible sigmoidoscopy, double-contrast barium enema (BE), or computed tomographic (CT) colonography every 5 years. Moreover, carcinoembryonic antigen (CEA) blood test and FOBT are often used in CRC screening. However, the current screening procedures have their own disadvantages and low predictability.

On the other hand, the potential application of miRNA as a novel predictive and diagnostic biomarker for human cancers including CRC has been more considered by researchers (Chen *et al.*, 2008; Masuda *et al.*, 2017).

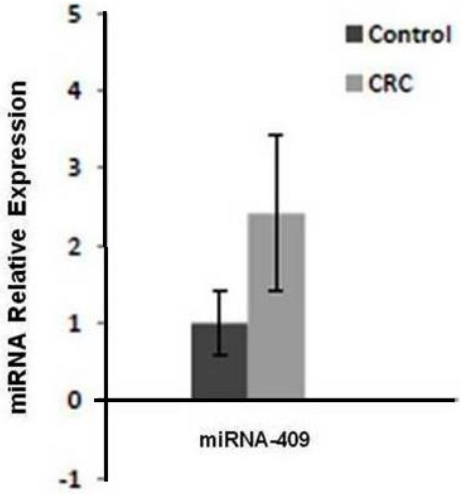

Fig. 3. The expression level of *miRNA-409* in plasma of colorectal cancer patients: Real-time RT-PCR was applied to assess the plasma level of *miRNA-409* in CRC patients. *miRNA-409* was significantly increased by 2.4 fold in CRC patients relative to the normal group ($p<0.05$).

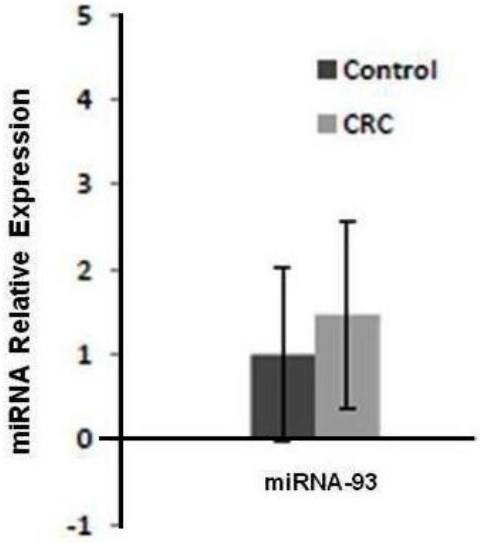

Fig. 4. The expression level of *miRNA-93* in plasma of colorectal cancer patients: Real-time RT-PCR analysis showed that the plasma level of *miRNA-93* was elevated in CRC patients relative to the normal group, while the difference was not significant ($p>0.05$).

The miRNAs as a class of small non-coding RNA molecules involved in the regulation of gene expression by promoting mRNA degradation or suppressing translation (Djuranovic *et al.*, 2012; Jonas and Izaurralde, 2015). Considering their tissue-specific aberrant expression in carcinogenesis process and their stability, they seem to be valuable candidates as therapeutic and diagnostic biomarkers (Catela Ivkovic *et al.*, 2017; Reddy, 2015). Additionally, they could be assessed via non-invasive and lower-priced approaches in a small number of different sample sources such as stool and blood (Masuda *et al.*, 2017; Ren *et al.*, 2015). In this regard, circulatory miRNAs have been considered as a preferable noninvasive screening marker.

Although the RNA-degrading activity of ribonuclease is extensive in blood, miRNAs are stable in the circulatory system which is due to their packing status in exosome/vesicle or their attachment to proteins (Turchinovich *et al.*, 2011; Valadi *et al.*, 2007; Vickers and Remaley, 2012). Therefore, they could be simply evaluated in blood-based samples applying a small quantity of blood sample (Ahmed *et al.*, 2012; Ren *et al.*, 2015). Both serum and plasma can be used as the source of blood circulatory miRNAs, while the plasma seems to be a more suitable source; because additional released RNA, via hemolysis, in serum may affect on target miRNA assessment (Wang *et al.*, 2012).

In the present study, the expression level of *miRNA-7*, *miRNA-93*, and *miRNA-409* was evaluated in plasma samples of CRC patients in comparison with healthy controls. Totally, 30 patients with colorectal cancer and 30 healthy donors were included in the present assessment. Our results have shown the significant upregulation of *miRNA-409* and *miRNA-7* in plasma samples of CRC patients compared with the healthy controls. Furthermore, rather increased expression of *miRNA-93* has been also identified in the plasma of CRC patients.

The miRNAs involvement in CRC was firstly specified by Michael *et al.*, that reported the significant downregulation of *miRNA-143* and *miRNA-145* in CRC tissues (Michael *et al.*, 2003). Subsequently, several studies have shown the altered expression of different

miRNAs in CRC tissue, blood and stool samples (Masuda *et al.*, 2017; Ren *et al.*, 2015). Upregulation of *miRNA-21*, *miRNA-203*, *miRNA-372*, *miRNA-92A*, *miRNA-130b* and downregulation of *miRNA-22*, *miRNA-93*, *miRNA-144*, *miRNA-16*, and *miRNA-132* are among identified detectable miRNAs in CRC (Dong *et al.*, 2014; Masuda *et al.*, 2017; Ren *et al.*, 2015). However, circulatory miRNAs, release by cancer cells (Hunter *et al.*, 2008), are preferable to be applied as a diagnostic and prognostic biomarker. Chen *et al.* have firstly reported the altered expression of a panel of 69 miRNAs in the serum of CRC patients (Chen *et al.*, 2008).

