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Original Article

miR-193a-5p Inhibits Migration and Invasion of Colorectal Cancer Cells Via Targeting Vimentin

Saeed Noorolyai[1,2], Behzad Baradaran[1]*

[1]*Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*
[2]*Department of Biochemistry, Higher Education Institute of Rab-Rashid, Tabriz, Iran*

Corresponding Author: Behzad Baradaran, E-mail: baradaranb@tbzmed.ac.ir

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ABSTRACT

Background and Purpose: Colorectal cancer is the third leading cause of cancer death worldwide. MicroRNAs are a group of post-transcription regulators whose expression are altered in various cancers and affect the expression of different genes and contribute to cancer progression. According to studies, the expression of miR-193a-5p in some cancers, including colorectal cancer, is reduced. But so far, the main effects and mechanisms of miR-193a-5p in colorectal cancer are ambiguous and unknown. The purpose of this study was to investigate the effects of miR-193a-5p mimic transfection on HT-29 cell line and investigate the expression of vimentin molecule on these cells. **Materials and Methods:** First, the HT-29 cell line from colorectal cancer was cultured in RPMI-1640 culture media. Then, miR-193a-5p was transfected with Jet-PEI reagent and using the qRT-PCR technique, the expression of miR-193a-5p and vimentin gene was evaluated in miR-193a-5p transfected HT-29 cells and control group. Statistical analyzes were performed using GraphPad Prism 6 statistical software. **Results:** In this study, the simultaneous effect of miR-193a-5p mimic on HT-29 cells was investigated. Expression levels of miR-193a-5p was increased after transfection, and then, vimentin expression was altered, which was evaluated by using qRT-PCR. **Conclusion:** The results of this study revealed that miR-193a-5p may act as a tumor-suppressor by affecting the molecules such as vimentin. This microRNA has an important role in tumor metastasis. Therefore, it may be suggested as a potential therapeutic target in the treatment of colorectal cancer.

INTRODUCTION

Carcinogenesis is a complex process that occurs step by step, involving the formation and progression of the tumor, the formation of blood vessels around the primary mass and ultimately the progression of cancerous cells. Separation of tumor cells from tumor mass, their migration and invasion to involved tissues through the blood vessels lead to the formation of a secondary tumor at a point far away from the original tissue (1).

One of the most commonly cited and highly prevalent cancers is colorectal cancer. It is among the most common types of cancers resulting in high mortality worldwide (2).

According to the World Health Organization (WHO) statistics in 2016, 1200,000 people suffer from colorectal cancer annually, of which about 600,000 resulted in dead and it is considered the third cause of cancer deaths (3).

The genetic and epigenetic changes of tumorigenic gene and tumor-suppressing genes result in the essential mechanism of carcinogenesis (4, 5).

These alterations lead to the false expression of these genes and affect their downstream signaling pathways, which may occur in various tumorigenic processes, including cell proliferation, cell death, metastasis, angiogenesis, multivariate resistance, genomic stability, and genetic involvement (4, 6).

Today, the role of a new group of different classes of non-coding RNAs, called microRNAs, has been shown to be carcinogenic (7-9).

MicroRNAs are a group of non-coding and regulatory RNAs with an approximate length of 18-22 nucleotides, which play a key role in regulating the expression of the target gene, after transcription, by binding to the 3'UTR regions of the protein coding mRNA. One microRNA alone can regulate multiple target mRNAs (10-12).

The first microRNA was discovered in Caenorhabditis elegans as a nucleotide RNA-22, essential for the development of cells during post-embryonic expression (13), after which researchers have identified miRNAs in many unicellular and multi-cellular organisms, which eventually were about 471 up to today, the microRNA was completely recorded in various organisms (14). Studies suggest that in the human genome, there are about 1000 microRNAs, while only a small number of them have been practically described

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and identified. Every microRNA has the ability to affect several different genes as the target gene that ultimately affects a number of these several thousand mRNAs, so that around 40% of human protein encoding genes is regulated by microRNAs (15).

Regarding the high prevalence and mortality rates of cancer, and also due to the severe complications of treatment in the future, and the relapse of the disease after the stages of treatment, today one of the methods of treatment is the use of microRNAs (16).

