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ORIGINAL RESEARCH

miR-21: a promising biomarker for the early detection of colon cancer

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Purpose: The aim of this study was to compare the expression of *miR-21* gene in stages II-IV of formalin-fixed paraffin-embedded (FFPE) tissue in patients with colon cancer and introduce *miR-21* as a potential molecular marker for detection of colon cancer in the early stages.

Introduction: Currently, identification of key molecules involved in the pathogenesis of cancer is one of the areas under consideration. miRNAs, are small RNAs which have been identified in many cancers. In this study, we investigated the expression of *miR-21* in three pathologic stages in patients with colon cancer in the north of Iran.

Patients and methods: A total of 40 FFPE samples were obtained from patients with stages II, III, and IV from hospitals in Mazandaran and Golestan provinces. After extraction of RNA, treatment with DNase I and cDNA synthesis was performed and *miR-21* expression was assessed by qPCR. Then, the data were analyzed using statistical software R (3.4.3).

Results: The expression of *miR-21* in stage II was significantly different from stage IV. However, no significant difference was observed between the other stages. In stage II, the level of *miR-21* expression was higher in men than women. Moreover, in the second pathological stage, miR-21 expression was reduced in patients with adjacent lymphoid tissue engagement. In addition, the expression of *miR-21* in grade I was significantly higher than grade II.

Conclusion: The results of this study suggest that *miR-21* can be a diagnostic marker for early stages of colon cancer, especially in men. It can also be considered as a good candidate for targeted treatment of colon cancer in the early stages of the disease. Furthermore, for the first time, we suggested that *miR-21* can be a good molecular marker for classification of the stages of colon cancer.

Keywords: colon cancer, pathological stages, miR-21, gene expression, biomarker, early diagnosis

Introduction

The exact diagnosis and effective treatment of cancer depends on the exact recognition of its molecular characteristics at different stages of the cancer, if this disease is diagnosed early, it can be treated efficiently.[1] Colon cancer is pathologically classified into five stages (0, I, II, III, IV). However, the same treatment for patients at each stage does not result in the same consequence. This may be due to the presence of different molecular features in tumor cells in different stages.[2] Therefore, better understanding of the molecular characteristics of cancer cells helps to form a better classification of tumors. Functional differences in the types of tumors and various stages of cancers are related to the expression of the miRNAs. Moreover, the expressions of the miRNAs are related to the clinical and biological features of the tumor, such as tissue type, differentiation, invasion and response to treatment.[3] Furthermore, since miRNAs are

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the key regulators of gene expression and have unique features among the molecular markers, the use of miRNAs to classify tumor progression stages is more appropriate than other molecular markers such as mRNA and proteins.[4] miRNAs are subgroups of non-coding RNAs, with a length of 17–22 nucleotides that are evolutionally protected and their roles in the onset and progression of various cancers has recently been confirmed.[3,4] These molecules, through their seed regions (2–7 nucleotide) attach to the microRNA response element (MRE) regions at the 3′-UTR of mRNA of targeted genes and regulate their expression by inhibiting translation or degradation and depending on the type of genes that they inhibit, they play the role of tumor suppressors or oncogenes.[5,6] Due to the coupling of miRNAs with a large number of target mRNAs, miRNAs can target the expression of many genes in multiple paths.[7] Bioinformatics analyses showed that more than 50% of the human genome is regulated by the miRNAs which include more than 1% of the human genome.[8,9] Altering *miR-21* expression as a crucial miRNA has been shown in many cancers. *miR-21* with targeting genes such as *PDCD4*,[10] *NF-KB*,[11] *RECK*,[12] *PTEN*,[13] *TPM1*[14] and by regulating apoptosis,[15] cell proliferation and migration[16] plays an important role in the types of cancers, including gastrointestinal cancers.[17,18] Our goal in this study was to evaluate and compare the expression of *miR-21* in colon cancer stages II–IV stages, in order to evaluate *miR-21* as a potential biomarker, for prognosis, and examining its expression in various stages of colon cancer.

