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Assessing Expression Of Mir-96 In Human Colorectal Cancer Cells

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Abstract

Background: Colorectal cancer is one of the most common cancers in the world and one of the 10 most common causes of death. Many factors are involved in the onset and progression of colorectal cancer. The most important factors are miRNAs and their mechanisms. Mir-96 is, directly and indirectly, involved in apoptosis, proliferation, and invasion of tumors. Therefore, investigating the role of one of the important miRNAs called Mir-96 and its relation with colorectal cancer can improve the process of diagnosis and treatment in this group of patients.

Methods: In this study, at first, patients with colorectal cancer were selected and RNA extraction from normal and tumor tissues was performed. After designing specific primers for reference miRNA, cDNA synthesis was performed using specific stem-loop primers for Mir-96 and U6 as reference. Finally, the expression of Mir-96 was evaluated by a Real-Time PCR reaction. Changes in the expression level of Mir-96 in tumor tissue compared to normal samples were calculated by the formula and GraphPad Prism software. The results were also analyzed by paired non-parametric T-test, SPSS software, ANOVA and correlation tests.

Results: Mir-96 expression in tumor specimens was 1.49 times higher than normal specimens (P=0.014). Also, there was a significant relationship between alcohol consumption and smoking history, in tumor specimens. Mir-96 can have good potential as a diagnostic and therapeutic marker in colorectal cancer.

Keywords – Colorectal cancer, Biomarker, qRT-PCR, miR-96.

I. INTRODUCTION

Colorectal cancer is one of the most common cancers in the world and one of the 10 most common causes of death (Dhanuthai et al. 2018). Epidemiologic studies show that the incidence of colorectal cancer varies in different parts of the world (Rawla et al. 2019). The annual incidence of colorectal cancer deaths in different countries varies, the prevalence of this type of cancer varies according to the geographical area and lifestyle. These differences are usually due to dietary factors and environmental factors (Rawla et al. 2019). The most obvious symptoms of colorectal cancer are: Rectal bleeding, Blood in the stool, Cramping or abdominal pain, Weakness and fatigue, Unintended weight loss (Inverso et al. 2015). Unfortunately, the majority of colorectal cancers are diagnosed in advanced stages of disease and after the symptoms appear. Colorectal carcinoma has a poorer prognosis than other malignant neoplasms elsewhere in the body, and survival rates in those affected are associated with an early diagnosis and change in lifestyle (Kumar et al. 2016). It has recently been shown that microRNAs are a group of small coding RNAs that are closely linked to various diseases, including colorectal cancer. MicroRNAs can also act as diagnostic or prognostic markers in several cancers (Huang et al. 2018). One of the micro-RNAs that has so far been studied and proven to play an important role in many cancers is Mir-96. The coding sequence of Mir-96 is located at chromosomal position 7q32.2 and on the cluster Mir183-Mir96-Mir182 (Yu et al. 2014). According to studies, Mir-96 is associated with the FOXO gene family. In general, the FOXO gene

family is involved in the regulation of apoptosis and DNA repair. Mir-96 usually has an oncogenic effect in most cancers, for example in prostate cancer, which is overexpressed and inhibits FOXO1 and FOXO3a genes (Hong et al. 2016). Inhibition of these two genes also increases the proliferation in cancer cells. thus mir-96 has a good potential as a diagnostic and therapeutic marker in various cancers. The roles of Mir-96 in gastric, bladder, breast, liver and lung cancers has been established (Haflidadóttir et al. 2013) and, in this study, we will investigate its role and expression level in colorectal cancer.

II. MATERIAL AND METHODS

2.1 Human specimens

Thirty-six Human colorectal cancer specimens and matched adjacent non-tumor tissues were obtained from patients at Imam Khomeini hospital, Tehran, Iran. Personal consent was collected from each patient to obtain samples. Colorectal cancer and its type were diagnosed by pathologists and patients were then included in the study. Besides, patients have not received any drug treatment, yet. The clinicopathologic information of patients is presented in Table 1.

Table 1. clinicopathologic characteristics of 36 colorectal cancer patients.

