Valizadeh *et al.*
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RESEARCH

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Evaluation of the biomarker potential of miR-650 and miR-663b in tumor tissues and plasma specimens of colon cancer patients living in northwest of Iran

Mehdi Valizadeh[1], Jabar Kamal Mirza Abdalla[2], Abbas Yazdanbod[3] and Esmaeil Babaei[2*]

Abstract

Background Colorectal cancer (CRC) is considered as one of the most common malignancy and the fourth leading cause of cancer-related deaths, worldwide. Here, we aimed to investigate the expression of miR–663b and miR–650 in CRC tissue and plasma specimens.

Methods In this case–control study, tumor specimens, non-tumoral adjacent tissues, and matched-plasma samples were obtained from forty patients with CRC living in the northwestern of Iran. Plasma of healthy patients was also collected as control. Total RNA was extracted from all specimens and studied by real-time PCR. Furthermore, the correlation between the expression of microRNAs and clinico-pathological features were also studied.

Results Our data illustrated that miR-650 and miR-663b are down-regulated and up-regulated in tumor samples compared to non-tumoral margins, respectively ($p < 0.001$). However, the results did not show any significant difference in patient's plasmas compared to controls. Further analysis disclosed that the expression of miR-663b is significantly associated with tumor size, lymph node metastasis, and tumor stage, while miR-650 is remarkably related to TNM stage, lymph node metastasis, distant metastasis, tumor size, and age. ($p < 0.05$) Furthermore, receiver operating characteristic (ROC) analyses revealed that miR-650 and miR-663b are potential biomarkers in differentiating CRC patients from healthy controls.

Conclusion In conclusion, our data illustrated the potential of miR-650 and miR-663b as biomarkers in colorectal cancer. However, further studies are needed to confirm the employment of these microRNAs in the diagnosis and/or prognosis of colorectal malignancies.

Keywords Biomarker, microRNA, Colon cancer, Quantitative Real-time PCR

*Correspondence:
Esmaeil Babaei
babaei@tabrizu.ac.ir
Full list of author information is available at the end of the article

Introduction

Colorectal cancer (CRC) is the third most common malignancy that leads to death in effected people [1].

Although CRC incidence and mortality rates are broadly different in the world, recent studies report that more than 1.8 million new cases as well as 881,000 mortalities occurred in 2018. Based on the current trends, the incidence of colon-related cancers are expected to rise up to 90.0% and 124.2% by 2030, respectively [2]. The five-year survival rate of CRC patients varies from 12.5 to 70.4% [2].

The development of CRC depends on many factors including lifestyle, family history, age, diet and bowel inflammations. A few cases are also affected by genetic background [3].

The availability of clinico-pathological features for cancer restricts the evaluation of patient's outcome. Therefore, new appropriate diagnostic and prognostic biomarkers for CRC are mandatory to improve the effective strategies and patient's survival rate. Although the current genetic markers have shown a promising clinical significance, more studies are demanded to confirm the clinical utility of these biomolecules. In recent years, a number of researches have focused on the biological function of non-coding RNAs (ncRNA), predominantly microRNAs (miRNAs) in the pathogenesis of CRC [1, 4].

MicroRNAs are endogenous and short single-strand RNA molecules with approximately 20–23 nucleotides that paly role in gene and protein expression by targeting messenger RNAs (mRNAs). These small non-coding RNAs bind to the 3'-UTRs and either destabilize RNAs or block its translation [5]. Increasing evidence suggests that miRNA deregulations are associated with several disorders such as cancer [6], psychiatric [7], neurodegenerative [8], immune responses [9], aging [10], kidney and liver diseases [11, 12], infertility [13], cardiovascular diseases [14] as well as diabetes mellitus [15].

Study of miRNAs in cancer has gradually shifted from profile studies to biological demonstrations of the potential role of these small cellular entities in tumorigenesis, promotion, and their consequences as biomarkers or therapeutic tools [16].

The miR-650 is located on chromosome 22q11.22, and its sequence is paralogous to the first exon of λ light chain (IgL λ) variable region gene [17, 20]. The miR-650 may play numerous roles in various malignancies. As an oncoMIR, the rearrangement of the immunoglobulin gene leads to the up-regulation of miR-650 in chronic lymphocytic leukemia [17]. Zheng et al. declared that increased expression of miR-650 is related to the progression of hepatocellular carcinoma [18, 19].

Abnormal miR-650 expression has previously been found to be linked to numerous cancers including melanoma, gastric cancer and non-small cell lung. A high level of miR-650 was found to be a prognostic marker for lymph node involvement and more aggressive clinical form of lung adenocarcinoma. However, the mechanism for miR-650-induced tumorigenesis is still unclear [20].

MiR-663b resides on chromosome 2q21.2 [21]. Currently, there is limited research on the role of miR-663b in cancer. The study of Cai et al. [22] shows that miR-663b has an anti-cancer effect in pancreatic cancer and can be controlled by HOTAIR. Another study [23] emphasizes that miR-663b can be considered as a new circulating diagnostic biomarker in bladder cancer. However, the role of miR-663b in colorectal malignancies are unknown until now.

Given the importance and role of miR-650 and miR-663b in cancer, this research was performed to study the expression profile of these miRNAs in colorectal cancer tissues and plasma specimens taken from patients living in northwest of Iran.

Materials and methods

Tumor and non-tumor adjacent tissues (NATs) as well as matched-plasma samples were taken from forty patients undergoing surgery resection in hospitals of the northwestern Iran, between 2019 and 2020.

In the control group, 35 blood samples were obtained from healthy Iranian Azeri who had already been diagnosed without any kind of malignant tumor. They also had no cancer patients in their first and second families. Participants in the control group were matched with the patients, by age, place of birth and lifestyle.