Materials and methods

Population studied (experimental design)

A total of 40 formalin-fixed paraffin-embedded (FFPE) samples from the stages of II, III and IV of colon cancer which pathologically approved were collected from Imam and Shafa hospitals in Sari city and Khatam al-Anbia hospital in Gonbad kavous city between 1996 and 2000 and their pathological information was recorded Table 1.

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Khatam al-Anbia hospital.

Extracting miRNA

First, sections with a diameter of 15 microns from FFPE samples were prepared by using a microtome system, and deparaffinization performed based on ethanol and xylene combination protocols. In order to extract the miRNA from the paraffin tissue, the miRNeasy FFPE kit (Qiagen NV, Hilden, Germany) was used, according to the manufacturer's protocol, which extracted RNAs with a length of less than 200 nucleotides, including miRNAs. Then with the Pico Drop (Pico200, Quantica) device, the

Table 1 Pathological characteristics of the patients

Variable	Levels	Stage II (n=17)	Stage III (n=13)	Stage IV (n=10)
Degree	GI GII GIII GIV	13 (76%) 4 (24%) 0 0	8 (61%) 4 (31) 1 (8%) 0	0 7 (70%) 0 3 (30%)
Age (years)	≤55 >55	10 (59%) 7 (41%)	9 (69%) 4 (31%)	5 (50%) 5 (50%)
Sex	Man Female	12 (71%) 5 (29%)	8 (62%) 5 (38%)	4 (40%) 6 (60%)
The size of the tumor	≤4 >4	11 (65%) 6 (35%)	11 (85%) 2 (15%)	9 (90%) 1 (10%)
The condition of lymph is involved	P* N**	2 (12%) 15 (88%)	12 (92%) 1 (8%)	9 (90%) 1 (10%)
Location of the tumor	Left colon Right colon	11 (65%) 6 (35%)	7 (54%) 6 (46%)	- -
Stage IV metastasis	L*** AW****			8 (80%) 2 (20%)

Notes: *Lymph involved; **no lymph involved; ***metastasis to the lymph nodes; ****metastasis to the abdominal wall.

extracted RNA concentration is assessed about 320 ng/μL and optical absorption ratio at wavelengths of 260 and 280 (A260/280) was 1.8–2 which indicates the proper quality of extracted RNAs.

Synthesis of cDNA and quantitative real time PCR

The study of *miR-21* proliferation was performed using the PARSGENOME MiR-Amp kit. The *RNU6B* was used as a reference gene to compare *miR-21* expression in different stages. Firstly, by the PolyA polymerase enzyme, at the 3′ end of all RNAs, polyA tail were added according to the protocol. In the second step, PolyA RNAs were used to synthesize cDNA using Reverse Transcriptase enzyme. In the third step, the study of gene expression with specific primers of *miR-21* and *RNU6B*, was performed using SYBR Green in qPCR technique. Each sample was amplified as a duplicated reaction which used, 2 μL cDNA (9-fold diluted) in each reaction and amplified as the following program by Step One Plus Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Wlatham, MA, USA): Initial denaturation for 5 minutes, at 95°C and 40 cycles, 95°C, 5 seconds, 63°C, 20 seconds, 72 °C, 30 seconds.

Statistical analysis

Analysis of Real Time PCR data was performed using R (3.4.3) software and all *p-values* were two-sided (inequality between groups was observed). Our primary goal was to investigate the difference in expression of the *miR-21* in various stages of colon cancer and its role as a molecular biomarker in identifying the pathological stages of colon cancer. The expression of *miR-21* in the specimens was calculated using the $2^{-\Delta CT}$. Data were analyzed by K-test and Mann Whitney (u-test). Then, the association of miR-21 expression with pathologic characteristics of patients was investigated.

