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Assessment of microRNA expression as a regulator in the serum of patients with colorectal cancer

Abstract

The present research investigates the treatment and colorectal cancer prevention by altering the growth factors present in the patient's RNA with colorectal cancer. We first prepare serum from blood samples taken from patients, then we remove the serum supernatant and incubate it with the kit solution; after centrifugation, the pellet containing the exosome is dissolved in PBS. To photograph the exosome by electron microscopy, we prepare the slide. For preparing the slide, we randomly removed it from a healthy sample and transferred it to a vial. We inserted the prepared vials into a sonicator, after drying the samples covered them with a thin layer of gold and used electron microscopy for the size and morphology of the exosomes. After isolating the RNAs, we recorded their information on a computer and performed polyadenylation and single-stranded cDNA synthesis. We proliferated the target DNA by real-time PCR. Finally, we used Excel software and a T-test for statistical analysis of the data. The results showed that the normalized expression ratio of miRNA140 in the serum exosome of colon carcinoma was significantly lower than the normal serum exosome ($p < 0.001$). The result is consistent with the tumor-suppressor function of this miRNA, which regulates the growth factor pathway of the beta-modifier. Therefore, it seems that the amount of microRNA 140 expression and its accumulation in the exosome is associated with the stage progression of the disease as well as the metastasis of cancer cells to the lymph nodes.

Keywords: *Cancer, Gene mutation, colorectal carcinoma, miRNA*

Nazanin Saber
Graduated with a master's degree in the field of cellular and molecular genetics from Islamic Azad University, Tehran branch The country of Iran, the city of Tehran
nazanin1993s@gmail.com

Introduction

Colorectal cancer (CRC) is the third most common cancer and one of the leading causes of death in the world. According to the World Health Organization in 2019, about 145,600 people in the United States are diagnosed with this cancer annually, of which about 51,020 have died (1, 2). The prevalence of colon cancer in Iran is 7-8 people per 100,000 men and women, which is very close to the statistics reported in other Middle Eastern countries but is lower than in western countries (3). In recent years, significant advances have occurred in the diagnosis and treatment of colorectal cancer. Methods used to treat this cancer include chemotherapy, surgery, radiotherapy, and immunotherapy. Despite their relative effectiveness, these methods also have disadvantages and are not completely successful. These methods destroy only the tumor bulk and do not affect cancer stem cells (4). In the meantime, miRNAs have attracted the most attention due to their frequent disorders in their expression in cancer (5). Studies show that microRNAs play a significant role in a variety of processes including growth, progression, and metastasis by targeting certain oncogenic genes or suppressor tumors. Colorectal cancer is treatable in the early stages, but many patients do not show any phenotypic symptoms at this stage. Therefore, circulating microRNAs (extracellular microRNAs found in body fluids) as potential biomarkers are very important.

Early diagnosis of colorectal cancer is key to preventing this cancer and affecting patients' longevity. The discovery that microRNA levels in body fluids such as plasma, serum, and exosomes are stable provides a unique opportunity to develop new strategies under these molecules as biomarkers for early detection (6).

MiRNAs and their role in various biological processes have been supposedly associated with CRC cancer. MiRNAs act as oncogenes or suppress tumors by setting different targets. For example, miR-18a, miR-155, and miR-205-5p suppress proliferation, migration, and invasion of CRC cells, while miR-494, miR-598, and miR-17-3p promote the capacities of Cell proliferation, migration, and invasion. MiR-106a and miR-7 concern CRC cell apoptosis or resistance to apoptosis. MiR-221 and miR-214 reduce autophagy in CRC cells. MiR-192/215 and miR-19b-1 play a regulatory role in metabolic pathways. Other miRNAs that regulate different targets are also below (7).

