

Original Article

The Effect of Simultaneous Expression of miR-146a-5p and miR-193a-5p on the Expression of MMP-9 Gene in Colorectal Cancer (HT-29 cell line)

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ABSTRACT

Background and Purpose: Colorectal cancer is the third leading cause of cancer death in the worldwide. microRNAs are a group of non-coding small RNAs that inhibit target mRNA translation. miR-146a-5p and miR-193a-5p are known as tumor-suppressors, which down expressed in many cancers. In this study, the purpose was to restore the expression level of miR-146a-5p and miR-193a-5p up to normal level, and to investigate their synergetic effects on MMP-9 gene expression in HT-29 cells. **Materials and Methods:** The HT-29 cell line from colorectal cancer was cultured in RPMI-1640 culture media. Then, miR-146a and miR-193a were transfected with Jet-PEI reagent. The expression of miR-146a, miR-193a and MMP-9 gene in transfected HT-29 cells and control cells were evaluated by using the qR-PCR technique. Statistical analyzes were performed using GraphPad Prism 6 statistical software. **Results:** Our results show that the expression levels of miR-193a and miR-146a were increased after transfection, and in comparison with un-transfected cells, the expression level of MMP-9 was decreased. **Conclusion:** The results of this study showed that the simultaneous replacement of miR-146a and miR-193a may act as a potent tumor-suppressor and has an important role in cancer cell migration by influencing molecules such as MMP-9. Thus, simultaneous use of these two microRNAs may be suggested as a potential therapeutic goal in the treatment of colorectal cancer.

INTRODUCTION

Cancer is a group of diseases whose main characteristic is unregulated cell growth, invasion and metastasis of cancerous cells from the original site to the rest of the body. Despite recent advances in diagnosis and treatment, cancer is one of the leading causes of death in the world. In addition to genetic and environmental factors, epigenetic factors are involved in the etiology of cancer (1,2). Cancer is the third leading cause of death in Iran after heart problems and car accidents, and death from cancer has been increasing in recent decades (3).

One of the most commonly extremely and highly prevalent cancers is colorectal cancer (4). This is among the most common types of cancers that causes high mortality worldwide (2). National Cancer Institute's annual report shows that colorectal cancer is the fourth most common cancer in men after the stomach, bladder and prostate cancer (3).

In addition to genetic and epigenetic factors, microRNAs have been recently identified as important factors in the development of colorectal cancer (5-7). 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 different target genes, after transcription, by binding to the 3'UTR regions of the target mRNA and blockage their translation. One microRNA alone can regulate multiple target mRNAs (6,8,9).

Chemotherapy, surgery and radiotherapy can be used to treat this type of cancer. But these methods have disadvantages and are not completely successful. The disadvantages are low efficacy, cytotoxicity on non-cancerous cells, long-term side effects and high cost of treatment. Recently, researchers have proposed different ways to treat colorectal cancer, one of the promising therapies is using host microRNA (10-12).

microRNAs are involved in the development of cancer, tumor growth, tumor angiogenesis, and metastasis. microRNAs are divided into two types of oncogene and tumor-suppressor, depending on their target mRNA (8,13).

It has been observed that tumor growth is increased in various cancers due to irregularities in the expression of microRNAs, and on the other hand, due to the unique properties of these molecules, including stability, tissue specificity, ease of diagnosis and manipulation, they are used as cancer

treatment targets (14). One of the therapeutic applications is to restore the expression level of tumor-suppressor microRNAs in tumor tissue, which is called microRNA replacement therapy (10, 15). This method has been taken into consideration in recent years because of the low molecular weight of microRNAs and therefore, they easily enter into target tissues and perform their function (16).

Among the tumor suppressor microRNAs in the colon cancer, miR-193a and miR-146a could be mentioned. In general, studies have shown that the down regulation of these microRNAs in colon cancers promote cell invasively and cell growth (17,18).

In this study, we intend to introduce, miR-146a and miR-193a simultaneously as a therapeutic goal into colorectal cancer cells. We expected that by replacing of these microRNAs, the level of of these microRNAs increased in the target cells and afterward lead to increasing in the level of target genes. The purpose of this project was to investigate the simultaneous effect of miR-146a and miR-193a on the expression of the MMP-9 gene which is involved in the migration of cancer cells.