The biological functions of microRNAs are heavily dependent on the cell in which they are located and vary according to the various gene transcripts in different tissues and cells. Therefore, considering the target gene transcript, the expression of some microRNAs in one cancer is increased and acted as oncogenes, while the same microRNAs in other types of tumors are reduced and acted as suppressors (17).

Currently, microRNAs are used as therapeutic targets for cancer treatment. Returning microRNAs to the normal level provides a significant potential for therapeutic interventions(17). Generally, microRNA-based therapies can be divided into two categories:

1. Restoring the tumor suppressor microRNA activity using microRNA-expressing gene vectors with double-strand microRNA mimic, called a microRNA replacement therapy.
2. Inhibition of the oncogenic microRNA using single-strand oligonucleotides that have chemical changes with an inhibitory effect (18).

MicroRNAs play an important role in the colorectal cancer biology, carcinogenesis, tumor progression, invasion, metastasis and angiogenesis (19).

Among the tumor suppressor microRNAs in the clone, miR-193a-5p can be mentioned. In general, studies have shown that the expression of this microRNA in human cancers is reduced, and this expression reduction is associated with advanced disease development (4, 20).

In this study, using microRNA replacement therapy, miR-193a-5p was introduced as a therapeutic target for colorectal cancer cells and by replacing this microRNA, it was expected that the level of expression of this miR-193a-5p would be increased in cells having reduced expression.

The purpose of this project is to investigate the effect of miR-193a-5p on the expression of the vimentin gene involved in the migration of cancer cells.

MATERIALS AND METHODS

Cell Culture

The HT-29 cell line of human colorectal cancer was purchased from the Pasteur Institute of Iran. This cell line was cultured in RPMI 1640 enriched with 10% FBS and 1% antibiotics (100 Unit/ml penicillin, and 100 µg/ml streptomycin) at 37° C, 5% CO2 and 95% humidity in incubator.

miRNA Transfection

To carry out the transfection, cells were firstly seeded onto 6-well plates, then stored for 24 hours in the incubator, and when the cells filled 50 to 70 percents of the wells, the transfection could be conducted.

According to the protocol of jetPRIME (Polyplus Co., Illkirch, France) in vitro microRNA transfection, miR-193a-5p transfection was performed at different doses.

In order to evaluate the amount of transfection, the control miRNA conjugated with fluorochrome, was also transfected to cells. Using the MACS Quant Authorized 10 flow-cytom-Germany, the efficiency of cell transfusion was obtained.

RNA Extraction and cDNA Preparation

Following the transfection of HT-29 cells with miR-193a-5p, the RNA extraction was done by using Trysol kit (Gene All, Korea) according to the manufacturer's instructions. RNA was isolated from chloroform and isopropanol and washed with 75% ethanol. Finally, the concentration of RNAs was measured using a Nano drop and the quality was measured. After determining the concentrations of RNA and performing the required calculations, cDNA synthesis for miR-193a-5p gene was done by the cDNA synthesis kits, miRCURY LNATM Universal CDNA Synthesis (Exiqon/Qiagen, Vedbaek, Denmark). To prepare the cDNA of the vimentin gene, the Korea Bio fact kit was used.

Quantitative Reverse Transcription PCR (qRT-PCR)

The miR193a and vimentin gene expression was evaluated by qRT-PCR technique using a standard SYBR Green PCR master mix (Takara, Korea) protocol on a Roche Light Cycler 96 system (Roche, Germany). The formula for the relative expression value was calculated as $2^{\Delta\Delta CT}$. Sequences of the primers are summarized in Table 1. The internal control primer (U6

Table 1. The sequence of primers

Gene	Sequence of the primer
miR-193a-5p	5′-UCA UCU CGC CCG CAA AGA CCC A-3′
U6 snRNA	Forward: 5′-CTTCGGCAGCACATATACTAAAATTGG-3′
	Reverse: 5′-TCATCCTTGCGCAGGGG-3′
Vimentin	Forward: 5´-AATCGTGTGGGATGCTACCT-3´
	Reverse: 5´-CAGGCAAAGCAGGAGTCCA-3´
GAPDH	Forward: 5´-CCTCGTCCCGTAGACAAAA-3´
	Reverse: 5´-AATCTCCACTTTGCCACTG-3´

snRNA) and miR-193a-5p was purchased from ORIGENE Company. The primer for the vimentin gene was designed by 5Prime software, using a primer blast design site at the NCBI.