Increasing and decreasing the expression of microRNAs can play an important role in the development of colorectal cancer. In other words, changing the expression of microRNAs can be a useful biomarker in screening, diagnosis, and control of the response to treatment in colorectal cancer (8). In a study (2019), Li et al. showed that miR-454-3p expression has significantly increased in colon cancer tissues compared to normal colon tissues (9). A meta-analysis (2018) identified miR-200 as a potential diagnostic marker of colorectal cancer. This study showed that serum miR-141 in the miR-200 family has overexpression in colorectal cancer patients and has good sensitivity for the diagnosis of colorectal cancer (10). Isolating the serum of colorectal patients, Matsumura et al. (2015) found that the expression of miR-17-92 cluster in serum was associated with recurrence of the disease, and patients with high serum miR-17-92 expression had a poor prognosis (11).

In 2017, it was reported that a disorder in miR-378, miR-375, miR-422a, miR-215, and miR-135 Border in CRC patients played an important role in CRC pathogenesis. The miR-422A molecule is an independent predictor in patients with colorectal cancer (12). In 2015, Qin et al. examined the association of miRNAs with hTERT (Human Telomerase reverse transcription) in colorectal cancer through screening. They found that miRNA422a among different miRNAs affects human telomerase reverse transcriptase activity (13).

The researchers found in 2018 that miRNA increased and miRNA decreased in purified exosomes of SW620 cell cultures. High expression levels of circulating exosomes miR-17-5p and miR-92a-3p are significantly associated with the pathology and grade of colorectal cancer patients' CRC (14). Bahnassy et al reported that, in patients with CRC in stage 2 cancer, expression of miRNAs miR-21, Let-7a-5p, miR-100-5p, miR-200c-3p, and miR-23b-3p were significantly usable as common diagnostic markers for CRC. (15)

Zhang et al. (2018) found that miR-150 targeted the expression of concentration Cyclin A to stop colorectal cell cancer and inhibit proliferation by inhibiting HMGA2 (16). Other results have shown that miR-150-5p may act as a tumor inhibitor in CRC (17). A study (2018) on patients with lung adenocarcinoma showed that simultaneously decreased exosomal expression of Exosomal let-7a-5p and high expression of BCL2L1 are biomarkers (18).

Nowadays, colorectal carcinoma is treated based on the stage of the tumor, the location of the tumor, and the rate of tumor proliferation, but studies show that the genetics of the disease is important in predicting response to treatment and not all patients respond equally to treatment. With the rapid development of genetics, biology, pharmacology, and pathology, we can manage treatment to the best response. Therefore, recognizing genetic markers in cancers is important. Suppressor tumor genes, oncogenes, and genes involved in angiogenesis, apoptosis, and cell proliferation can be therapeutic targets. Drugs such as fluorouracil, irinotecan, and oxaliplatin are some selected drugs for the treatment of clone cancer. Molecular studies have shown different toxicity of these drugs in genetic modifications (19). Accordingly, the present research evaluated the expression of microRNA as a regulator of the pathway of beta-modifying growth factors in the serum of patients with colorectal cancer.

Materials and methods

1. Separation of serum from blood samples

The maximum allowable time for serum or plasma separation is 2 hours after sampling. An important point is to allow serum samples to be separated from completely clotted blood before centrifugation. We have to wait about 20 minutes for the blood to clot completely, or put it at 37 degrees for 10 minutes to form a clot.

2. Separation of exosome from serum through deposition

First, we removed a certain amount of serum and centrifuged it at 300 xg for 10 minutes. We take the solution from the surface of the serum and add the kit solution (5 (serum) to 1 (kit solution)). We incubate it at 4 ° C for 20 minutes. We centrifuge it (1500 g (rpm) for 15 minutes at 4 ° C). Then we separate and discard the supernatant and the pellet containing the exosome (the exosome pellet may not be visible but dissolves in PBS). Next, we transfer the solution to a vial (optional step: placing it on the heater for 5 minutes).

3. Scanning electron microscopy (SEM)

To prepare the slide for exosome imaging by electron microscopy, we first randomly isolate 10 µl of a healthy sample and transfer it to a new vial, then add 20 µl of PBS solution to each vial. After these steps, we placed the samples in a blue sonicator (we use this device to separate the exosomes through the vibrations in it). After removing the vial from the sonicator, we take 10 µl of the sample and spread it on a slide (smear), then dry it on the slide glass surface, cover it with a thin layer of gold and photograph it with a scanning electron microscopy (SEM).

