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Serum overexpression of microRNA-454-3p in patients with colorectal cancer and determine its correlation with pathological characteristics

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ABSTRACT. This study aimed to evaluate serum overexpression of microRNA-454-3p in patients with colorectal cancer and determine its correlation with pathological characteristics. Exosomes isolated from the serum of 40 CRC patients and 30 healthy subjects by Exoquick kit solution, were characterized using SEM. The expression of miR-454-3p was assessed in both healthy and CRC patients using the RT-PCR method. Then, by ROC analysis, the capability of this microRNA as a biomarker was determined. P-value < 0.05 was determined as significant. The results of the study showed that the rate of microRNA-454-3pexpression in the samples of CRC patients was significantly higher than in the control population ($p < 0.01$). Also, the expression of microRNA-454-3pin stage III of the disease showed a significant increase, compared to stages I and II ($p < 0.05$).miR-942-5p expression in CRC patients with lymphatic metastasis was significantly higher than those with no metastasis to their lymph nodes (P-value < 0.05). Also, the sensitivity and specificity of miR-454-3p were found to be 79 and 99%, respectively (with an area under the ROC curve [AUC] of 0.89). It seems that the increased expression of the microRNA-454-3p gene has a role in the pathogenesis of CRC and also the expression of this gene is associated with the stage of cancer.

Keywords: CRC; Colorectal cancer; microRNA-454-3p; Real-time PCR; Exosome.

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Introduction

Colorectal cancer (CRC) is the third most common cancer in the world with an annual number of 1.2 million cases, accounting for about 10 to 15% of all cancers, and is the second leading cause of cancer death in Western countries (Jeon et al., 2018). CRC affects the colon and rectum of the gastrointestinal tract. Currently, one of the most common cancers of the gastrointestinal tract in Iran is CRC and the results of studies indicate that the incidence and prevalence of this cancer in Iran are increasing (Shetleret al., 2018). CRC is the fourth most common cancer in men in Iran after gastric, bladder, and prostate cancers and the second most common cancer in women after breast cancer. The prevalence of CRC in Iran is much higher than the global average of this disease, so its prevalence in the population of Iran was 160 per 100,000, while the global prevalence for men and women is 66.8 and 73.9 per 100,000, respectively (Siegel et al., 2017; Shadmani et al., 2017).

Genetically, CRC is a multifactorial disease and the product of complex interactions between a variety of genetic and environmental factors (Rafiemanesh et al., 2016). Diagnostic methods for colorectal cancer include flexible sigmoidoscopy (Segev et al., 2018), colonoscopy (Moreno et al., 2016), double-contrast barium enema (Brady et al., 1994), computed tomographic colonography (Simon, 2016), fecal occult blood test (FOBT) (Winawer et al., 1993), stool DNA test (Imperialeet al., 2004) and microsatellite instability testing in CRC (Vilar & Gruber, 2010), each with its advantages and disadvantages. The use of non-coding RNAs in cancer diagnosis is a new approach in cancer research. Also, the biomarker potential of extracellular microvesicles (exosomes) due to the rich content of their non-coding RNAs is a field of new research in recent years that has provided new opportunities in the diagnosis of diseases.

MicroRNAs (miRNAs) belong to a class of small non-coding RNAs which regulate gene expression and protein-coding genes. Improper expression of miRNA is associated with human diseases including cancer. Tumors analyzed by determining miRNA profiles show different profiles of miRNAs compared to normal cells

from the same tissue. Overexpression of miRNAs has an oncogenic role. In addition, several miRNAs in cancers have been identified functionally as oncogenes or suppressors (Sinicrope, 2018). One of the studied miRNAs in the field of colorectal cancer is miR-454-3p, which is a member of the miR-130-3p / 301-3p / 454-3p miRNA cluster, which has been shown to play a critical role in the progression and malignancy of various cancers (Liao et al., 2021). According to reports, miR-454-3p plays different roles in different types of cancer. For example, miR-454-3p promotes cell proliferation by targeting the PI3K/AKT signaling pathway in triple-negative breast cancer (Sun et al., 2018) but miR-454-3p inhibits cell growth by targeting STAT3 and ATG12 in chondrosarcoma (Bao et al., 2017). Some studies have shown that miR-454-3p acts as a suppressive miRNAin several cancers, including glioblastoma (Zuo et al., 2019), lung cancer (Jin et al., 2019), bladder cancer (Wang et al., 2018), and pancreatic ductal adenocarcinoma(Fan et al., 2017).