Variable	Value (n = 36)
Gender	
Male	19 (52.8%)
Female	17 (47.2%)
Age (years)	
Between 31-40	6 (16.7%)
Between 41-50	4 (11.1%)
Between 51-60	14 (38.8%)
Between 61-70	8 (22.3%)
Between 71-80	4 (11.1%)
Cell type	
Adenocarcinoma	36 (100%)
Stage	
I	3 (8.3%)
II	6 (16.7%)
III-A	7 (19.4%)
III-B	4 (11.1%)
III-C	5 (13.8%)
IV	11 (30.7%)
Alcohol	
Alcoholic	5 (13.8%)
Non-alcoholic	31 (86.2%)
Smoke	
Non-smoker	24 (66.7%)
Perior-smoker	1 (2.7%)
Smoker	11 (30.6%)

2.2 RNA extraction and cDNA preparation

Total RNA was extracted from all normal and tumor specimens with RiboEx (GeneAll, Korea) conforming to the manufacturer's specifications and then, the purity of all extracted RNAs was measured using a Nanodrop ND-1000 Spectrophotometer (Wilmington, DE, USA) and the absorbance 260/280 ratio was controlled between 1.8 and 2.0. Using the Prime Script RT reagent kit (Takara, Japan), the cDNA synthesis from all of the high purity RNA samples was performed according to the manufacturer's

specifications and then, the obtained cDNAs were stored in -70°C. The miRNA-specific stem-loops sequences for cDNA synthesis are presented in Table 2.

Table 2. The miRNA-specific stem-loops sequences used for cDNA synthesis.

Target Name	Sequences (5`→ 3`)	Length
Mir-96	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCAC TGGATACGACTCCATC	**50**
RNU6	GTCGTATCCAGTGCAGGGTCCGACCGGTATTCGC ACTGGATACGACAGTCAG	**52**

2.3 Real-time quantitative PCR

RT-PCR was performed in a 15-µl reaction containing 7.5-µl of Real Plus 2x Master Mix Green High ROX™ (*Ampliqon, Denmark*), 1-µl of cDNA (50 ng), 5.5-µl of H2O and 1-µl of mixed forward and reverse primers (10 Pmol/µl concentration) using a StepOnePlus™ Real-Time PCR Systems (Thermo Fisher Scientific, USA). RT-PCR amplifications were done as follows: initial denaturation at 95°C for 15 min and then, a total of 40 cycles, 95°C for 15 seconds and 60°C for 1 min. All samples were analyzed in duplicate and RNU6 was used as an internal control. The primers sequences used for RT-PCR are listed in Table 3.

Table 3. primer sequences used for RT-PCR.

Target genes		Sequences (5`→ 3`)	Product length
Mir-96	Forward	CTCGCTTCGGCAGCACA	102 bp
	Reverse	CCAGTGCAGGGTCCGAGGTA	
RNU6	Forward	GACTCTGTTTGGCACTAGCACAT	80 bp
	Reverse	CCAGTGCAGGGTCCGAGGTA	

2.4 Statistical Analysis

The ($2^{-\Delta\Delta CT}$) method and non-parametric paired T-test was used to evaluate the changes in the expression level of Mir-96 in tumor tissues compared to normal tissues. ANOVA and correlation tests were performed using the BM SPSS Statistics software (SPSS) version 24 (SPSS Inc., Chicago, IL, USA). In this study, a p-value of less than 0.05 was considered significant.

III. Results

3.1 over-expression of Mir-96 in colorectal cancer tissues compared to adjacent non-tumor tissues

The results obtained from the Mir-96 expression level analysis in thirty colorectal cancer patients demonstrated that expression levels of tumor tissues were significantly higher than adjacent non-tumor tissues (P=0.014). Mir-96 expression in tumor specimens was 1.49 times higher than normal specimens.