4. Total RNA extraction

In this study, we used Trizol (Invitrogen) reagent to extract RNA. In this method, Trizol solution forms RNA complexes with guanidinium and water molecules but prevents the hydrophilic binding of RNA to DNA and proteins. DNA and proteins are separated from the separated aqueous phase, while RNA molecules remain in this part.

5. Quantitative evaluation of the extracted RNA

In the next step, after RNA extraction, we examined its quality and quantity by UV spectrophotometric methods and in some cases, using a Nanodrop device. It is noteworthy that we obtain the purity of the RNA sample by spectrophotometric method and through the light absorption at 260 and 280 nm. As for this step, we dissolve 5 µl of RNA in 495 µl of distilled water and determined its adsorption and concentration at 260 and 280 nm.

6. Polyadenylation and reverse transcription reaction; Single-stranded cDNA synthesis from the miRNA pattern

Initially, adding some material to a nuclease-free microcentrifuge tube triggered the polyadenylation reaction.Then, we mixed all the mentioned materials gently with the help of a pipette (without vortex). We then incubated the mixture at 37 ° C for 10 minutes.

To synthesize single-stranded cDNA from the miRNA pattern at the beginning, we prepared the following reaction mixture in a separate microtubewe incubated the mixture for 5 minutes at 65 ° C, added again the following mixture to the above reaction, and mixed.

Then, we immediately incubated the reaction mixture for 10 minutes at 42 ° C. We immediately incubated the mixture at 25 ° C for 5 minutes. It is noteworthy that to inactivate the RT enzyme, we incubated the reaction mixture for 5 minutes at 70 ° C. We should note that the product obtained from this reaction is storable at -20 ° C or -70 ° C for a longer period.

7. Real-time PCR reaction; reaction mixture and temperature and time conditions

In this stage, we provided the reverse general primer and miRNA-specific forward primer by Kit (Exiqon). To begin this stage, we prepared firstly the following reaction mixture to a final volume of 18 μl by adding materials per sample:

- 5 microliters SYBR Green I Master Mix
- 2 microliters of primers
- cDNA (2 microliters)
- Water up to a volume of 9 microliters

Then, we poured the reaction mixture (18 μl) into each of the 48-well plates or 8-tube strips. We covered the surface of the plate with a transparent cover or, in the case of using strip, covered their door with a special lid. Next, we placed a plate or strip in the device and executed the temperature and time program .

We should note that using a dissociation step consisting of 95 ° C for 15 s, 60 ° C for 30 s, and 90 ° C for 15 s, each complete proliferation step continued for analyzing the melt curve.

8. Data analysis through the comparative threshold cycle method

We performed the data analysis of this study through the comparative threshold cycle method according to the table 5. We compared the normalization of changes in miRNA expression levels with endogenous U6 small nuclear RNA expression levels.

Data analysis was done through the comparative threshold cycle method, using the following equations.

[(Control) mCT reference gene - (control) mCT gene] = ΔCT: control (1)

[(Test) mCT reference gene - (Test) mCT gene] = ΔCT: Test (2)

(Test) ΔCT- (control) ΔCT = $\Delta\Delta$CT (3)

Then, the following equation determined the ratio of expression changes between the two test and control samples.

(Expression) Ratio = $2^{-\Delta\Delta CT}$

We performed the data analysis through ABI step one Real-Time PCR Software v2.0.2 (Applied Biosystems, UK). Statistical analysis included the calculation of mean, standard deviation, and correlation coefficient (R^2) through Excel software (Microsoft, 2010) and SPSS software (Version.19). We normalized also the changes in mRNA expression levels compared to the expression levels of the reference gene.

