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The Expression Level of Mir-210 and ZEB1 Gene in CRC Patients

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Abstract

Background: Colorectal cancer is the fourth leading cause of cancer deaths around the world. This type of cancer, like other cancers, is caused by the influence of environmental and genetic factors. Several miRNAs and genes are involved in the initiation and development of colorectal cancer which among them can point to Mir-210 and ZEB1 gene. Recently molecular studies have been conducted on early diagnosis of colorectal cancer. In this study, expression levels of Mir-210 and ZEB1 gene were reported in CRC patients.

Methods: The expression levels of Mir-210 and ZEB1 were checked by Real Time-PCR method in 60 CRC tissues and 60 adjacent normal tissues after RNA extraction and cDNA synthesis. The comparative threshold cycle ($2^{-\Delta\Delta CT}$) method and T-test was used to compare expression in tumor and controls group.

Results: This study for Mir-210 reported that 75% of tumor samples have increased of expression levels comparison with normal samples (P=0.001) and 60% of tumor samples showed an increase in expression level for ZEB1 gene (P=0.007). Finally, there is a significant difference between the increase of the Mir-210 expression level and stage of cancer (P>0.05).

Keyword - Colorectal cancer, Mir-210, ZEB1, Real Time-PCR method.

I. INTRODUCTION

Colorectal cancer is one of the most common cancers in the gastrointestinal tract that is the second leading cause of death in women (9.4% of all cancer) after breast cancer and in men it is the third leading cause of death (10% of all cancers) after lung and prostate carcinoma, also it is the fourth cause of death of cancers in the world (Duraes et al. 2018). The annual incidence of this cancer in North America and Europe is reported to be between 30 and 50 cases per 100,000 people so that estimated at 3 to 7 per 100,000 in the Middle East. In 2016, 49,190 people were diagnosed with this cancer in the United States (Luo et al. 2018). The symptoms of colorectal cancer include intestinal problems such as diarrhea and constipation, dark stools, intestinal bleeding and weight loss. The cause of colorectal cancer, as with other cancers, is unclear, but evidence and experience indicate that two important environmental and hereditary factors contribute to its formation and sometimes both factors with together can cause cancer (Dolatkhah et al. 2015). In addition, the risk factors for developing colorectal cancer include age, personal history of adenomatous polyps, personal history of inflammatory bowel disease, family history of colorectal cancer or adenomatous polyps, inherited genetic risk, also of the environmental factors can be mentioned to nutritional practices, physical activity and obesity, cigarette smoking, heavy alcohol consumption (Wang et al. 2016). According to recent studies, several miRNAs and genes are involved in the initiation and development of colorectal cancer which among them can point to Mir-210 and ZEB1 gene. microRNA-210 has been largely studied in the past several years and has been identified as a major miRNA induced under hypoxia. A variety of miR-210 targets have been identified pointing to its role, not only in mitochondrial metabolism, but also in angiogenesis, the DNA damage response, cell proliferation,

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and apoptosis (Ye et al. 2019). One of the most important genetic targets of Mir-210 is ZEB1. Highly expressed Zinc-finger E-box binding protein 1 (ZEB1) is significantly associated with the malignancy of various cancers. Signal transduction and activation of ZEB1 play important roles in cancer transformation and epithelial-mesenchymal transition (EMT) (Lehmann et al. 2016). Emerging evidence suggests that ZEB1 drives the induction of EMT with activation of stem cell traits, immune evasion and epigenetic reprogramming (Zhang et al. 2015). As an ideal target for EMT research, ZEB1 has been extensively studied for decades.

II. Material And Methods

2.1 Human specimens

Sixty Human colorectal cancer specimens and matched adjacent non-tumor tissues were obtained from patients at Masih Hospital, Tehran, Iran, with informed consent from each patient. These patients didn't receive any treatment such as chemotherapy or radiation therapy, yet. Clinico-pathologic information's of patients are presented in Table 1.

2.2 RNA extraction and cDNA preparation

Total RNA was isolated from all tissues by using RiboEx (GeneAll, Korea) according to the manufacturer's specifications. The concentration of total RNA in the final eluate was determined by spectrophotometry and the absorbance 260/280 ratio was controlled between 1.8 and 2.0. The cDNA synthesis process was performed using the Prime Script RT reagent kit (Takara, Japan) according to the manufacturer's specifications. The obtained cDNAs were stored in -20℃ until use. The sequences of miRNA-specific stem-loops used for synthesis of cDNA are listed in Table 2.

Table 1. Clinicopathologic characteristics of sixty colorectal cancer patients.