Early detection of CRC is one of the most important challenges in cancer management (Bao et al., 2017). Due to the importance of identifying non-coding RNAs as biomarkers in cancer diagnosis, the present study was performed to evaluate the expression level of miR-454-3p in serum exosomal vesicles in patients with colon carcinoma. For this purpose, the correlation between miR-454-3p expression level and clinical and pathological features of the disease such as stage of the disease and metastatic involvement of lymph nodes was assessed.

Material and methods

Subjects and sample collection

In this study, a total of 40 serum samples from patients with Colorectal cancer and 30 serum samples from healthy individuals without any reported disease were evaluated. Patient samples were prepared in collaboration with a specialist physician from Imam Khomeini Cancer Institute and Shariati Hospital. From all participants, written informed consent was taken. Clinical parameters of patients such as stage and grade of cancer and lymph node metastasis were evaluated (Table 1).

Table 1. Clinical and pathological features of patients with colon cancer

Number	Characteristics
40	Total
	Gender
29	Male
11	Female
	Age
64/4	Average
45-78	Age range
	Tumor location
18	Right side
22	Left side
	TNM staging
26	I+II
14	III
	TNM indexPrimary tumor (T stage)
11	T1 ≥ :2 cm
18	T2< :2 cm ≥5
11	T3< :5 cm
	Local lymph nodes (stage N)
3	NX
14	N0
12	N1
7	N3
4	N4
	Distant Metastasis (stage M)
9	MX
15	M0
15	M1

TNM,tumor-node metastasis.

Solation of exosomes

First, serum was collected from blood samples. Precipitation with Exoquick kit solution was used to separate the exosome from the serum of CRC patients and healthy individuals. To this purpose, the following was done:

Serum centrifuge at 300 × g for 15 minutes, removed the serum supernatant after centrifugation and the kit solution was added in a ratio of 5 (serum) to 1 (kit solution) and mixed well, incubated at 4°C. Centrifuged at 15000 × g for 30 minutes at 4°C. The supernatant was removed and the pellet containing the exosome was dissolved in PBS. Stored at -20°C (for more than 6 months at -80°C).

Examination of isolated exosomes using scanning electron microscopy (SEM)

For microscopic observation, a small volume of the purified exosome was fixed with 2.5% glutaraldehyde and washed with PBS. Then, the sample was dewatered by using ethanol and covered with a thin layer of gold on a dry glass surface. The size and morphology of the exosomes were evaluated by scanning electron microscopy (Digital SEM, KYKY-EM3200, China).

Total RNA extraction

The Total RNA containing small RNAs was extracted from the exosome by using TRIzol (Invitrogen) according to the manufacturer's instructions. The quality and quantity of extracted RNA were determined by using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Polyadenylation and reverse transcription reaction; single-stranded cDNA synthesis from the miRNA template

To perform the polyadenylation reaction, the reagents listed in (Table 2) were added to a nuclease-free microcentrifuge tube. The reaction mixture was then spun and incubated at 37°C for 10 minutes. cDNA synthesis was performed using the 1st-strand miRNA cDNA synthesis kit (Parsgenome) according to the manufacturer's instructions.

Table 2. Reagents required for polyadenylation reaction

Volume	Reagent
1-2.5 µL	total RNA
1 µL	ATP) mM10(
4 µL	polymerase A buffer
Up to a final volume of 20 µL	RNase-free water

Real-time quantitative RT-PCR

Real-time qPCR was performed using miScript SYBR Green PCR kit (QIAGEN) in ABI 7500 Real-time PCR system (Applied Biosystems; Foster City, CA, USA). The miRNA-specific primer sequences for qRT-PCR were designed based on the miRNA sequences obtained from the miRBase database (http://microrna.sanger.ac.uk/). Reverse universal primer and direct miRNA-specific primer are provided by Exiqon Kit Each. The sample was run in triplicates for analysis under the following conditions: an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 5 sec, and annealing at 62 -65°C for 20 sec and extension at 72°C for 30 sec at a ramp rate of 1.6°C/s. The expression levels of miRNAs were normalized to U6snRNA as a housekeeping gene. Data were analyzed by using the comparative Ct method ($2^{-\triangle\triangle Ct}$).