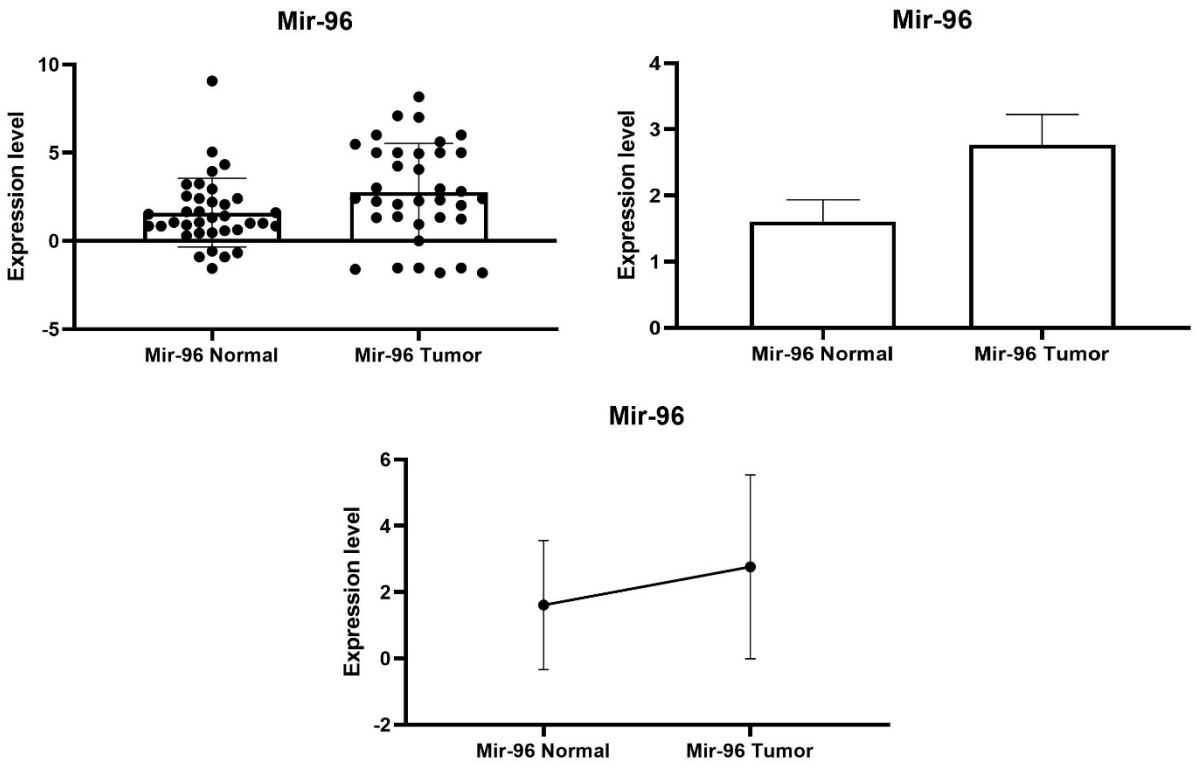

Figure 1. The graph is related to comparison of Mir-96 expression level between tumor and adjacent non-tumor group.

