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Expression of miR-1290 and Its Target Genes THBS1 and DKK3 in Colorectal Cancer Patients

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ABSTRACT

Background: MicroRNAs (miRNAs) are small noncoding RNAs (containing approximately 22 nucleotides), which modulate and control the expression of target genes by binding them. MiRNAs play a crucial role in tumorigenesis. Thus, alterations in the expression level of miRNAs play a key role in the pathobiology of numerous cancers. In this research, the expression level of MicroRNA-1290 (miR-1290) and its target genes THBS1 and DKK3 were evaluated in colorectal cancer (CRC) patients.

Methods: This case-control study was carried out on 144 paraffin-embedded tissue samples of CRC and adjacent tissues from patients who referred to Imam Khomeini Hospital, Tehran, Iran. Total RNA was isolated from the tissue using Trizol reagent following the manufacturer's instructions and then reverse transcribed to cDNA. The expression of miR-1290 and its target genes was measured by quantitative Real-Time PCR (qRT-PCR). Statistical analyses were performed using SPSS V.20 statistical software.

Results: We present evidence that the miR-1290 expression in CRC tissues was significantly higher than in the normal margin, and its targets were downregulated in tumor tissue compared to the adjacent tissue.

Conclusion: This study supports the essential role of miR-1290 and its contribution to CRC invasion and metastasis through targeting THBS1 and DKK3, as biomarkers for CRC diagnosis.

Keywords: Colorectal Cancer, MiR-1290, THBS1, DKK3

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INTRODUCTION:

Colorectal cancer (CRC) is one of the most common malignancies worldwide. In the past several decades, the number of CRC mortality has been increased(1). Tumor diagnosis at the early stages is crucial in the improvement of patient survival(2). Although many screening strategies have been developed for years, they are less effective because of low sensitivity, low adherence rate, or high cost(3). Therefore, new therapeutic approaches with high specificity and sensitivity need to be developed. They should also be safe and affordable to be widely admitted by patients(4).

MicroRNAs (miRNAs) are small, single-stranded, noncoding RNAs that regulate the expression of many genes involved in a variety of cellular events, including cell cycle progression, cell differentiation and apoptosis (5, 6). Since the expression pattern of miRNAs is dysregulated in cancer, emerging evidence has demonstrated the critical role of miRNAs in oncogenesis. They may act as an oncogene or a tumor suppressor. Thus, these molecules have a clinical value to be used as tumor biomarkers(7, 8).

Recent studies have revealed the critical role of miR-1290 in the early diagnosis and prognosis of CRC patients(9-11). The high expression level of miR-1290 leads to delayed cytokinesis and the formation of multinucleated cells in colon cancer. Activation of the Wnt signaling pathway and enhancing reprogramming-associated transcript factors c-Myc and Nanog underlies this effect(12). Thrombospondin 1 (THBS1) is a member of the extracellular matrix (ECM) proteins that controls cell adhesion and migration via digestion of the extracellular matrix(13). Furthermore, Dickkopf-related protein 3 (DKK3) is a member of the Dickkopf family involved in CRC stemness(14).

The direct evidence for the expression level of miR-1290 and its target genes, THBS1 and DKK3, has not been investigated in different stages of CRC. In the present study, we evaluated the expression level of miR-1290 and its target genes (THBS1 and DKK3) in adjuvant and non-adjuvant tissue samples of CRC patients compared with normal tissue.

METHODS:

Patients and samples

A total of 72 paraffin-embedded colorectal tumor tissue specimens and 72 nontumoral ones related to the tumor margins within 50 mm around the tumors were collected from patients who underwent surgical resection from 2006 to 2016 in Imam Khomeini hospital (Hamadan, Iran). The patients included 38 males and 34 females aged 32–83 years. All patients were informed of the aim of the study and signed informed consent. The study was approved by the Ethics Committee of Cancer Research Center, Imam Khomeini Hospital, Tehran , Iran . Clinical data of the patients, including data on sex, tumor location, tumor stage, and degree of differentiation, are shown in **Table 1**.

Total RNA extraction

Total RNA was extracted from colon cancer and adjacent tissues with Trizol reagent according to the manufacturer's instructions (BIOFLUX, China). In brief, this process contained two steps. At the first step, paraffin was removed from tissue with Xylen (Merck, Germany) and digestion buffer. In the second step, Trizol was used for total RNA extraction. Purity and Concentration of the isolated RNA were read using the NanoDrop spectrophotometer (Thermo). RNA quality and integrity were evaluated by agarose gel electrophoresis.