Our finding identified that the plasma level of *miRNA-7* and *miRNA-409* is significantly higher in the CRC patients compared with the healthy controls. So, it could be concluded that *miRNA-7* and *miRNA-409* involve in CRC tumorigenesis procedure and they might be applied as CRC diagnostic biomarker.

Altered expression of mentioned miRNAs has been recognized in some cancers. Downregulation of *miRNA-7* was shown in several cancers (Horsham *et al.*, 2015). Supporting to our data, increased expression of *miRNA-7* has also been found to be associated with tumor invasion and progression in breast cancer, urothelial carcinoma and cervical cancer patients (Horsham *et al.*, 2015; Rao *et al.*, 2012). Furthermore, the serum level of *miRNA-7* has been shown to be upregulated in patients with epithelial ovarian cancer (Meng *et al.*, 2015). Similarly, upregulation of *miRNA-7* was found in advanced CRC and some colorectal cell lines (Nakagawa *et al.*, 2015). More importantly, increased expression of *miRNA-7* was also found in stool samples of CRC patients (Ahmed *et al.*, 2013). Additionally, Yang *et al.*, have identified the significantly increased expression of *miRNA-7* in postoperative CRC patients with early relapsed compared with non-early relapsed patients and it was associated with worse survival of the CRC patients (Yang *et al.*, 2016). In contrast, Suto *et al.*, have shown that low expression of *miRNA-7* in colorectal cancer is associated with poor prognosis (Suto *et al.*, 2015). An elevated level of serum *miRNA-7* was also found in other diseases such as acute pancreatitis that represents its high potential as a new diagnostic and prognostic biomarker (Lu *et al.*, 2017).

Altered expression of the *miRNA-409* family has been shown in some cancers. Upregulation of *miRNA-409-3p* was found in the plasma of breast cancer patients (Cuk *et al.*, 2013a; Cuk *et al.*, 2013b), while its downregulation was observed in breast cancer tissues and cell lines (Cao *et al.*, 2016; Zhang *et al.*, 2016). Increased expression of *miRNA-409-5p* was reported in both breast cancer tissues and cell lines (Yu *et al.*, 2017). The upper level of *miRNA-409-3p* and *miRNA-409-5p* was also observed in prostate cancer tissues and metastatic prostate cancer cell lines (Josson *et al.*, 2014). Additionally, *miRNA-409-3p* has been shown to be overexpressed in the serum of high-risk prostate cancer patients compared with low-risk patients (Nguyen *et al.*, 2013). Josson *et al.*, have shown that upregulation of stromal fibroblast-derived *miRNA-409* induces tumorigenesis and epithelial-to-mesenchymal transition in prostate cancer (Josson *et al.*, 2015). However, in contrast to our results, the downregulation of *miRNA-409-3p* was shown in CRC tissues compared to adjacent non-tumor tissues (Bai *et al.*, 2015). In this concept, the different expression pattern of the same miRNA in various cancers further support the cell or tissue specificity of miRNAs due to their involvement in different molecular pathways.

Our results indicated a little upregulation of plasma *miRNA-93* in CRC patients compared with the control group, however, the difference was not significant. Overexpression of *miRNA-93*, as a promising biomarker, was found in gastric cancer that was associated with tumor progression and metastasis (Guan *et al.*, 2017). In contrast, decreased expression of *miRNA-93* has been reported in early relapsed post-operative CRC patients (Yang *et al.*, 2016). Recently, Zedan *et al.,* have shown the overexpression of *miRNA-93* in prostate cancer tissue and plasma. Furthermore, the plasma level of *miRNA-93* is considerably decreased after treatment in local advanced prostate cancer patients which more signified its potential as a prostate cancer biomarker (Zedan *et al*., 2018). Our data indicate that the increased expression of *miRNA-409* and *miRNA-7* are basically involved in CRC carcinogenesis procedure. Overexpression of

miRNA-93 may also be contributed in CRC. More notably, *miRNA-7* and *miRNA-409* may be applied as a new diagnostic biomarker for CRC.

Conclusion

As it was mentioned, the incidence and mortality of CRC are still high in the world. Thus, new reliable screening and detection approaches are demanded. Regarding this, miRNAs that play an important role in the initiation and progression of CRC, possess significant potential as biomarkers as well as therapeutic targets. Considering the sever upregulation of both *miRNA-7* and *miRNA-409* and the appropriate sample source of plasma which was used for miRNA screening, our data suggest the potential application of *miRNA-409* and *miRNA-7* as a diagnostic biomarker in CRC. However, we recommend these data could be combined with other related analysis to introduce a panel of candidate miRNAs for CRC screening, treatment monitoring, and therapeutic approaches. Otherwise, the application of circulatory miRNA biomarkers as a clinical routine test needs further large-scale evaluation for finding the optimal miRNA panel and standardization of the extraction and measurement protocols.

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