Variable	Value (n = 60)
Gender	
Male	41 (68.3%)
Female	19 (31.7%)
Age (years)	
Under 50 (43.7)	7 (11.6%)
Between 50-60 (57.1)	18 (30%)
Between 60-70 (63.9 ± 0.08)	21 (35%)
Over 70 (79.6 ± 0.06)	14 (23.4%)
Cell type	
Adenocarcinoma	60 (100%)
Stage	
I	8 (13.3%)
II	20 (33.3%)
III	11(18.4%)
IV	21 (35%)

Table 2. Sequences of miRNA-specific stem-loops used for synthesis of cDNA.

Target Name	Sequences (5`→ 3`)	Length
Mir-210	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCAC TGGATACGACTCCATC	**50**
RNU44	GTCGTATCCAGTGCAGGGTCCGACCGGTATTCGC ACTGGATACGACAGTCAG	**52**

2.3 Real-time quantitative PCR

Real-time PCR was performed using a StepOnePlus™ Real-Time PCR Systems (ABI Applied Bio-systems, Thermo Fisher Scientific, USA) in a 15-µl reaction containing 7.5-µl of Real Plus 2x Master Mix Green High ROX™ (*Ampliqon, Denmark*), 1-µl of cDNA, 5.5-µl of H2O and 1-µl of mixed forward and reverse primers (6 Pmol/µl concentration). Real-time PCR amplifications were done as follows: for selected miRNAs and ZEB1 gene, PCR amplification was set to an initial 95°C for 15 min and then, a total of 35 cycles, 95°C for 15 seconds and 59°C for 1 min. All samples were analyzed in duplicate and RNU44 and GAPDH was used as an internal control. The primers used for real-time PCR are reported in Table 3.

Table 3. Primer sequences for selected targets.

Target genes		Sequences (5`→ 3`)
Mir-210	Forward	CGTACCAGTGCGTGTGACAG
	Reverse	CCAGTGCAGGGTCCGAGGTA
ZEB1	Forward	AGCAGTGAAAGAGAAGGGAATGC
	Reverse	GGTCCTCTTCAGGTGCCTCAG
GAPDH	Forward	CATCAAGAAGGTGGTGAAGCA
	Reverse	GCGTCAAAGGTGGAGGAGTG
RNU44	Forward	CCTGGATGATGATAGCAAATG
	Reverse	TCGTATCCAGTGCAGGG

2.4 Statistical Analysis

Gene expression was estimated using the comparative threshold cycle ($2^{-\Delta\Delta CT}$) method and T-test was used to compared expression in tumor and controls group. All tests, was performed using the GraphPad Prism software v7.03 (GraphPad Software Inc., USA) and *P* value <0.05 was considered statistically significant.

III. RESULTS

3.1 Comparison of Mir-210 and ZEB1 expression between CRC tissue and adjacent non-tumor tissues

The results obtain of Mir-210 expression in 60 normal and tumor tissues demonstrated that there is a significant difference between the expression level of this microRNA in tumor tissues compared with normal tissues (P=0.001). For Mir-210, 45 (75%) samples of 60 tumor samples showed an increase in Mir-210 expression level. Whilst, 15 (25%) tumor samples reported a decrease in Mir-210 expression level. Also, the increase of ZEB1 expression level were observed in 36 (60%) tumor tissues and ZEB1 expression levels were significantly higher in CRC tissues than adjacent non-tumor tissues (P=0.007)

