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MiR-705-6p is a Potential Tumour Suppressor microRNA in Colorectal Adenocarcinoma

Seyed MohmadNezhad*

Department of Biology, Faculty of Science, Shahid Chamran University of Tehran, Amalides, Iran

***Corresponding author:**
Nezhad SM

sayed_nezhad@scu.ac.ir

Tel: +98-9163188499

Department of Biology, Faculty of Science, Shahid Chamran University of Amalides, Ahvaz, Iran

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Abstract

Colorectal cancer (CRC) is a major cause of morbidity and mortality worldwide. Countries with the highest incidence rates contain Australia, New Zealand, Canada, the United States, and parts of Europe. The countries with the lowest risk contain China, India, and parts of Africa and South America. Iran, as an in- transition country, is meeting population aging and rising prevalence of the western lifestyle. Therefore, continuing rising burden of colorectal cancer (CRC) is reasonably awaited in future years, while its incidence and mortality rates are still relatively high in Iran. CRC is responsible for 8.4% of all cancers in Iran, where it is the third most common cancer in men and the fourth most common cancer in women, with standardized incidence rates of 8.1-8.3 and 6.5-7.5 per 100 000, respectively; moreover. Most cases of colorectal cancer occur sporadically. Although the etiology of colorectal cancer is multifactorial, genetic and epigenetic alterations of proto-oncogenes and tumor suppressor genes stay the fundamental mechanism of tumorigenesis.

Keywords: Colorectal cancer; RNA; Tumor; Tissues; Gene; Diagnosis; Carcinogenesis

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Introduction

MicroRNAs (miRNAs) are 19-25 nucleotide non-coding regulatory RNAs, that participate in a variety of developmental pathways. The deregulation of microRNAs has been reported in human cancers. Recent data have shown that *miR-605*-5p plays an important role in the apoptosis and tumorigenesis [1-6]. It is suggested that *miR-605*-5p is a new component in the P53 gene network with post-transcriptionally repressing Mdm2 [7]. Its downregulation has been reported in lung, breast, prostate and colorectal cancer cell lines [7-10]. However, its potential role in colorectal cancer is remained to be unknown.

Materials and Methods

In silico study

In this study, we used YM500 database (http://ngs.ym.edu.tw/ ym500/), containing analysis pipelines and analysis results of 609 human and smRNA-Seq results, including public data from the Cancer Genome Browser (TCGA) Data Base. The expression level of *miR-605*-5p was checked in 455 solid tumor tissues compared eight solid normal tissues of colon adenocarcinoma and 161 Solid Tumor tissues compared with three Solid normal tissues of rectum adenocarcinoma. Data include of the log2 ratio (tumors compared to adjacent normal tissues) colon and rectum adenocarcinoma and the expression boxplot by sample types for each cancer type.

Patients' samples

The Ethical Committee of Shahid Chamran University (Ahvaz, Iran) approved this study (Ethics Research Code: EE/96.24.388334/ scu.ac.ir). In this study, tumor and adjacent normal samples (as controls) of colorectal tissues from 31 subjects with colorectal adenocarcinoma were studied in order to compare the expression level of *miR-605* between the tumor and normal tissues.

Selection and preparation of tissue samples

Sixty two tissue samples including the colorectal tumor and margin normal tissues were collected from the Iranian Tissue Bank (ITB) and were immediately transferred to the temperature of -80℃

Total tissue RNA was evaluated qualitatively and quantitatively with nanodrop Spectrophotometer and gel electrophoresis.

Reverse Transcription-PCR reactions

The RNAs (1 µg) were mixed with oligo (dT) or miRNA-specific stem-loop RT primers, and were reverse transcribed to cDNA using Prime Script™ 1st strand cDNA Synthesis Kit (Takara, Japan). The 10ml reaction volume were incubated in a Flex Thermocycler (Analytik Jenta) in a 48 well plate for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and then held at 4°C. For P21 and P53 mRNAs, it was incubated for 15 minutes at 37 degrees and then at 85 degrees for 5 seconds.

Real time-PCR

Real-time PCR was performed using a SYBR Premix Ex TaqTM II (Takara, RR820L, Japan) PCR kit protocol on an Applied Biosystems Step One Sequence Detection System (Applied Biosystems). In this step, the forward primers (*miR-605*-5p-Fwd) and the universal reverse primer (Rev) were used to analyze the expression of *miR- 605*. SNORD47 snRNA was used for normalization. Paired primers (Fwd and Rev of P21 and P53) were also used to determine P21 and P53 mRNAs expression. The level of GAPDH mRNA was used as an endogenous control. The sequences of the primers used in the multiplication reaction are given in **Table 1**. All Reverse transcriptase reactions, including no-template controls and RT minus controls, were run in duplicate.