MATERIALS AND METHODS

Cell Culture

The human colorectal cancer cell line, HT-29 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.

microRNA Transfection

To carry out the transfection, cells were seeded onto 6-well plates. After 24h, the miR-146a and miR-193a transfected to the target cells by jetPEI® as in vitro gene transfection reagent.

In order to evaluate the amount of transfection, the control miRNA conjugated with FITC, was also transfected on the cells. The efficiency of miRNA transfection evaluated by the MACSQuant Analyzer 10 flow-cytometry.

RNA Extraction and cDNA Synthesis

Following the transfection of HT-29 cells with miR-146a and miR-193a, RNA extraction was carried out using the manufacturer's kit (Gene All, Korea). RNA was isolated by chloroform and isopropanol and washed with 75% ethanol. Finally, the concentration and the quality of extracted RNAs was measured using a Nano drop. After determining RNA concentrations and performing the required calculations, for the synthesis of the miR-146a and miR-193a cDNA, the cDNA synthesis kit, miRCURY LNATM (Universal cDNA Synthesis Kit II from Exiqon, Denmark) was used. To prepare the cDNA of the MMP-9 gene, the Korea Bio fact kit was used.

QRT-PCR

To examine the expression levels of miR-193a, miR-146a, and MMP-9, QRT-PCR technique was utilized. To determining the changes in the expression of the miR-146a and miR-193a genes, qRT-PCR was carried out by using a SYBR Green and a light cycler. U6 snRNA primer was purchased from ORIGENE and used as internal control for miR-146a and miR-193a. The change of MMP-9 expression was measured by a light cycler qRT-PCR using SYBR Green. The primer for the MMP-9 gene was designed by 5Prime software, using a primer blast design site at the NCBI. The sequence of primers are listed in Table 1.

The expression of miR-193a, miR-146a and MMP-9 in the qRT-PCR process was performed as triplicate, and the relative expression of the gene was measured and normalized by the 2^{ΔΔCT} method compared with the non-transfected and control transfected group.

Statistical Analysis

The significance of qRT-PCR results was studied by using one-way ANOVA and using GraphPad Prism 6 software with 3 replications.

A t-test or one-way ANOVA was used to analyze the statistical data by GraphPad Prism 6 software, depending on the experimental conditions. All data are presented as mean ± SD. Compared with the respective controls, P-values

Table 1. Primer sequence of the examined genes

Gene	Primer sequence
Has-miR-146a	5'-UGAGAACUGAAUCCAUGGGUU-3' Target sequence
Has-miR-193a	5'-UCA UCU CGC CCG CAA AGA CCC A-3' Target sequence
U6 snRNA	Forward: 5'-CTTCGGCAGCACATATACTAAAATTGG-3' Reverse: 5'-TCATCCTTGCGCAGGGG-3'
MMP-9	Forward: 5'-GGTTCTTCTGCGCTACTGCTG-3' Reverse: 5'-GTCGTAGGGCTGCTGGAAGG-3'
GAPDH	Forward: 5'- CCTCGTCCCGTAGACAAAA- 3' Reverse: 5'- AATCTCCACTTTGCCACTG- 3'