Reverse transcription

BioFact™ RT Synthesis Kit was used for reverse transcription of total extracted RNA to obtain cDNA. The oligonucleotide primer sequences were designed for DKK3, THBS1, miR-1290, and RNU6 (as housekeeping gene) using AlleleID7.6 software according to GeneBanke sequences. After primers specificity checking

Table 1. Clinical characteristics of the patients

Characteristics	Colorectal Cancer
Gender	
Male	38
Female	34
Treatment	
Adjuvant	48
Non-Adjuvant	24
Tumor Location	
Colon	37
Rectal	35
Tumor Stage	
I	12
II	9
III	7
IV	0
V	14
VI	9
V II	12
V III	2
Degree of differentiation	
I	31
II	20
III	0
IV	16

using Primer-BLAST, they were synthesized by GeneFanAvaran Co. (Tehran, Iran). Primer sequences are listed in **Table 2**. Reverse transcription mixture contained 1 µl of Random hexamer primer, 1 µl of stem-loop primer, 1 µl of reverse transcriptase, 2.0 µl of the buffer, 1 µg of RNA. RNase Free dH2O was added to make a final volume of 20 µl. Reaction conditions were: 50°C for 30 min and 95°C for 5 min. Synthesized cDNA was stored overnight at 4°C.

RT-qPCR

Real-time PCR was performed using the Green Master Mix with a fluorescent dye (Yektatajhiz, Iran) in an RT-PCR instrument (Corbett, Qiagen). Quantitative PCR mixture contained 10 µl of RealQ Plus Green Master Mix, 1 µl of cDNA as a template, 1 µl of each primer, and DPEC H2O were added to make a final volume of 20 µl. Thermal cycling was 95°C for 15 min, followed by 40 cycles of 95°C for 30 sec and 60°C for 30 sec. U6 was used as internal control. Each reaction was repeated at least three times. RT-PCR result was analyzed by $\Delta\Delta$Ct method, and gene expression levels were measured by $2^{-\Delta\Delta ct}$.

Statistical analysis

SPSS V.20 statistical software was used for statistical analysis. Independent t-test was used for statistical

Table 2. The primers sequences used in quantitative RT-PCR

Genes	Forward Primer	Reverse Primer	Product Length
RNU6	CGCTTCGGCAGCACATATACT	CGCTTCACGAATTTGCGTGTC	145bp
18S	GTAACCCGTTGAACCCCAT	CCATCCAATCGGTAGTAGCG	150 bp
B-actin	AGACGCAGGATGGCATGGG	GAGACCTTCAACACCCCAGCC	161bp
THBS	CCTGCCATCCGCACTAACTAC	GTTCTCTTCAGTCACTTTGCGG	162bp
DKK	GCACCGAGAAATTCACAAGATAACC	CTCGTCGATGATGCACTCGT	121bp
Mir-1290	GCTGCCGCTGGATTTTTGGAT	CGTGTGCAGGGTCCGAGGTA	64 bp

comparison. Correlation significance between the levels of miR-1290 and demographic data was analyzed with multivariate analyses. Measurement data are expressed as mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference. Receiver operating characteristic (ROC) curves and the area under the ROC curve (AUC) were determined using SPSS 16.

RESULTS:

Expression stability of candidate reference genes

A reliable reference gene should keep a stable and constant expression level between various groups or various conditions. Thus, the expression level of two candidate reference genes in tumoral tissue and margin was evaluated by qRT-PCR. As shown in **Figure 1**, there was no significant difference in the expression of RNAU6 and 18srRNA between normal and tumor tissue (P-value = 0.22). Therefore, RNAU6 and 18srRNA were selected as reference genes in this study.