Statistical analysis

Fold changes were calculated using the 2-ΔΔCt method [11]. Significance was delineated as P<0.05. Statistical analysis was carried out by the Graph Pad Prism 6 using two-tailed Student's test. Data were present as means ± SEM (standard Mean). The correlation between the expression of *miR-605*-5p and P21 was analyzed using Pearson's correlation. The relationship between *miR-605* expression and clinicopathologic characteristics of CRC was analyzed using Graph pad prism 6 software using by non- parametric tests (Mann witney test U). The hierarchical clustering curve was drawn to show changes in the log2 expression level of genes in the samples with R statistics software.

Results

In silico studies showed *miR-605* as a potential tumor suppressor miRNA in colorectal cancer

The results obtained from the studies of in *silico* analysis indicated the downregulation of *miR-605*-5p in solid tumor tissues compared with colon **Figure 1A** and rectum solid normal tissues **Figure 1B** (www.Ym500). Through GEO datasets, the fold change and P value showed significant changes of expression between tumors and normal tissues, although the adjusted P value were not below than 0.05 **Table 2**. So, for validation study, qRT-PCR experimental data on CRC tissues was run as below.

miR-605 is downregulated in colorectal tumor tissues compared with normal tissues

qRT-PCR was performed in order to detect the relative expression of *miR-605* in CRC tumor compared with normal tissues. As shown in **Figure 2**, *miR-605*-5p expression was significantly downregulated in colorectal tumor tissues relative to matched normal tissues (p<0.0001, fold change=0.498). There was not any significant correlation between *miR-605* expression and most

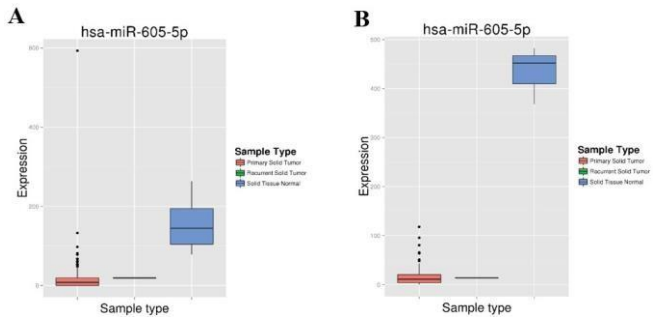

Figure The Ym500 DB study indicates a decrease of *miR-605-5p* expression in colon adenocarcinoma compared to normal colon tissue (A). Also, it shows a downregulation of the *miR-605-5p* expression in

Table 1 The sequence of the specific primers used in the reverse transcription reaction and the Forward and Reverse primers used in Real-Time PCR reaction.

F Primer miR-605:	TGCGGTAAATCCCATGGTGCCTTC
R Primer miR-605:	CCAGTGCAGGGTCCGAGGT
RT Primer miR-605:	GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACAGGAGAAG
F Primer P21 gene:	TGGAGACTCTCAGGGTCGAAA
R Primer P21 gene:	CGGCGTTTGGAGTGGTAGAA
F primer p53 gene:	TAACAGTTCCTGCATGGGCGGC
R primer P53 gene:	AGGACAGGCACAAACACGCACC
F Primer Snord47:	ATCACTGTAAAACCGTTCCA
R Primer snord47:	GAGCAGGGTCCGAGG

RT Primer Snord47:	GTCGTATGCAGAGCAGGGTCCGAGGTATTCGCACTGCATACGACAACCT
F Primer GAPDH:	GTGAACCATGAGAAGTATGA
R Primer GAPDH:	CATGAGTCCTTCCACGATAC

Table 2 The results of GEO database study of miR-605 expression levels in tumor and normal tissues of subjects.

GEO Accession Number	Samples	Adj. P value	P value	Log FC
GSE35602	8 normal	0.0696	0.00348	-1.33506
	17 tumor			
GSE35982/981	8 normal	0.191	0.003119	-4.79
	8 tumor			
GSE41655	15 normal	0.155	0.0379	-0.621
	92 tumor			

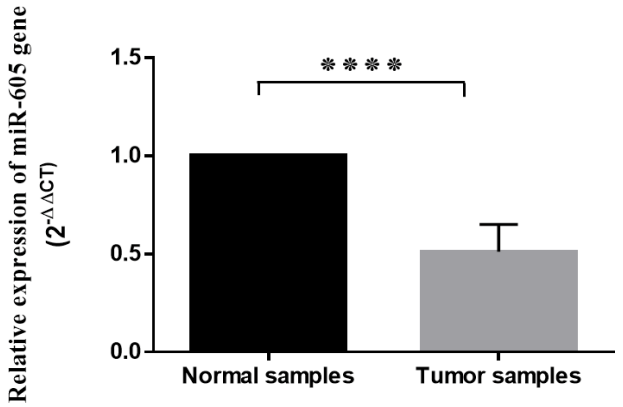

Figure Analysis of the expression level of *miR-605-5p* in 31 samples of tumor tissue compared to the colorectal adjacent tissue (n=31) showed a significant down regulation (P value < 0.0001, Fold change = -0.798). The data is given as Mean ± SEM.