Statistical analysis

Data were presented as mean ± standard deviation (SD) from two or three independent experiments and a t-test was used for statistical analysis of data changes. $P < 0.05$ were considered statistically significant. As a diagnostic test, Receiver Operating Characteristic (ROC) curve analysis was applied to determine the sensitivity and specificity of miR-454-3pexpression in the serum of CRC patients.

Results

Identification of isolated exosomes by SEM

Exosomes isolated from the serum of patients with colorectal cancer were completely spherical with a range size between 30 and 100 nm (Figure 1).

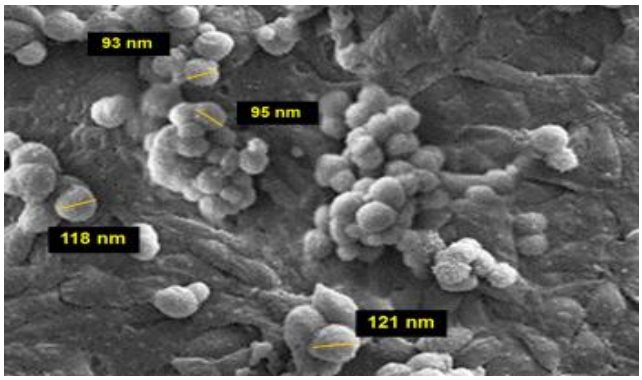

Figure 1. Examination of isolated exosomes by SEM: Exosomes isolated from the serum of CRC patients are spherical are spherical.(scale bar= 30-100 nm).

Real-time PCR results

Exosomal miR-454-3p expression level in serum samples

After checking the specific amplification (Figure 2), the exosomal miR-454-3p expression level was determined in serum samples from CRC patients and healthy control subjects. As shown in (Figure 3), the expression of miR-454-3pin samples from CRC patients was significantly higher than in normal subjects (p-value < 0.01). This result is consistent with the oncogenic function of miR-454-3p**.**

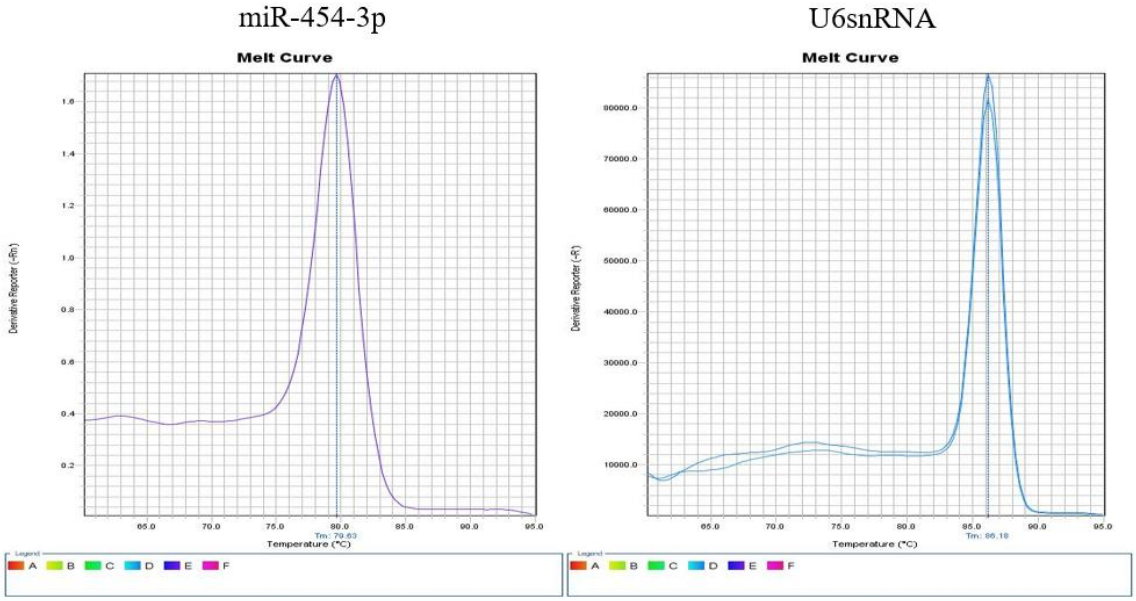

Figure 2. The miR-454-3p and U6snRNA amplicons shows a single peak, representing a pure, single amplicon.