The expression of miR-193a-5p and vimentin in the qRT-PCR process was performed as triplicate, and the relative expression of the gene was measured and normalized to the control group by $2^{-\Delta\Delta CT}$.

Statistical Analysis

The significance of the results of qRT-PCR test was compared by using one-way ANOVA and GraphPad Prism 6 software with 3 replicates. p values of 0.05 or less were considered as significant.

RESULTS

Transfection Efficiency in Transfected Cells

The results of the evaluation of HT-29 cell transfection showed that this transfection was done with the efficiency of 80.2% (Figure 1).

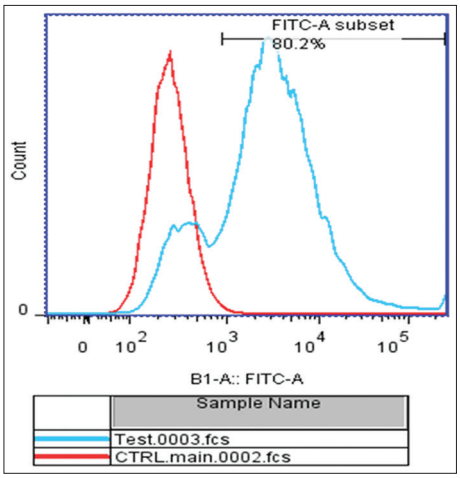

Figure 1. Transfection efficiency in transfected cells. The transfection efficiency of transfected cells was 80.2%

qRT PCR Results

Changes in expression of miR-193a-5p in transfected HT-29 cells

According to Figure 2, the analysis of Ct from the proliferation curves showed that the expression of miR-193a-5p in transfected HT-29 cells was significantly higher than the control group (***P < 0.0001).

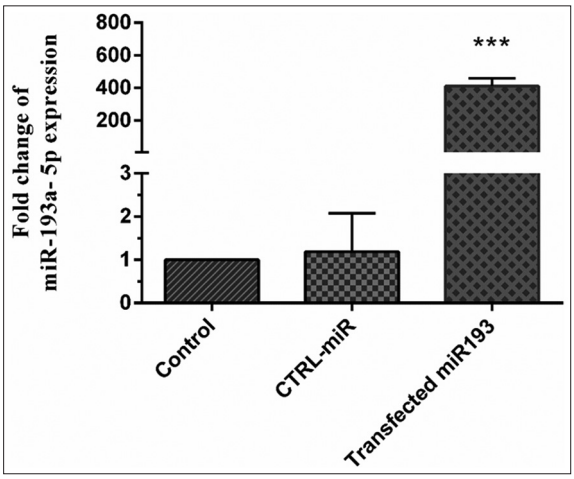

Figure 2. Changes of miR-193a-5p expression in transfected HT-29 cells. The expression of miR-193a-5p in transfected cells was significantly increased compared with control cells. (*** P <0.0001)

Changes in vimentin gene expression in miR-193a-5p transfected cells

The analysis of Cts from the proliferation curves showed that the expression of vimentin in transfected HT-29 cells was significantly reduced in comparison with the control groups (Figure 3).

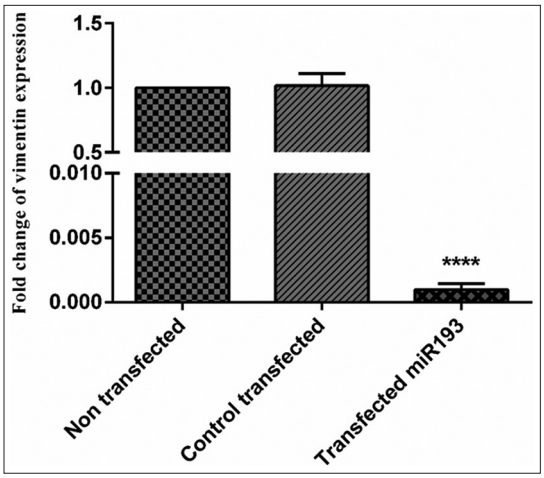

Figure 3. Changes in vimentin gene expression in miR-193a-5p transfected HT-29 cells compared to control cells. Vimentin expression in transfected HT-29 cells was significantly reduced (**** P <0.001)

DISCUSSION

Disturbance in the expression of microRNA has been reported in a variety of cancers (21). The tumor-suppressor microRNA that affects oncogene genes are associated with reduced expression in a variety of cancers (22).