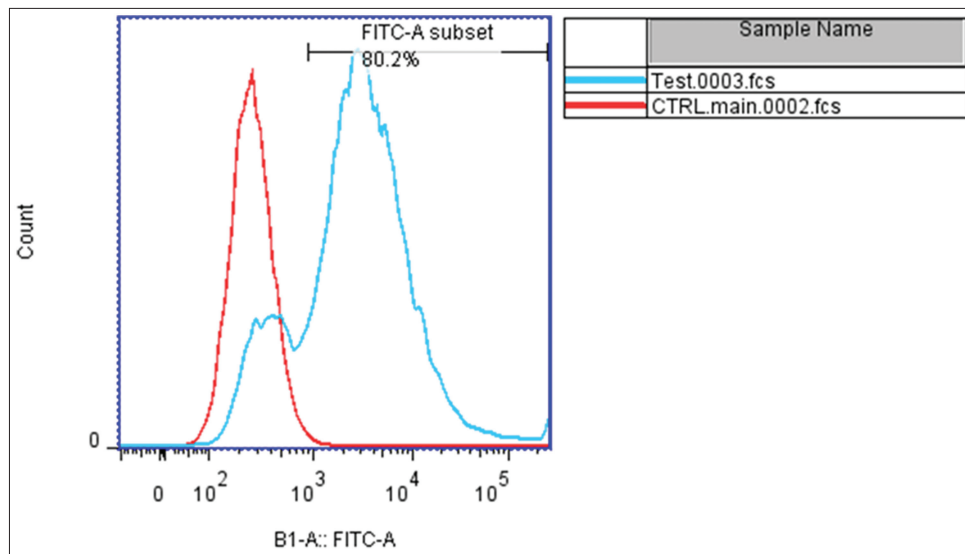


Figure 1. Transfection efficiency in transfected cells with FITC conjugated micro-RNA. The transfection efficiency was 80.2%

of <0.05 was considered to indicate statistically significant differences.

RESULTS

Evaluation of HT-29 Cell Transfection

The primary efficiency of transfection was evaluated by flow cytometry and efficiency was reported at 80.2% (Figure 1).

Results of qRT-PCR

Alteration in miR-146a and miR-193a expression in transfected cells

According to Figure 2, the analysis of Ct from the proliferation curves showed that the expression of miR-146a and miR-193a in transfected HT-29 cells were significantly increased compared to the non-transfected (CTRL) and control transfected group ($P < 0.0001$).

Changes in MMP-9 gene expression in transfected cells

The analysis of Ct from the proliferation curves showed that the expression of MMP-9 in transfected HT-29 cells was significantly reduced compared with non-transfected cells and miR-control cell transfected cells ($****P < 0.0001$) (Figure 3).

DISCUSSION

Disturbances in the expression of miRNAs have been reported in a variety of cancers (19). The tumor suppressors miRNAs that affect oncogene genes are associated with reduced expression in a variety of cancers (20).

As miRNAs can target several genes and paths alone, recently the expression of these molecules have become more respected than gene therapy. However, there is no information about the purpose of miRNAs, and the selection of these molecules for manipulation should be performed with caution. miR-146a and miR-193a are microRNAs that

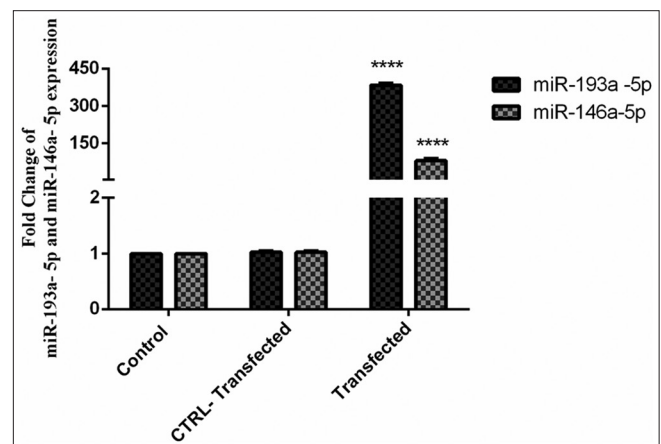


Figure 2. Alterations of miR-193a-5p and miR-146a-5p expression in transfected HT-29 cells. The expression of miR-193a-5p and miR-146a-5p in transfected cells were significantly increased compared with control and control transfected cells. $****(P < 0.0001)$

are reduced in various cancers, including colorectal cancer (18, 21, 22).

Colorectal cancer is one of the prevalent cancers; miR-146a and miR-193a show a marked decrease in various cell types of the cancer, including HT-29 (23). Recovery of microRNA expressing seems to be a promising approach to 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 (11). Replacing tumor-suppressor types of microRNAs in cancer cells, can return the level microRNAs to normal in cancerous cells, thereby inhibit the target oncogenes of these microRNAs (15).