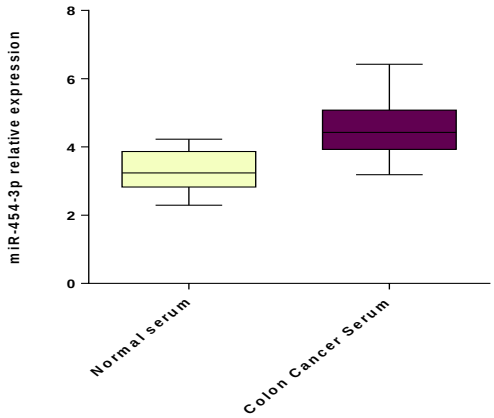

Figure 3. Evaluation of exosomal miR-454-3p expression level in the serum of CRC patients compared to healthy individuals.

Evaluation of exosomal miR-454-3p expression in CRC patients at different TNM stages

The expression level of exosomal miR-454-3pin patients with stage III CRC was significantly higher than those with stages I and II (P < 0.05) (Figure 4). This difference may indicate the importance of the prognosis of miR-454-3p in colorectal cancer.

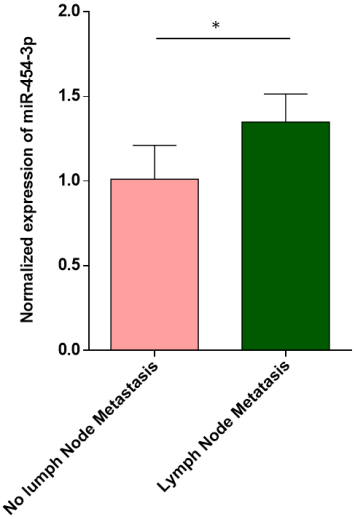

Figure4. Exosomal miR-454-3p pexpression level in serum based on patients' clinical TNM stages. Up-regulation of exosomal miR-454-3pis evident in the serum of patients with Stage III compared with Stage I and II.

Evaluation of exosomal miR-454-3p expression in patients with lymph node metastasis

The expression level of miR-454-3p in the CRC patients with lymph node metastasis showed increased expression compared to CRC patients with no lymph node metastasis (p-value < 0.05) (Figure 5).

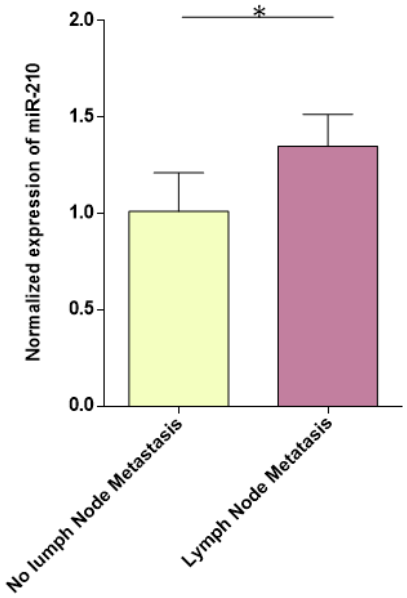

Figure 5. Evaluation of exosomal miR-454-3p expression in CRC patients with lymph node metastasis and non-metastasis. Upregulation exosomal miR-454-3p is evident in patients with colon carcinoma with lymph node metastasis than in patients without lymph node metastasis.

ROC curve analysis to evaluate the value of miR-454-3p secretory microRNA biomarker for the diagnosis of colon carcinoma

ROC curves were developed to evaluate the diagnostic potential of exosomal miR-454-3p as a non-invasive biomarker candidate for CRC diagnosis (Figure 6). ROC curve analysis indicated that the exosomal miR-454-3p expression level could be considered a promising marker for the diagnosis of CRC patients with a sensitivity and specificity of 79 and 99%, respectively (an area under the ROC curve, AUC: 0.89).