Findings

1. Bioinformatics of miR-140

In this research, we examined data and sources in the field of microRNAs in the metastasis process. We also selected the miR-140 microRNA for further investigation in this study. We should note that the miR-140 microRNA is on chromosome 16 of the long arm. Its ring stem sequence as well as its mature miRNA sequence is 21 nucleotides(Figure 1)

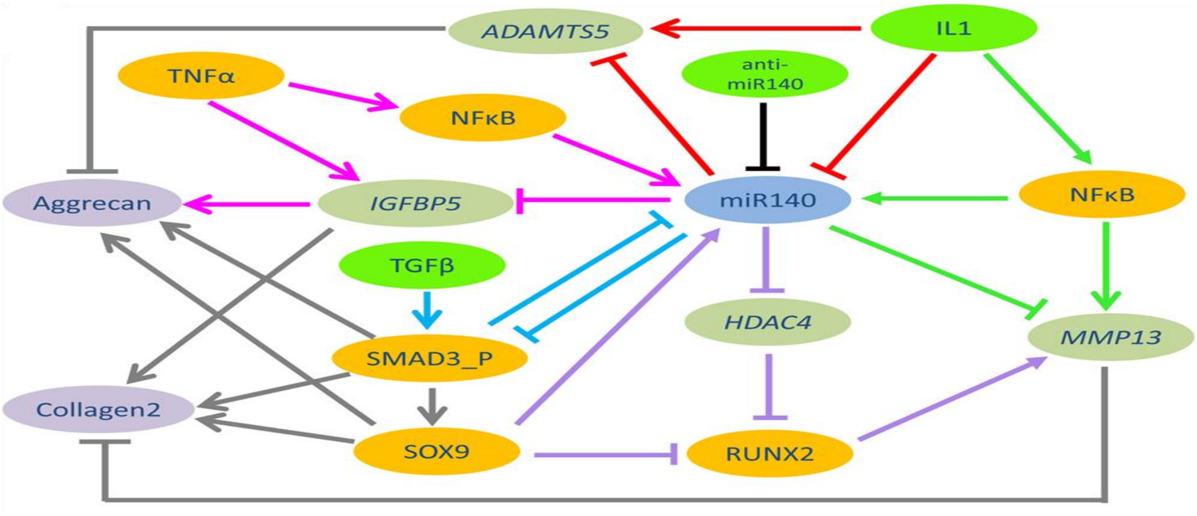

Figure 1: Regulatory correlation of miR-140 with signaling pathways involved in the pathogenesis of colon cancer, especially SMAD3 gene from TGF-β pathway components

2. Examination of exosome vesicles isolated by scanning electron microscopy

Figure (2) shows an example of exosomes isolated from the serum of a person with colon carcinoma. We assessed the size and shape of the isolated exosome vesicles through Scanning Electron Microscopy (SEM). In the continuation of the study, the results showed that the isolated exosome microvesicles have a spherical appearance with a size range of 30 to 150 nm.

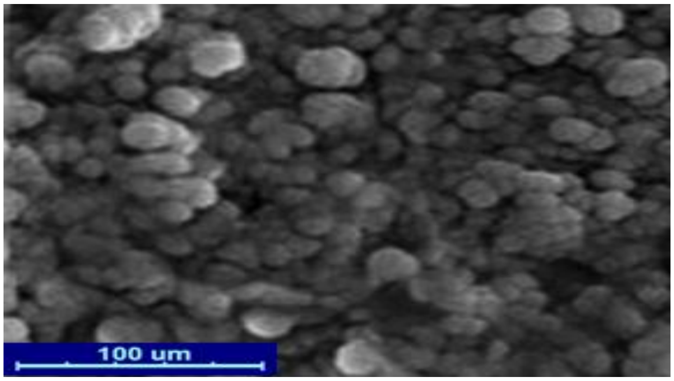

Figure 2: Examination of exosome vesicles isolated by electron microscopy

3. Quantitative analysis of total RNA extracted from exosome microvesicles

As we can see in Table (1), the extracted total RNA concentration and its light absorption ratio are in the desired range of 260 nm to 280 nm (A260 / A280).