Ethics approval and informed consent

All participants were informed about the purpose of the study and all signed the written informed consent. The study was approved by the Ethics Committee of Khatam al-Anbia hospital and the study was conducted in accordance with the Declaration of Helsinki.

Results

The study of the distribution of *miR-21* expression data in all three stages by Kruskal-Wallis test indicated a lack of normal distribution of data and the comparison of the mean of expression of *miR-21* in the three stages was statistically significant (p-*value*=0.04). This means that the average expression of this gene is different at least in one of the stages, therefore, the u-test was used. The results of this test showed that the mean expression of *miR-21* gene in the studied patients had a significant difference in stage II compared to stage IV (p-*value*<0.05), However, there was no significant difference between stages II and III and stages III and IV (p-*value*>0.05) (Figure 1).

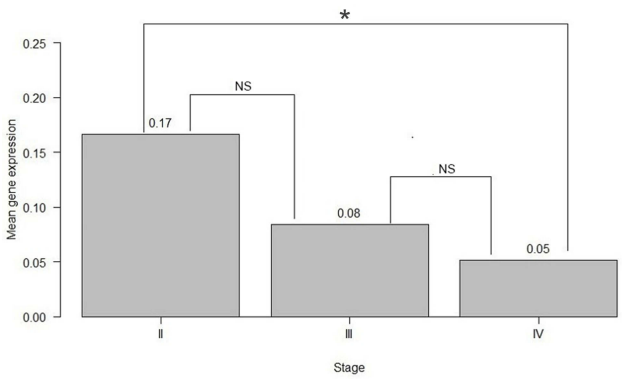

Figure 1 Expression of miR-21 in different stages of the disease. The expression of miR-21 in stage IV was significantly reduced compared to stage II (*p-value <0.05).
Abbreviation: NS, non-significant.

Assessment of *miR-21* in all stages showed that the expression was higher in men than women, with the ratio of 2.27 fold-change (p-*value*=0.006) (Figure 2). This is due to the apparent difference in expression of *miR-21* in stage II (p-*value*=0.0003). The expression of this gene in stages III and IV does not show a significant difference between men and women (Figure 3). Investigating the expression of *miR-21* in different degrees of disease showed that expression of this gene in grade I specimens was greater than grade II (p-*value*=0.00002) (Figure 4).

Investigating the lymphatic vessels involved in the tumor with the disease stages showed that in most patients in stage III and IV, lymphatic vessels were involved, but in a few patients with stage II, the lymph's vessels were involved. In this regard, the evaluation of the association of *miR-21* expression with the state of the lymph involved in stage II disease indicates that *miR-21* expression in patients with no lymph nodes is higher than those with lymph nodes involved in the tumor (p-*value*=0.03) (Figure 5).

In almost all patients in stage II and III, the tumor tissue involved the colon sigmoid (left colon), and patients

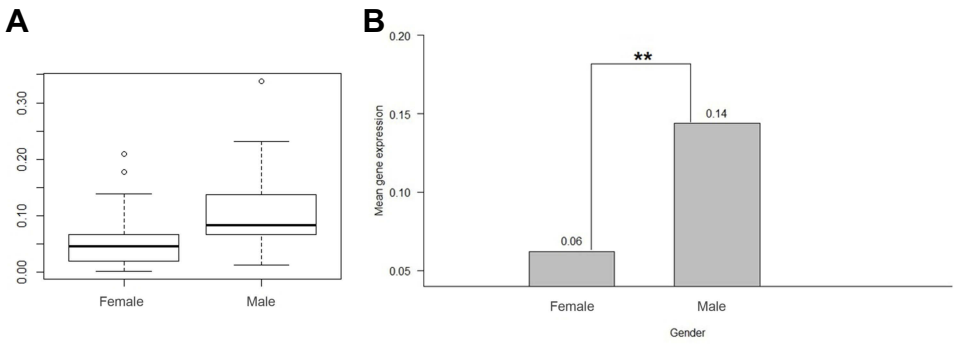

Figure 2 Expression of miR-21 in men and women in all stages. (**A**) Distribution of miR-21 gene expression. (**B**) The mean expression of miR-21 is significantly higher in men than women (**p-value <0.01).