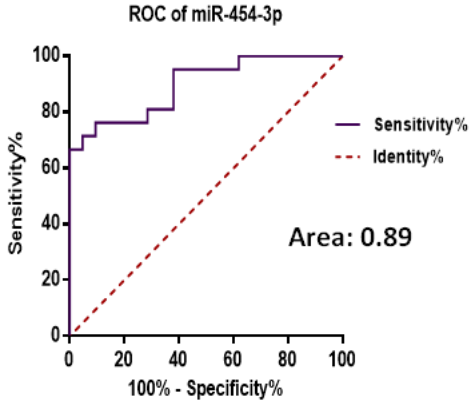

Figure 6. ROC curve constructed based on the expression levels of miR-454-3p in CRC patients and healthy subjects. Analysis results demonstrated that of miR-454-3p could discriminate between CRC patients and healthy individuals, with the AUC of 0.89, combing with the sensitivity of 79% and the specificity of 99%.

Discussion

Colorectal cancer (CRC) is the third most common cancer and one of the leading causes of death in the world. In recent years, there has been significant progress in the diagnosis and treatment of colorectal cancer. Diagnostic methods used to treat this cancer include chemotherapy, surgery, radiotherapy, and immunotherapy. These methods, despite their relative effectiveness, also have disadvantages and are not entirely successful (Desai et al., 2019). The use of miRNAs has attracted the most attention due to their frequent expression disorders in cancer (Friedman et al., 2009). Studies have shown that microRNAs play a significant role in various processes, including tumor growth, progression, and metastasis, by targeting certain oncogene or tumor suppressor genes. CRC is treatable in the early stages, but many patients do not show any clinical signs at this stage. Circulating microRNAs (extracellular microRNAs found in body fluids) can be a potential biomarker. Early diagnosis of CRC plays an important role in preventing this cancer and patient survival. The discovery that microRNA levels in body fluids such as plasma, serum, and exosomes are stable has provided a unique opportunity to develop new strategies based on these molecules as biomarkers for early detection (Mohammadi and Safaralizadeh, 2019), as changes in the expression of microRNAs can play an important role in the development of CRC. In other words, changes in the expression of microRNAs can be a useful biomarker in screening, diagnosis, and control of response to treatment in CRC (Ye & Cao, 2014). According to the results of previous studies (Griffiths-Joneset al., 2008; Ogata-Kawata et al., 2014) and the role of circulating microRNAs in the diagnosis of CRC, the present study was performed to evaluate the expression level of miR-454-3p in the serum exosomal vesicles of CRC patients. Cancer metastasis is a major cause of cancer mortality in patients. Recent studies have focused on the role of exosomes in metastasis. Therefore, new opportunities have been provided for diagnostic, therapeutic, and disease prediction approaches (Zhao et al., 2016). By extracting miRNAs from exosomes produced by cancer cells, their presence in tumor tissue can be estimated (Alvarez et al., 2012; Melo et al., 2014). In the present study, to evaluate the potential biomarker of miR-454-3p in colorectal cancer, its expression levels in serum extracellular vesicle samples of patients and healthy individuals were investigated. The expression of this miRNA was significantly higher in CRC patients compared to healthy individuals (p-value < 0.01). Also, the increased expression of secretory miR-454-3p in the group of patients in stages III was observed compared to the group of patients in stages I and II (p-value < 0.05). Also, the increase in miR-454-3p expression was significantly associated with lymph node metastasis (p-value < 0.05). Also, the increase in miR-454-3p expression was significantly associated with lymph node metastasis (p-value < 0.05), and the area below the ROC diagram was calculated to be 0.89, suggesting that this miRNA can be a potential biomarker for CRC. This result is consistent with the oncogenic function of miR-454-3p, which is also shown in the study conducted by Yan et al., (Yan et al., 2017) and Li et al (Li et al., 2018). Exosome-derived miR-454-3P acts as an anchor miRNA by activating the NOTCH pathway regulated by MIER1. Clinically, high miR-454-3P expression was associated with poor survival in CRC patients, especially if MIER1 expression was low (Li et al., 2019). Overall, current data suggest miR-454-3P as a biomarker in the diagnosis of CRC.

Conclusion

According to the results of the present study, it seems that the increasedexpression of microRNA-454-3p gene has a role in the pathogenesis of CRC and also the expression of this gene is associated with increasing the stage of cancer.

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