Table 1: Concentration results and light absorption ratios of 260/280 for several RNA samples

Sample	Concentration (µg/ mL)	OD 260/280
1	250	1.83
2	210	1.98
3	245	2.00
4	256	1.84
5	283	1.94

4. Determining the specificity of real-time PCR reaction through the melting curve

In the study of melting curves due to temperature changes that occur after the PCR process, each existing DNA fragment, based on the length and content of GC bases at its specific melt temperature, is single-stranded and the system displays this state change as a peak. Peaks at lower temperatures indicate smaller fragments. Examination of the generated peaks shows that these peaks at low temperatures are directly associated with the amount of non-specific products generated at the end of the PCR process. We used melt curve analysis to confirm the results. As shown in Figure (3), each gene understudy has a unique melt temperature, indicating that the product of real-time PCR proliferation was unique.

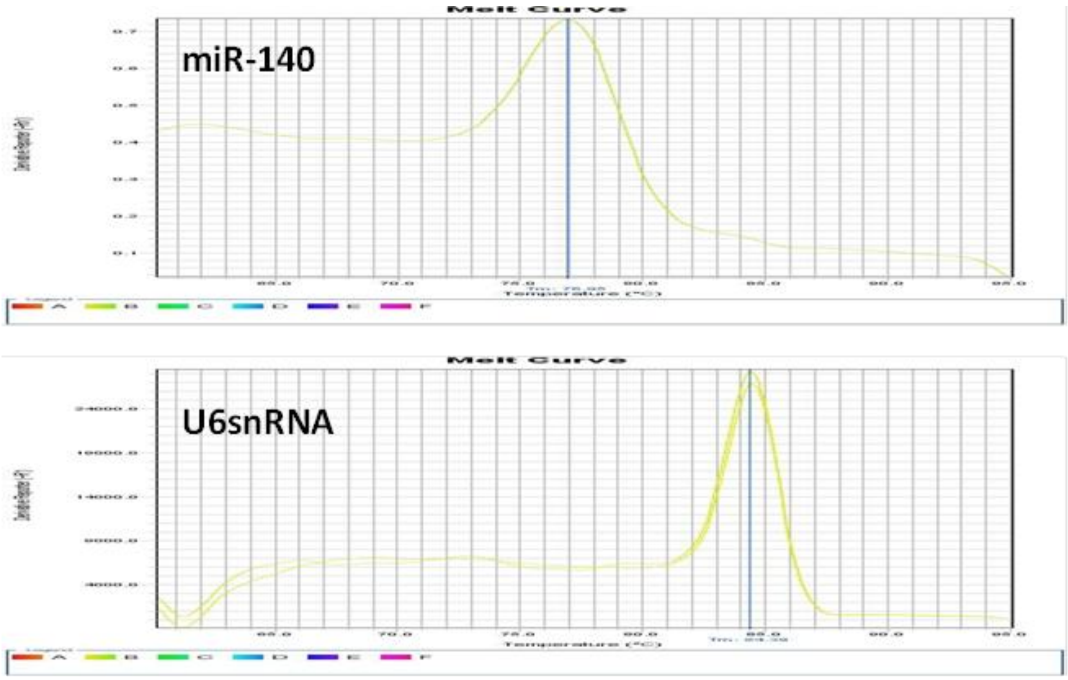

Figure 3: Post-proliferation melting curves for miR-140 and U6snRNA. Being a single peak is a sign of specific proliferation.

5. Determining the accuracy of Real-time PCR reaction specificity through agarose gel electrophoresis

To confirm the results of the melt curve, the proliferation products were also electrophoresed on agarose gel after PCR. As we can see, the single band determines the specificity of the proliferation. The size of the bands is also between 100 and 200 bp, which is expectable due to the polyadenylation performed on the microRNA that increased its length (Figure 4).