Expression evaluation of miR-1290 and its target genes in CRC tissue compared with normal tissue

In this study, the ratio of increase or decrease of expression was calculated based on the numerical value of fold change. So that, fold change > 1 was considered as an increase in the level of gene expression changes in the tumor tissue relative to the tumor margin. The miR-1290 expression in CRC patients was significantly higher than the expression level in normal tissue (P<0.0001, **Figure 2a**), while the targets of miR-1290, THBS1 (P<0.0001, **Figure 2b**) and DKK3 (P<0.0001,

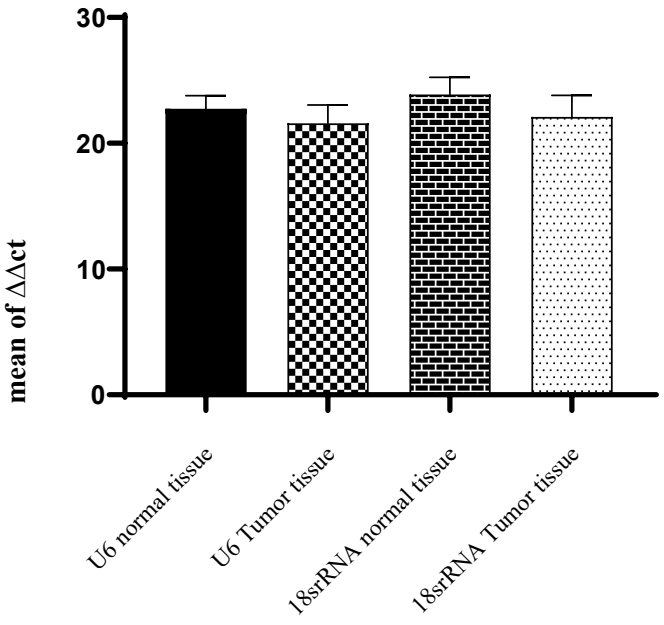

Figure 1. U6 and 18srRNA expression in normal and tumor tissue. Expression levels displayed no significant change in the two groups.

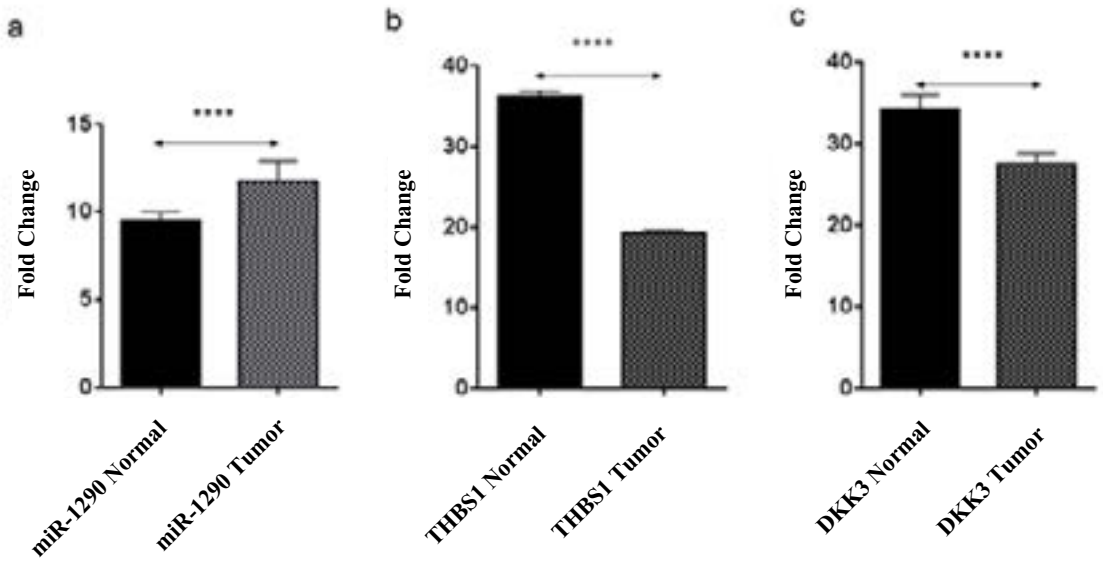

Figure 2. The expression of miR-1290 (a), and its target genes THBS1 (b) and DKK3 (c) in the CRC and normal tissues.

Figure 2c), were down-regulated.

Expression evaluation of miR-1290 and its target genes in adjuvant and non-adjuvant chemotherapy samples

The expression level of miR-1290 in CRC patients who received adjuvant chemotherapy and those without adjuvant chemotherapy showed no significant difference (**Figure 3a**). Analysis of Real-time PCR data indicated that 53 out of 72 (73%) showed a decrease in THBS1 gene expression, and 34 out of 72 (34%) indicated a reduction in DKK3 expression ($P < 0.0001$).