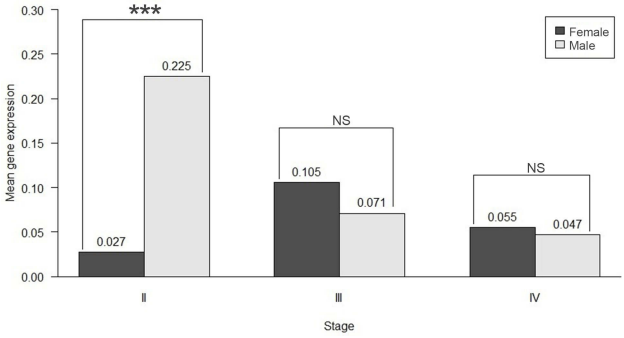

Figure 3 Relationship between expression of miR-21 and the sex of the subjects in different stages of the disease. In the second stage of the disease, the expression of miR-21 in men is significantly higher than women (***p-value <0.001), which is not significant at other stages.
Abbreviation: NS, non-significant.

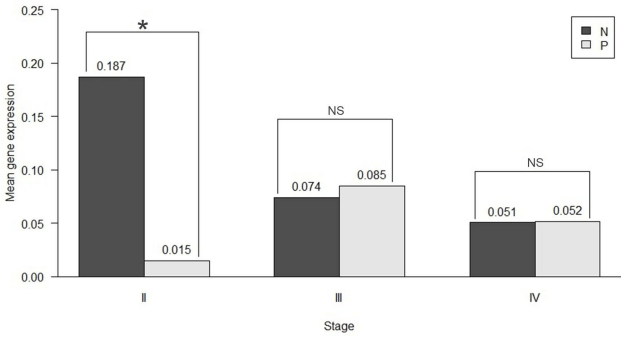

Figure 5 Expression of miR-21 with the condition of the lymph involved (*P*) in various stages of the disease. Expression of miR-21 in stage II in tissues without lymphatic vessels (N) is more than those involved (*p-value <0.05), which is not significant in other stages.
Abbreviation: NS, non-significant.

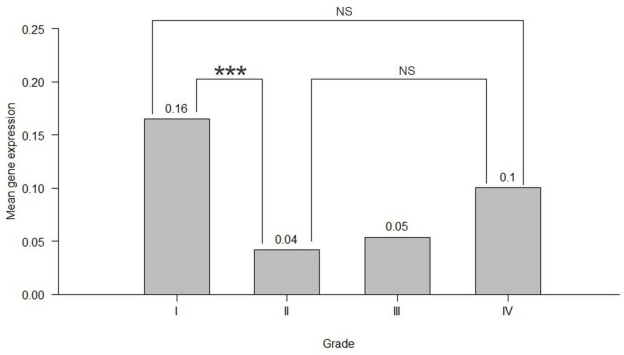

Figure 4 Gene expression with different degrees of disease. Using the u-test, it was shown that the increase of expression of miR-21 in grade I was significantly higher than grade II (***p-value <0.001). Because of the low number of people with grade III no comparisons were made between this stage and other stages.
Abbreviation: NS, non-significant.

in stage IV more often metastasis to the lymph nodes. However, the expression of *miR-21* did not reveal any relationship with the tumor site at different stages. Also, in metastatic samples (stage IV), no relationship was found between the expression of *miR-21* and the presence of secondary tumor. In addition, the expression of *miR-21* did not show any association with tumor size.