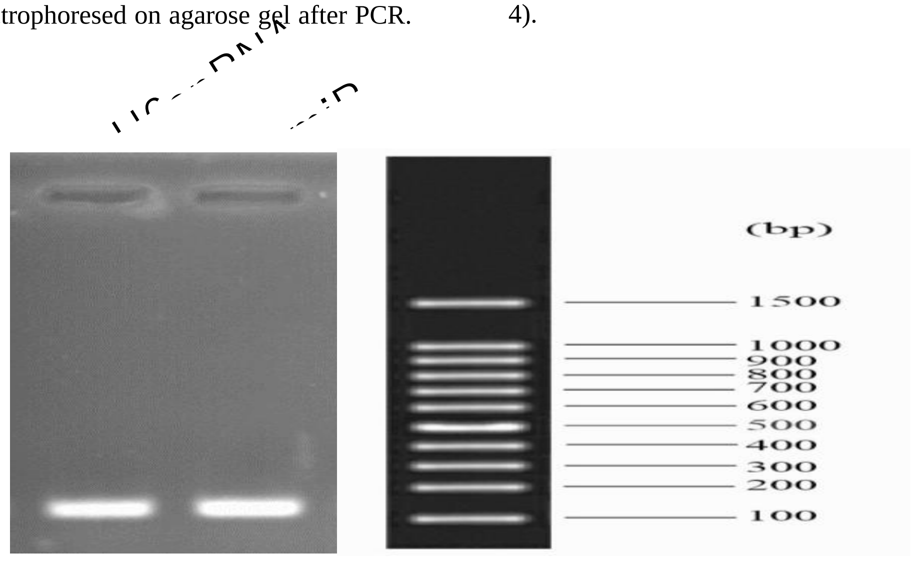

Figure 4: Post-proliferation gel electrophoresis for miR-140 and U6snRNA. Being single-band in the expected size range is a sign of specific proliferation.

6. Evaluation of miR-140 expression levels in serum exosome vesicles of colon carcinoma patients compared to serum exosomes of healthy individuals

As shown in Figure (5), based on the results of this study, the normalized expression ratio of miR-140 in the serum exosome of colon carcinoma was significantly lower than the normal serum exosome (*** P <0. 001). We can say that this result is consistent with the tumor suppressor function of miRNA, which regulates the growth factor of the beta-modifying factor. The image on the right shows a selected view of the miR-140 proliferation curve in the Real-time PCR reaction.

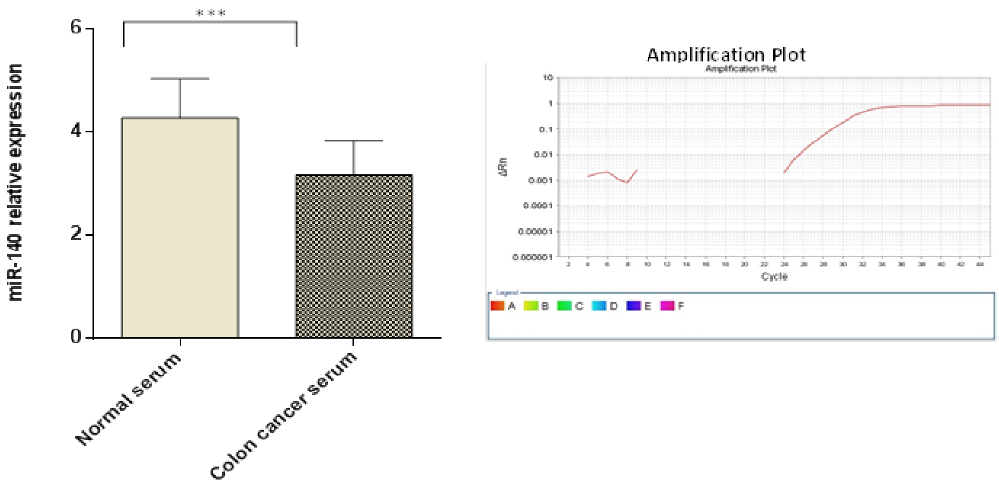

Figure 5: Changes in the expression level of miR-140 in the serum exosome of colon carcinoma compared to the normal serum exosome. Left Figure shows miR-140 expression changes in both healthy and unhealthy serum groups.