Correlation of miR-1290 expression with clinicopathological factors

The high expression level of miR-1290 was correlated with lymph node metastasis and advanced clinical stage in the non-adjuvant group (p-values were 0.005 and 0.001, respectively). There was no correlation between miR-1290, DKK3, and THBS1 expression with age, gender, and tumor size (p-value > 0.05). The results of our study showed that DKK3 downregulation was correlated with lymph node metastasis in the adjuvant therapy group (p-value = 0.001). Besides, THBS1 was associated with lymph node metastasis in non-adjuvant therapy tissue samples (p-value = 0.002)

Area Under the ROC Curve (AUC): a Measure of Overall Diagnostic Performance

To determine the diagnostic accuracy of the miR-1290 between CRC tissue and normal tissue, the AUC was calculated (0.717, p=0.0001) (**Figure 4**).

DISCUSSION

CRC is one of the leading causes of cancer-associated death. The CRC frequency and fatality in Iran have increased quickly in the past several decades (15, 16)Early detection of CRC and precancerous lesions is crucial

for reducing the mortality rate(2). Molecular biomarkers play an important role in the development of cancers(17). Using biomarkers is an effective way of early detection and following disease progression or response to treatment. MiRNAs have been proposed as novel diagnostic or prognostic biomarkers with high specificity and sensitivity for clinical application(18, 19). Several studies have shown that alterations in miR-1290 expression in CRC tissue and serum, suggesting that miR-1290 has clinically significant diagnostic value in CRC. Therefore, the upregulation of miR-1290 may be involved in CRC progression(20-22). The findings of this study indicated that miR-1290 was significantly upregulated in colon cancer tissue compared to adjacent normal tissue and has been suggested to promote tumor progression. It has been reported that upregulation of miR-1290 causes cytokinesis failure, and leads to the multinucleated cell formation and cellular reprogramming in colon cancer(23). Although the mechanism of miR-1290 involvement in CRC remains elusive, there are several pieces of evidence demonstrating that miR-1290 induces EMT and subsequently increases cancer invasion and metastasis. EMT is relevant to the maintenance of stem cell-like properties through the Wnt signaling pathway. DKK3, as one of the validated targets of miR-1290, is a secreted protein that belongs to the DKK protein family with a vital role in the Wnt signaling pathway(12). Our result indicated a reverse correlation between miR-1290 and DKK3 expression. It has been shown that DKK3 suppression could activate the Wnt pathway and act as a tumor suppressor in CRC(14). The result of the present study revealed that DKK3 downregulation is correlated with lymph node metastasis in the non-adjuvant therapy group. A correlation between DKK3 downregulation and lymph node metastasis has also been shown

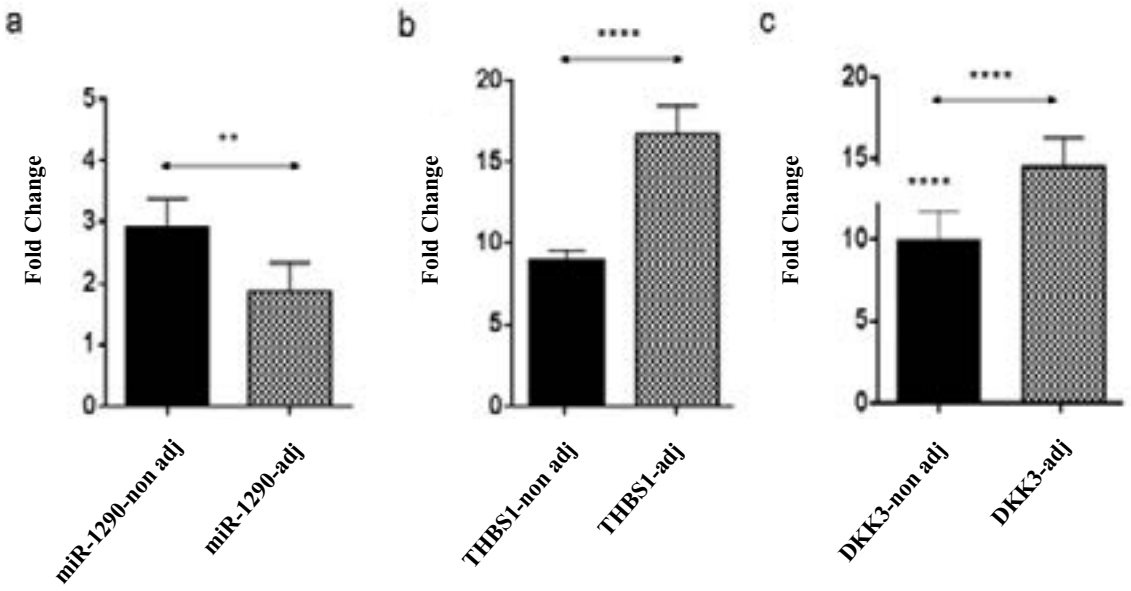

Figure 3. Expression level of miR-1290 (a), THBS1 (b), and DKK3 (c) genes in tumoral tissues *in patients who received adjuvant therapy versus the control group.*