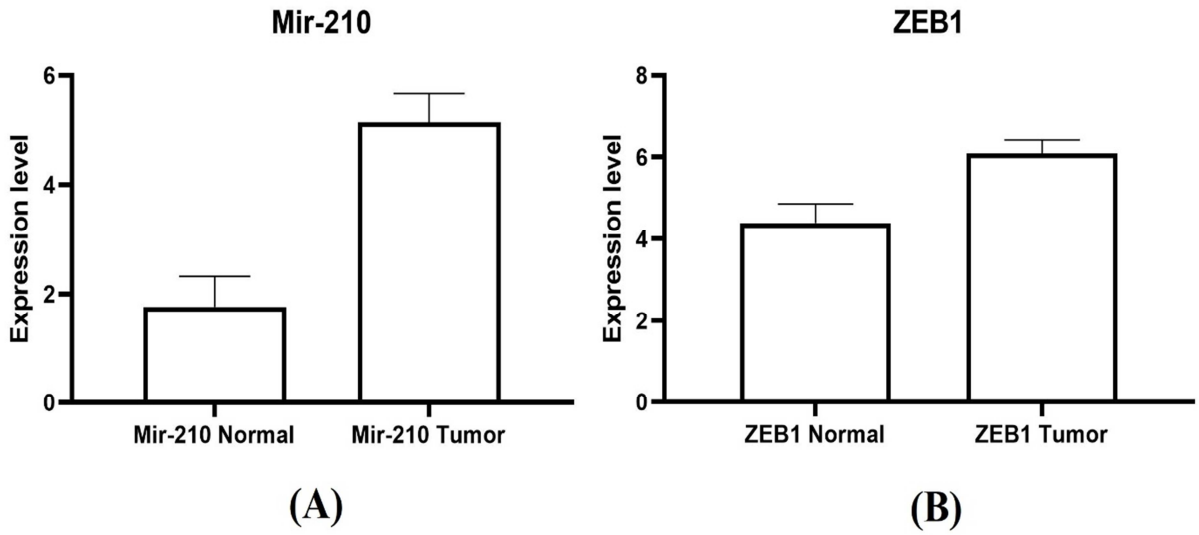

Figure 1. The graph is related to (A): comparison of Mir-210 expression level between tumor and adjacent non-tumor tissues, (B): comparison of ZEB1 expression level between tumor and adjacent non-tumor tissues.

3.2 Correlation between Mir-210 and ZEB1 expression and clinic-pathologic parameters

Using statistical analyzes, the correlation was examined between the expression level of Mir-210 and ZEB1 against gender, age, and TNM-stage of CRC. According to the results of Mir-210 (Table 4), there is a significant difference between the increase of the Mir-210 expression level and stage of cancer. It means that samples which have been at the advanced stage of cancer, illustrated the higher expression than early-stage samples (P<0.05). The ZEB1 expression level was not correlated with any clinicopathological parameters (P>0.05).

Table 4. Correlation between Mir-210 and ZEB1 expression and clinicopathological parameters in CRC patients.

Variable	N0. of cases	Mir-210 expression		P value	ZEB1 expression		P value
		High (45)	Low (15)		High (36)	Low (24)	
Gender				0.110			0.144
Male	41	23	18		20	21	
Female	19	12	7		8	11	
Age (years)				0.087			0.061
Under 60	25	18	7		14	11	
Over 60	35	23	12		19	16	
Stage				0.017			0.071
I- II	28	9	19		13	15	
III- IV	32	23	9		18	14	

IV. DISCUSSION

Over the last few years, a great number of studies have reported aberrant patterns of miRNAs expressions in various cancers including colorectal cancer. Therefore, understanding of the different mechanisms involved in the onset and progression of colorectal cancer can provide the basis for better treatment. In this study, we have analyzed the expression level of mir210 and ZEB1 gene in sixty colorectal cancer patients by real-time quantitative PCR.

Earlier researches on these targets determine different results in various cancers. Puissegur et al. demonstrated that the expression rate of mir210 was significantly increased in lung cancer tissues rather than normal tissues (Puissegur et al. 2011). Also, Yang et al. indicate that miR-210-3p plays an important role in the regulation of bladder cancer growth and metastasis in vitro and in vivo through targeting FGFRL1 (Yang et al. 2017). In this study, we analyzed the expression level of Mir-210 in 60 colorectal cancer patients and we demonstrated that Mir-210 was upregulated in CRC tissues compared to control group. It's clear that dysregulation of Mir-210 can disturb the normal cellular functions and causes the onset and progression of the CRC. Analysis of the clinicopathological parameters showed that the miR-210 expression level was associated with advanced TNM stage.

Another gene that was investigated in this study was ZEB1, and it has already been studied in various cancers. Hasuwa et al. (2013) described anovulation and infertility in female mice lacking the microRNAs miR200b and miR429. Both miRNAs are strongly expressed in the pituitary gland, where they suppress expression of the transcriptional repressor ZEB1 (Hasuwa et al. 2013). Park et al. (2008) found that expression of the miR200 family of microRNAs in human cell lines was associated with an epithelial phenotype and with expression of E-cadherin, an epithelial cell marker. They identified multiple miR200 target sequences in the 3-prime UTRs of the E-cadherin transcriptional repressors ZEB1 (Park et al. 2008). The results showed that ZEB1 expression is higher in samples of higher malignancy and that overexpression of ZEB1 was able to induce epithelial–mesenchymal transition by upregulating the mesenchymal marker Vimentin and downregulating the epithelial marker E-Cadherin. On the contrary, ZEB1 silencing repressed Vimentin expression and upregulated E-Cadherin. These data suggest the Mir-210 and ZEB1 has an important and crucial role in the initiation and progression of CRC and it can be used as a diagnostic marker and potential molecular therapeutic target.

V. Conclusions

In this paper, we analyzed the expression level of Mir-210 and ZEB1 in 60 colorectal cancer patients and we demonstrated that Mir-210 was upregulated in CRC tissues compared to control group which is associated with advanced TNM stage. Moreover, the expression level of ZEB1 was significantly increased in CRC tissues compared to normal colorectal tissues. Mir-210 and ZEB1 can also be used as a diagnostic and therapeutic marker if it has other features.

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