7. Evaluation of miR-140 secretory microRNA expression in patients with colon carcinoma at different stages of the disease

In this research, we considered the miR-140 expression among different groups of patients grouped according to the stage of the disease to investigate the relationship between miR-454-3p secretory microRNA expression levels and pathological clinical parameters of the disease. The results showed that the expression level of miR-140 secretory microRNA in stage III of the disease was of less expression than the group of patients in stages I and II (** $P < 0.01$).

8. Evaluation of miR-140 secretory microRNA expression in patients with lymph node metastasis

To determine the relationship between miR-140 expression levels and other clinical-pathological parameters of the disease such as lymph node metastasis (LNM), we studied the expression of miR-140 among patients in different groups grouped based on lymph node involvement. The results showed that the expression level of miR-140 was lower in the group of patients with lymph node involvement than in the group of patients without lymph node metastasis (* $P < 0.05$).

9. Evaluation of secretory miR-140 as a potential biomarker through ROC curve analysis for the diagnosis of colon carcinoma

The results of our study showed that the expression of miR-140 in the serum exosome decreased compared to the healthy sample. As for this result, we calculated the area under the curve (AUC) for miR-140 to be equal to 0.74. It showed that the miRNA can be a biomarker. (Figure 6)

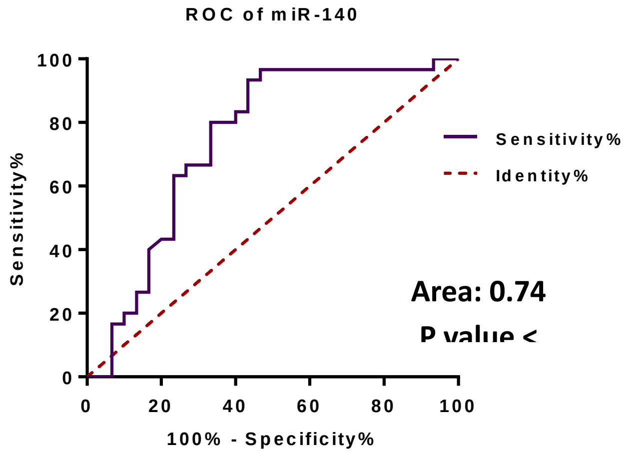

Figure 6: ROC analysis to measure the secretory miR-140 as a potential biomarker

Discussion

This study evaluated the expression of miR-140, a regulator of the beta-modifying growth factor pathway, in the serum exosome vesicles of patients with colon carcinoma. We evaluated the expression levels of miR-140 in the serum exosome of colon carcinoma patients compared with normal serum exosome to assess the possible role of circulating miR-140 in the pathogenesis of the disease and the potential of its diagnostic role. The results showed that the normalized expression ratio of miR-140 in the serum exosome of colon carcinoma was significantly lower than the normal serum exosome (*** $P < 0.001$). This result is consistent with the tumor-suppressor function of this miRNA, which regulates the pathway of beta-modifying growth factor. In 2009, Song et al. conducted a study on the role of miR-140 in colorectal cancer on the HCT 116 cell line. They observed that overexpression of miR-140 in colorectal cancer cells HCT 116 (wt-p53) inhibited cell proliferation. They saw that miR-140 caused the expression of p53 and p21 and stopped the G1 and G2 phases. They also showed that histone deacetylase 4 (HDAC4) is one of the important targets of miR-140 (20). Zhai et al. conducted a study similar to ours. In this study, they showed that hsa-miR-140-5p directly targets Smad2. Overexpression of hsa-miR-140-5p in CRC cell lines reduced Smad2 expression level, leading to a reduction in cell invasion and proliferation and an increase in cell cycle arrest. Ectopic expression of hsa-miR-140-5p in colonic CSCs disturbed autophagy and inhibited CSC growth in vitro (21).