in gastric cancer(24). Hence, DKK3 suppression may contribute to poor survival and tumor progression. Furthermore, our findings indicate that the upregulation of miR-1290 in tumors compared to normal tissue is associated with suppression of THBS1. We have shown that THBS1 is associated with lymph node metastasis in non-adjuvant therapy tissue samples. THBS1 is a tumor-specific ECM protein associated with migration and invasion of cancer cells and induces the expression of Matrix metallopeptidases (MMPs) through integrin signaling, therewith favoring CRC metastasis(25).

On the other hand, adjuvant therapy is the first-line treatment option for colon cancer treatment and improves the survival of patients with advanced colon cancer. In our study, following the adjuvant therapy in CRC patients, it was found that THBS1 and DKK3 were significantly increased in tissue samples compared with the non-adjuvant chemotherapy group, which may indicate the beneficial effect of chemotherapy at this stage of cancer. Chemotherapy might exert its effect by inhibiting miR-1290 expression, an oncomiR, in cancer progression, and by enhancing the THBS1 and DKK3 tumor suppressor genes as inhibitors of cancer progression.

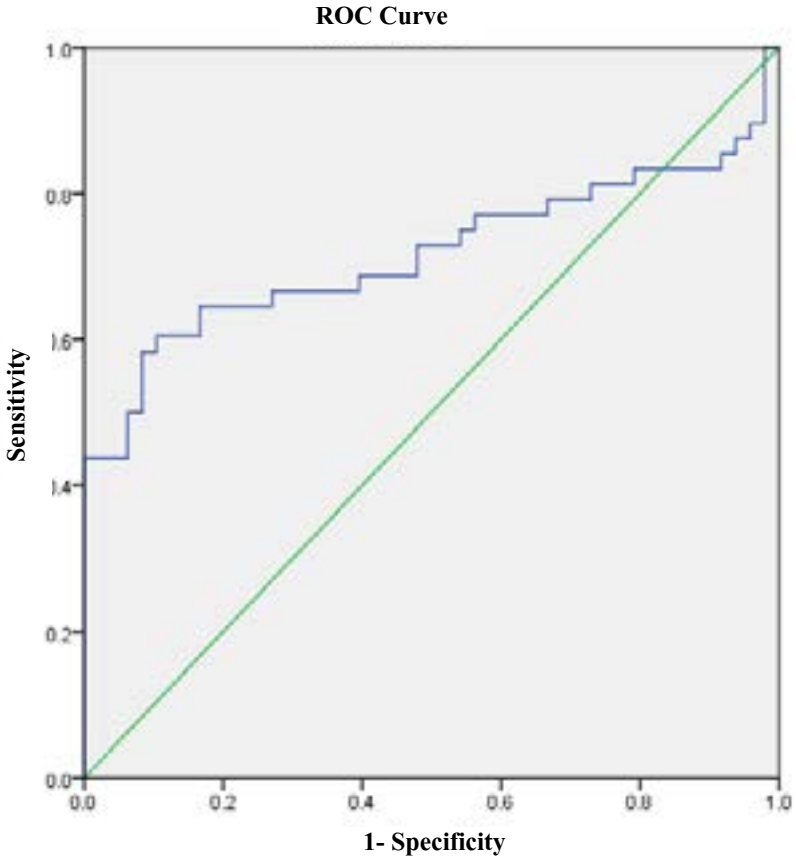

Figure 4. ROC curve analysis of a miR-1290 expression study on 73 patients with CRC (AUC=0.717).

CONCLUSION

In summary, the present study provides evidence of the relationship between response to adjuvant chemotherapy and miR-1290 downregulation in CRC tissue. Moreover, we confirmed the biomarker role of miR-1290 in CRC diagnosis and identified the THBS1 and DKK3 genes as targets in CRC. These findings provide a hypothesis for the future use of the miR1290/THBS1/DKK3 panel in CRC diagnosis and prognosis.

CONFLICT OF INTEREST

The authors declared no conflicts of interest.

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