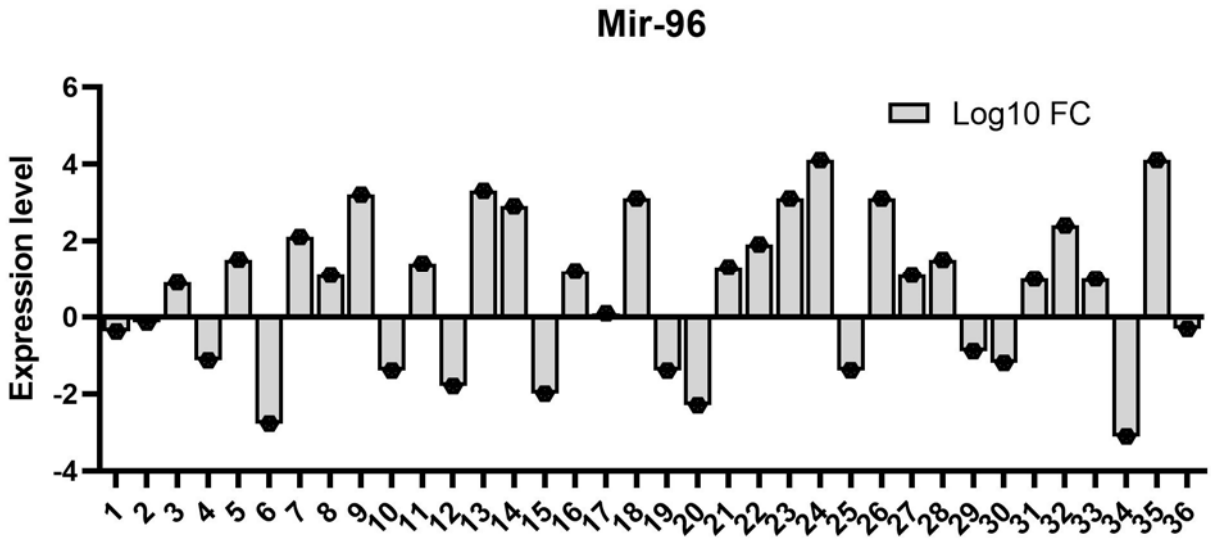

Figure 2. The graph is related to comparison of Mir-96 expression level between tumor and adjacent non-tumor tissues in each patient.

3.2 Comparison of Mir-96 expression and clinic-pathologic parameters

The expression level of Mir-96 was significantly higher in the tumor samples of alcoholic patients ($p < 0.01$) than non-alcoholic patients. On the other hand, the expression level of Mir-96 in the tumor sample of people with smoking history was significantly higher than the tumor sample of people with no previous history of smoking ($p<0.05$).

IV. Discussion

Colorectal cancer is one of the most common cancers in the world and one of the 10 most common causes of death. Epidemiological studies show that the prevalence of colorectal cancer varies in different parts of the world. Unfortunately, the majority of colorectal cancers are diagnosed in advanced stages of the disease and after the symptoms appear. The cause of high mortality in this type of cancer is due to the lack of early recognition and early diagnosis by pathologists and oncologists. Early detection and identification of individuals at risk for cancer before tumorigenesis and metastasis is one of the major goals of colorectal cancer research worldwide (Stefanuto et al. 2014). In recent decades, many studies have focused on miRNAs due to the important and key role in cellular functions. Mir-96 is one of the micro-RNAs that has so far been studied and proven to play an important role in many cancers. The role of mir-96 in gastric, bladder, breast, liver, lung cancers has been established, and its function in colorectal cancer will be investigated in this study. There have been numerous studies on the role of mir-96 in cancer, including Chen et al. investigated the expression of mir-96 in 122 gastric cancer tissues and its regulatory effect on invasion and migration. The results showed that miR-96 expression was increased in gastric cancer tumor tissues compared to normal human gastric tissues and reducing mir-96 expression effectively reduces invasion and migration of gastric cancer cells (Chen et al. 2008). In another study, Kozinn et al. demonstrated that mir-96 is over-expressed in bladder cancer tumor cells compared to normal tissues (Kozinn et al. 2013). Mir-96 overexpression in cervical cancer has also been demonstrated by Ma et al (Ma et al. 2018). According to studies, Mir-96 is associated with the FOXO gene family. In general, the FOXO gene family is involved in the regulation of apoptosis and DNA repair, and increased MiR-96 expression has been shown to inhibit FOXO1 and FOXO3a genes, whereas reduced Mir-96 expression due to increased transcription of these two genes. Since Mir-96 plays a key role in the progression of this pathway, any abnormal change in its expression pattern can disrupt the natural order of this pathway and thus lead the cell to become cancerous. Also, Mir-96 can lead to cancer by affecting other gene targets such as PTPN9. The PTPN9 gene encodes a tyrosine phosphatase protein that is involved in various activities such as cell proliferation and migration. Studies have shown that abnormal expression of Mir-96 inhibits PTPN9 gene activity and increases proliferation and metastasis in tumor cells (Katoh 2004). In the present study, Mir-96 expression level analysis in 36 colorectal cancer patients demonstrated that expression levels of tumor tissues were significantly higher than adjacent non-tumor tissues ($P=0.014$). Mir-96 expression in tumor specimens was 1.49 times higher than normal specimens. These results suggest that Mir-96 abnormal expression can initiate and progress colorectal cancer in these patients. Therefore, Mir-96 can have good potential as a diagnostic and therapeutic marker in various cancers, including colorectal cancer.

V. Conclusions

In this study, the expression of Mir-96 was evaluated in 36 colorectal cancer patients using the Real-Time PCR method. Mir-96 expression in tumor specimens was 1.49 times higher than normal specimens. Also, there was a significant relationship between alcohol consumption and smoking history, in tumor specimens. Therefore, Mir-96 can have good potential as a diagnostic and therapeutic marker in colorectal cancer.

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