Due to the fact that microRNAs can target several genes and paths alone, recently, the expression of these molecules has become more respected than gene therapy. However, there is no information about the target of microRNAs and the selection of these molecules for manipulation should be conducted with caution (23). miR-193a-5p is one of the few microRNAs that can be expressed in various cancers, including colorectal cancer (24).

Colorectal cancer is one of the prevalent cancers, and miR-193a-5p shows a marked decrease in various cell types of the cancer, including HT-29 (24).

Recovery of tumor suppressor microRNA expressing seems to be a promising approach in cancer treatment. Among the many approaches that have been taken so far, a new approach called microRNA Replacement Therapy has

been considered in recent years. By replacing tumor suppressor microRNA in cancerous cells, microRNA can be restored to normal levels in cancer cells in order to inhibit the oncogenes (25).

The advantages of microRNA Replacement Therapy include the normalization of the tumor-suppressors and their stability in the normal tissues of the body, to control several cancer pathways and a large number of oncogenes. Also, due to the abundance of this molecules in normal cells, the side effects are reduced and on the other hand, the sensitivity of tumor cells is increased. This method of treatment, like other therapies, still faces high costs of synthesis and production, with challenges such as engineered constraints, vector design and carriers, poor cellular absorption, and the proper placement of agents at the target site (26).

The restoration of microRNA expressions was first introduced in 2007 by Yong son lee and colleagues. They showed that the restoration of the expression of Let-7 mimic in lung cancer could result in limited tumor growth in in vitro and in vivo conditions (17). In 2009, Janaiah and colleagues used the miR-26a gene for the first time to investigate the inhibitory effect of miR-26a on the proliferation of liver cancer cells. Most of the mice survived and the results showed that proliferation of tumor cells was reduced (22).

The first drug that was prepared using the microRNA Replacement Therapy technique was MRX34. This drug was introduced by Mirna Pharmaceutical Company in 2012. The Nov340 Liposome vector containing miR-34a was used in this drug (27).

In this study, miR-193a-5p was selected as the candidate for restoring miRNA expression in colorectal cancer cells. miR-193a-5p was transfected into HT-29 cell lines using Jet PEI reagent and the primary transfection was reviewed and approved using Flow-cytometry. The results of qRT-PCR showed a significant increase in the expression of miR-193a after transfection, confirming the accuracy and efficiency of transfection.

After the increase of the miR-193a-5p expression in transfected cells, the amount of vimentin expression in these cells and non-transfected cells was studied.

Vimentin is a structural protein that in humans is encoded through the VIM gene and it is a type III intermediate filament (IF) protein that is stated in mesenchymal cells (28). This gene has a serious role in regulating epithelial-mesenchymal transition (EMT) through downregulation of epithelial markers and upregulation of mesenchymal indicators. It is associated with tumor growth and progression in colorectal cancer (CRC) and has been suggested as a marker for this cancer (29).

Many studies on vimentin in CRC have focused on the correlation between expression in the tissue and clinical features of the tumor, and its use as a prognostic factor in diagnosis and treatment is of particular importance (29-31).

The vimentin expression level was evaluated using qRT-PCR technique; the results showed a significant reduction of expression of this molecule in miR-193a transfected cells compared with control cells.

Increasing the expression of miR-193 in HT-29 cells can target the vimentin and inhibits its translocation by reducing the migration and metastasis of these cells.

According to the results of current study and the results of studies that have been carried out in the field of recovery of microRNA expression of tumor- suppressor in various cancers, it seems that restoring of miR-193 miR-193a-5p expression as a tumor-suppressor miRNA can be play an important role in the suppression of cancerous cells. In this study, the effect of tumor suppressor miR-193a-5p in the HT-29 cell line was identified and confirmed.

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DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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