Discussion

The best way to diagnose and provide therapy for colon cancer is to pay attention to pathogenesis and its molecular events.[19] miRNAs as the key molecules regulating gene expression plays an important role in the tumorigenicity and progression of cancers. *miR-21* as an oncomir is widely expressed in tumor tissue of colon cancer, which increases cell proliferation, invasiveness and cancer progression.[20]

Several *in vivo* and *in vitro* studies have been performed to investigate the role of oncogenicity of *miR-21*.[21,22] For example, a study in 2009 in the United States by using mouse models and anti-miR-21 injections or siRNAs CDC25A showed that *miR-21* could inhibit *CDC25* and inducing tumors in the colon.[23] In another study Muppala et al[24] showed that c-SRC inhibition with siRNA-SRC ultimately inhibited *miR-21* and thus did not inhibit PTEN and PDCD4

tumor suppressor proteins, thereby inhibiting tumor growth and progression in colon cancer.

However, few studies have been conducted on the pattern of expression of miRNAs in various stages of cancer. The present study is a part of the early studies to classify stages and grades of colon cancer based on the *miR-21* gene expression. The pattern of *miR-21* gene expression in different stages of colon cancer has shown different results. However, most studies have reported the expression of *miR-21* in stage II more than the other stages, and no significant difference has been reported in other stages. For example, Conev et al[25] showed that, if the *miR-21* gene expression is high in patients with colon cancer stage II, they are prone to recurrence.

Higher prevalence of colon cancer in men than women is reported in most parts of the world.[26] The gender-dependency of *miR-21* and *miR-16* expression was disclosed in human colorectal cancer by Hasáková et al.[27] Our findings showed a significant increase of *miR-21* gene expression in stage II colon cancer in males than in females (Figure 3). In agreement with our report, two cohort studies used a hybridization analysis to demonstrate higher expression of *miR-21* in men than women.[28,29] This tendency might be due to the activity of male hormones, for example, steroid male hormones such as testosterone, can affect the synthesis of Pri-miR-21.[27] In addition, this increase could be caused by some risk factors such as unhealthy diet, obesity, and tobacco consumption which men are more exposed to.[30] The significant difference in the lower stages can result from difference in access to medical care and their knowledge about colorectal cancer.[31,32] This may also be due to the different expression of *miR-21* isomirs in different stages of colon cancer.[33] However, this trend was not observed in all studies.[34]

The amount of lymph involved in various stages of colon cancer was another pathologic factor assessed. One of the factors that has a crucial role in cancer development is inflammation.[35] Some inflammatory pathways cause up-regulation of *miR-21* such as S100P/RAGE and COX-2.[36,37] Moreover, *miR-21* expression, significantly activates various types of immune cells.[38–40] Sacchi et al[41] revealed that activated mast cells could destroy lymphatic vessels to prevent cancer metastasis. Furthermore, the amount of lymph involved could be a suitable predictor of lymph node metastasis of submucosal colorectal cancer.[42] Based on the results obtained from patients in stage II, the current study showed higher *miR-21* expression in non-lymph specimens (p-*value*=0.03) (Figure 5). Accordingly, a high level expression of *miR-21* can be indicative of the early stages of colon cancer and a low possibility of metastasis. So, it seems that *miR-21* is one of the markers that is useful for sub classifying colon cancer stage II.

Although some studies suggest a direct relationship between the expression of *miR-21* and tumor size[14,43] the present study is unanimous with other studies in Iran, and rejects this relationship.[44,45] Also, our data is consistent with another study in Iran which showed that there is no association between *miR-21* expression and location of tumor and type of metastasis in colon cancer.[45] These studies may suggest that the expression of *miR-21* in Iranian colon cancer patients is not related to the occurrence of tumor and the type of metastasis, which is not unanimous with some studies in the world.[46] In summary, the results of current studies indicate that the characteristics of colon cancer in Iranian patients are different than other communities.

Conclusion

Our findings reinforce the probability that *miR-21* can be a diagnostic molecular marker in the early stages of colon cancer, especially in men. In addition *miR-21* can be considered as a good candidate for molecular classification of colon cancer stage II as well as an appropriate therapeutic goal.

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Disclosure

The authors report no conflicts of interest in this work.

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