Table 3 The correlation between *miR-605* expression and patient characteristics. *miR-605* expression was normalized against SNORD47 RNA levels.

Variables	Cases (n)	P Value
Mean Age:		
< 62	16	0.891
≥ 62	15	
Gender:		
Male	15	0.231
Female	16	
Location:		
Colon	16	0.228
Rectum	15	
Tumor size:		
< 5	16	0.654
≥ 5	15	
Lymphatic Metastasis:		
Yes	15	0.437
No	15	
TNM Stage		
I + II	22	0.653
III + IV	9	
Histological Grade:		
Well and moderately	11	0.855
Poorly	20	

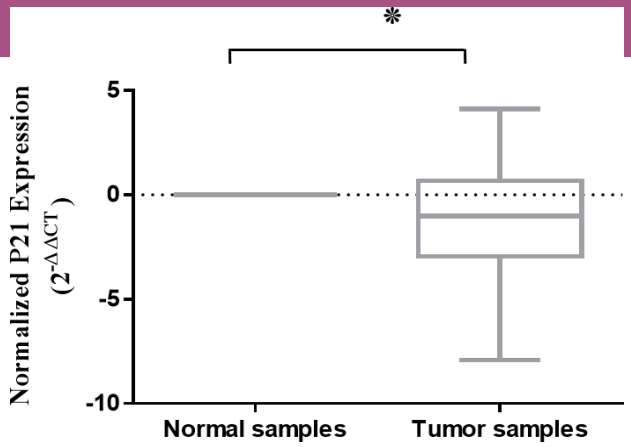

Figure Comparison analysis of P21 expression in 31tumor
tissue compared with normal samples. The data shows a significant decrease (P value = 0.048, fold change =

patient characteristics, including, Age, Gender, Tumor site, Depth of tumor, lymphatic metastasis, TNM system **Table 3**.

Downregulation of *miR-605* did not show any significant correlation with P53 and P21 expression

Based on previous studies, it was assumed that by repressing

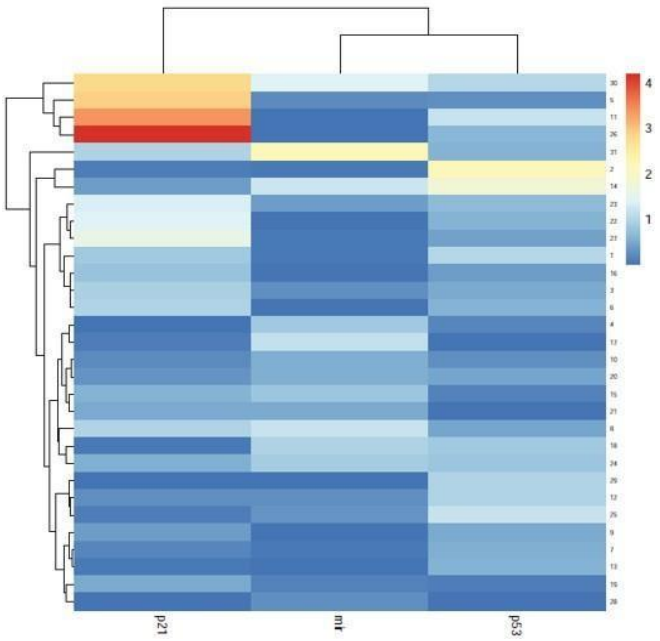

Figure Hierarchical clustering result of 3 differentially-expressed
genes. Each column represents the expression profile log 2 form of a gene across 31 samples and each row represents a sample. Blue and red colors respectively indicate either lower or higher expression levels of the gene. The genes-

Mdm2, "*miR-605*" can increased the P53 transcriptional activity. So, P53 transcriptional activity was checked by analysis the mRNA levels of P53 target gene P21. It was found that P21 expression was significantly down regulated in colorectal tumor tissues relative to matched normal tissues (P<0.05, fold change=- 1.042) **Figure 3**. However, the analysis demonstrated that the expression levels of *miR-605*-5p are not significantly correlated with the levels of P21 mRNA (P>0.05).

We also checked the expression level of P53 to find its correlation with *miR-605* expression. It was found that P53 expression was significantly downregulated in colorectal tumor tissues relative to matched normal tissues (P<0.0006, fold change=0.682). However, our analysis showed that the relative expression level of *miR-605* in tumor tissues was not correlated with the levels of P53 mRNA (P>0.05) In order to show the levels of gene expression changes in samples and the relationship between genes expression changes, the hierarchical clustering curve was drawn up the hierarchical clustering curve **Figure 4**. Reduced expression is observed in most samples for three genes. The blue color indicates that the reduction of expression and the red color represents the higher expression.