The results showed that the expression level of microRNA-140-5p secretory microRNA in Stage III showed a significant decrease compared to the group of patients in Stages I and II (* $P < 0.05$). This difference may indicate the importance of the prognosis of miR-210 in colorectal cancer. Li et al. conducted a similar study in 2018. They observed that miR-140 regulates Smad3 (a member of the SMAD family), a key factor subordinate to the TGF-β signaling pathway, at the translational level in CRC cell lines (22). The ectopic expression of miR-140 inhibits the epithelial-mesenchymal transmission (EMT) process, at least by targeting Smad3 and suppresses the ability of CRC cells to migrate and invade in vitro. MiR-140 also reduces CRC cell proliferation through Samd3 down-regulation. Moreover, overexpression of miR-140 inhibits tumor formation and CRC metastasis in vivo, and knocking out Smad3 has a similar effect. MiR-140 expression decreases also in clinical CRC prototypes and appears to be a gradual decrease in metastatic specimens, while Smad3 increases its expression in CRC specimens.

In the present research, the expression level of miR-140 in the group of patients with lymph node involvement showed less expression than in the group of patients without lymph node metastasis (* $P < 0.05$). This difference may be a reason for the importance of the prognosis of miR-140 in colon cancer and is consistent with previous reports of various types of cancer (23). Zhang et al. (2015) came to a similar conclusion. Thus, the expression of miR-140-5p in CRC tissues and cell lines decreased significantly. Decreased miR-140-5p expression was significantly associated with advanced CRC and decreased patient survival. Studies on function enhancement and loss have shown that miR-140-5p acts as a tumor suppressor by inhibiting cell proliferation, migration, and invasion (24).

Considering the significant difference observed in the expression of miR-140-5p secretory microRNA in both patient and healthy groups and due to a significant relationship between the miR-140-5p secretory microRNA expression and some pathological clinical features like disease stage and lymph node metastasis, we evaluated the capability of this microRNA as biomarker through the ROC curve. In the present study, the expression level of miR-140-5p in the serum exosome of patients increased compared to the healthy sample. We calculated the area under the curve (AUC) to be 0.74, which indicates the potential of secretory microRNA miR-140-5p to be a biomarker.

In general, aberrant expression of miRNA is associated with human diseases like cancer. Tumors analyzed by determining miRNA profiles show different profiles of miRNAs compared to normal cells of the same tissue. Proliferation and overexpression of miRNAs have an oncogenic role. Moreover, several miRNAs in cancers have been identified as functionally oncogenic or superconducting (25). In a study (2012), Chiba et al. showed that exosomes secreted from human colorectal cancer cell lines contained natural mRNAs, microRNAs, and antisense RNAs that could enter Hepatoma cell lines HepG2 and lung cancer A549. This finding suggested that cancer cell secretions can enter normal cells in the body and possibly alter gene expression and regulation of cellular processes. This factor can partially explain the metastasis process (26).

In general, studying the miR-140-5p microRNA expression levels in the extracellular exosome micro-vesicles of serum of colon cancer patients and determining the relationship between its expression and clinical features of the disease is an innovative aspect of our research.

Conclusion

The extraction of circulating exosomes may eliminate the need for more invasive biopsies in a variety of cancers, especially colorectal cancer. MiRNAs like microRNA-140-5p also can diagnose and evaluate good prognosis in colorectal cancer patients and are usable as diagnostic and prognostic biomarkers.

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There is no conflict of interest in this research.Ethics Statement

The main goal of any research should be to improve people's health along with respecting their dignity and rights. In this research, all the steps have been done with the personal consent of the people, and it should be noted that this article has not been published in any magazine before and at the same time it is in the refereeing process. There is no other journal. There is no plagiarism or citation distortion in this article, and the subject researched in this article is not repeated in any way.The results of this study have been published honestly, accurately and completely. The results, whether negative or positive, as well as funding sources, organizational affiliation, and conflict of interest, if any, will be fully disclosed. The research method will not contradict the social, cultural and religious values of the society. Finally, all ethical principles have been tried to be considered in this article.

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