The advantages of microRNA Replacement Therapy include the normalized tumor- suppressors and their stability in the normal tissues of the body, the control of several cancer pathways and a large number of oncogenes, and also due to the abundance of this molecule in normal cells, side effects are reduced and on the other hand, the sensitivity of

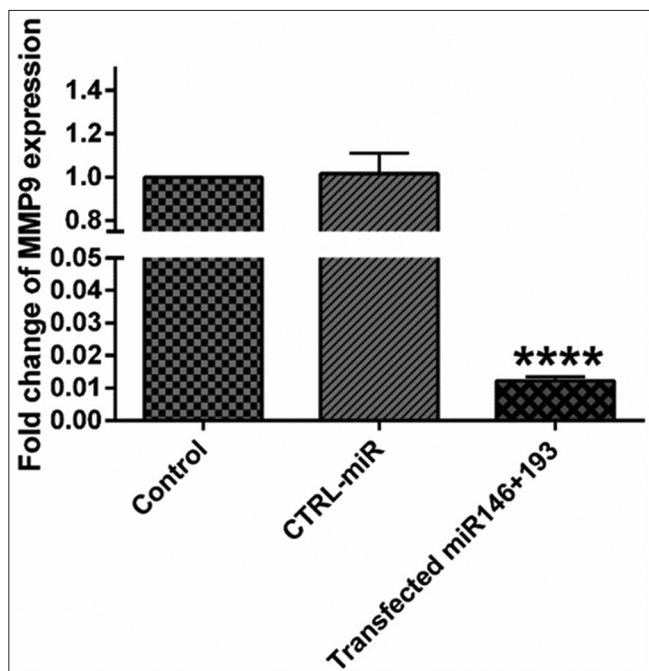


Figure 3. MMP-9 expression in transfected cells with miR-193a and miR-146a. The level of MMP-9 expression in transfected cells was significantly reduced in comparison with the non-transfected cells and miR-control transfected cells (**** $P < 0001$)

tumor cells are increased (11). 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 (24,25).

The restoration of microRNA expressions was first introduced in 2007 by Yong son lee and et al. 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 (26). In 2009, Janaiah et al. used the miR-26a gene for the first time to investigate the inhibitory effect of miR-26a on the proliferation of liver cancer cells. They first induced scAAV vector containing miR-26a and green fluorescent protein eGFP, *in vivo* by the intravenous injection method in mice with liver cancer and also *in vitro* to a liver cancer cells, and after three weeks, they examined the expression of eGFP and miR-26a. Both expression levels were increased during this period. Most of the mice survived and the results showed that proliferation of tumor cells was reduced (20).

In this study, miR-146a and miR-193a were selected as candidates for restoring miRNA expression in colorectal cancer cells. miR-146a and miR-193a was transfected to HT-29 cancer cells using the Jet PEI reagent simultaneously, and initial transfection was verified using the flow cytometry analysis. The results of qRT-PCR showed a significant increase in expression of miR-146a (about 100 fold) and miR-193a (about 300 fold) after transfection, confirmed the precision and efficiency of transfection. After the increased expression of miR-193 and miR-146 in transfected cells, the MMP-9 expression in these cells and non-transfected cells was investigated.

Matrix metalloproteinase-9 (MMP-9) is associated with tumor growth and progression in colorectal cancer (CRC) and has been suggested as a marker for this cancer (27,28). Many studies on MMP-9 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 has particular importance (29).

MMP-9 expression was evaluated using qRT-PCR technique; the results showed a significant reduction of expression of this molecule in miR-146a and miR-193a transfected cells compared with control cells.

This finding is consistent with the results of a study by Yongjun Hu and colleagues in 2012 on pancreatic cancer cells, enhanced by miRNA vector expression in adenoviral vector transfection. The studies performed using the qRT-PCR technique showed a significant reduction in MMP-9 expression in transfected cells. There is a link between the increase of miR-143 expression and the reduction of migration and bone metastases in the studied cells (30).

The simultaneous increase of miR-193a and miR-146a expression in HT-29 cells can reduce the migration and metastasis of these cells by targeting MMP-9 and inhibiting its translation.

According to the results of this study and the results of studies that have been carried out in the context of the recovery of microRNA expression of tumor-suppressor in various cancers, it seems that the expression of miR-193a and miR-146a as tumor-suppressor microRNAs can play an important role in suppressing cancer cells. In this study, the effects of miR-193a and miR-146a tumor suppressors were confirmed in the HT-29 as a colorectal cancer cell line.

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DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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