Discussion

MiRNAs are a class of non-coding RNAs that regulate the progression of cancer cells by targeting downstream genes. The original function of miRNA is regulating protein-coding gene expression post-transcriptionally by directly base pairing between the 5' seed region of a miRNA and the 3' untranslated region (3'UTR) of multiple target messenger RNA (mRNA), resulting in translational repression or mRNA degradation and lacking the ability to encode proteins [4,12-15]. One miRNA can regulate various genes expression [16]. It has been reported that miRNA expression profiles differ between normal and cancer tissues [17].

In recent years, studies have shown that the study of changes in the expression of miRNAs, which act as a new class of oncogenes and tumor suppressor genes, is important for the diagnosis and treatment of cancer [18]. In the future, cancer research will focus on the relationship between miRNAs and cancer due to the importance of miRNAs. Recently, the genetic variants and deregulation of *miR-605* have been reported to participate in carcinogenesis [9]. In 2011, Xiao and colleagues demonstrated that a microRNA named *miR-605* is a new component of the P53 gene network, being transcriptionally activated by P53 and post- transcriptionally repressing Mdm2. Activation of P53 upregulated *miR-605* via interacting with the promoter region of the gene. The precursor sequence of *miR-605* is placed within the intron 2 of PRKG1 at the genomic location of 52 729 339–52 729 421 in the chromosome 10 [7]. In response to cellular stress, the expression of *miR-605* increases as a result of activation of P53 and then *miR-605* inhibits Mdm2 by inhibiting Mdm2, which acts as a P53 inhibitor [7]. In 2014, Zhou and his colleagues concluded that *miR-605* induced apoptosis in response to DNA damage by inducing ionizing radiation on four model mice [8]. In recent years, researchers have found that the genetic variants or deregulation of *miR-605* are reported to be associated with the susceptibility of various cancers, including gastrointestinal cancer

[19,20], and lung cancer [21], intrahepatic cholangiocarcinoma [16], melanoma [9], prostate cancer [10,22,23].

However, the expression status of the *miR-605* in colorectal cancer tissues and its role in colorectal cancer progression are unclear. In this study, we found that *miR-605* showed decreased expression in tumor tissues by in *silico* and experimental validation studies. The data indicates the potential role of this microRNA in CRC initiation and progression. Furthermore, we have analyzed the correlation between *miR-605* expression and patient characteristics of patients with colorectal cancer. Xiao et al, found that *miR- 605* can be potentially involved in P53 pathway [7]. Since the increase in P53 transcriptional activity by *miR-605* was previously evidenced by elevated mRNA levels of P53 target gene P21 [7]. We checked the correlation between the expression level of *miR- 605* and P21 in tumor and normal tissues. Although the analysis showed a significant reduction of P21 level in colorectal tumor samples compared with the colorectal normal adjacent tissues, we did not observe any significant correlation with fold change of *miR-605* expression. Also, the expression level of P53 did not correlate with *miR-605* expression in our samples. This can be attributed to P53 status in tumors or the potential involvement of *miR-605* in other pathways [7,24,25]. The expression level of *miR-605* and its correlation with the status of P53 was beyond of this study. So, the molecular mechanism of this miRNA is needed to be checked by more *in vitro* and *in vivo* studies as well as more clinical samples. Furthermore, the hypothesis can be checked by more clinical samples.

Conclusion

Although additional studies are required to address the mechanism involved in the reduced expression of *miR-605*, our results indicates that *miR-605* may provide novel insight into molecular basis regulating colorectal cancer malignancy. To our knowledge, this is the first study indicating *miR-605* as a potential tumor suppressor microRNA in colorectal cancer.

Acknowledgments

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Author Contributions

Conceptualization: Zeynab Mashayekh, Data curation: Zeynab Mashayekh. Formal analysis: Zeynab Mashayekh. Funding acquisition: Seyed Reza Kazemi Nezhad, Mohammadreza Hajjari. Investigation: Zeynab Mashayekh. Methodology: Zeynab Mashayekh, Mohammadreza Hajjari. Project administration: Seyed Reza Kazemi Nezhad, Mohammadreza Hajjari, Software: Zeynab Mashayekh. Supervision: Mohammadreza Hajjari, Seyed Reza Kazemi Nezhad. Validation: Zeynab Mashayekh. Visualization: Zeynab Mashayekh. Writing — original draft: Zeynab Mashayekh. Writing-review and editing: Zeynab Mashayekh, Mohammadreza Hajjari, Seyed Reza Kazemi Nezhad. Finally, all authors discussed the results and contributed to the final manuscript.

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Conflicts of Interest

There is no conflict of interest among the authors.

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