This study was approved by the Biomedical Research Ethics Committee (IR.IAU.TABRIZ.REC.1400.009). Informed written consent was provided for each patient and health volunteer. Clinical data were obtained prospectively for all participants. The healthy specimen was > 2 cm away from the CRC tissue. The inclusion criteria for the study participants were as follows: (i) CRC patients who have histological slides identified by two independent pathologists; and (ii) patients with CRC who had not been treated before surgery. Otherwise, patients were excluded.

Sample preparation and total RNA isolation

The blood samples from all patients and control groups were collected in EDTA tubes and followed by plasma isolation. Briefly, blood samples were precipitated at 1500 g for 10 min to spin out the blood cells, and the supernatants were transferred into the new sterile and RNAse free tubes, followed by a second centrifugation at 3000 g for 5 min. Finally, a third centrifugation was performed at 4000 g for 5 min. The supernatant containing plasma was transferred to RNase/DNase-free tubes and stored at

− 80 °C until use. The biopsy samples also were sectioned using a cryostat microtome, and hematoxylin–eosin stained slides were examined by a pathologist for tumor content. Total RNA was isolated from 100 mg of tumor tissue and 1 ml of plasma sample using BRIzol reagent (Faragene Co., Tabriz, Iran) according to the manufacturer's instructions. The concentration was determined by Nanodrop 100 Spectrophotometer (Thermo Scientific Co., Wilmington, USA). We employed 1% agarose gel electrophoresis to evaluate the purity of RNA. The primer sequences are summarized in Table 1.

Real-time PCR

For all RNAs, the first-strand cDNA was synthesized using PrimeScript RT Reagent Kit (Parsgenome MiR-Amp, Iran). The expression of miR-650 and miR-663b were quantified by quantitative real-time PCR system (CFX96, Biorad, USA) and U6 was used as internal control. The qRT-PCR was carried out using a SYBR Premix Ex Taq Kit (Takara, Japan) in Thermal Cycler Dice Real Time System TP990 (Takara, Japan). The amplification specificity was validated by melting curve analysis and agarose gel electrophoreses. All experiments were performed in triplicate.

Statistical analysis

The GraphPad Prism version 6 (GraphPad Prism Software Inc., San Diego, California) and SPSS version 18.0 (SPSS Inc., Chicago, IL) were employed to do statistical analyses. P-values less than 0.05 were considered as statistically significant. The comparative CT method was performed to measure the relative expression levels of both miR-650 and miR-663b genes in the tumor and non-tumor samples. Furthermore, the expression levels were normalized to U6 internal control ($2^{-\Delta CT}$ method, $\Delta CT = CT_{miR\text{-}650/miR\text{-}663b} - CT_{U6}$). Associations between miRNAs expression and clinico-pathological features were explored using t test and one-way ANOVA. The biomarker potential of miR-650 and miR-663b was also evaluated by receiver operating characteristic (ROC) analysis.

Results

Demographic Information

Forty patients were included in this study that 11 (27.5%) of them were younger than 40 years old. The age of all study participants ranges from 26 to 78 years old, with a mean of 58.5 ± 6.8 years old. All personal information, including all patient's clinico-pathological data, are summarized in Table 2.

Table 1 Sequences of primers used in this study

Name (gene)	Sequences
miR-650	F 5′- AGGAGGCAGCGCTCTC-3′ R 5′- GAACATGTCTGCGTATCTC-3′
miR-663b	F 5′- CCGGCCGTGCCTGAG-3′ R 5′- GAACATGTCTGCGTATCTC-3′
U6	F- 5′- GCTTCGAGGCAGGTTACATG-3′ R- 5′- GCAACACACAACATCTCCCA-3′

F Forward, *R* Reverse

Table 2 Clinico-pathological features of specimens

Factors	MiR-650 (p value)	MiR-663b (*p* value)
Tissue (tumor: 40, non-tumoral: 40	0.0001	0.0001
Gender (male: 27, female: 13)	0.461	0.205
Plasma (patient: 35, control: 40)	0.137	0.152
Age (≤ 65: 11, > 65: 29)	0.004*	0.458
TNM Staging (I–II: 27, III–IV: 12)	0.001*	0.004*
Tumor location (colon: 12, rectum: 22, unknown: 6)	0.187	0.751
Tumor size (cm; ≤ 3: 25, > 3: 15))	0.002*	0.015*
Lymph node metastasis (negative: 28, positive: 12)	0.001*	0.003*
Distal metastasis	0.003*	0.822
Smoking history (yes: 16, no: 24)	0.624	0.230

p values show the expression ratio between all factors considered in this study

*Statistically significant ($p < 0.05$)

The melting curve analyses detected single peak curves for all genes.

Down-regulation of miR-650 in CRC tissues versus non-tumoral adjacent specimens

Here, we studied the expression of miR-650 by quantitative RT-PCR in CRC samples and paired non-tumoral adjacent tissues. As shown in Fig. 1, the expression level of miR-650 (tumor, non-tumor: 0.022 ± 0.107, 0.240 ± 0.769, respectively, $p < 0.0001$) decreases significantly in tumor samples compared to non-tumors. However, we did not detect any meaningful changes in plasma of patients compared to healthy group (Fig. 1A).

The expression of miR-663b is increased in CRC tissues

Our data for miR-663b showed that this non-coding gene is up-regulated in tumor specimens in comparison with non-tumoral margins (tumor, non-tumor: 0.120 ± 0.10, 0.035 ± 0.56, respectively, $p < 0.0001$). Furthermore, we evaluated the expression of miR-663b in plasma of CRC patients and healthy group. We could not find any significant alteration in the expression level of miR-663b in the plasma samples (Fig. 1B).

Relationship between miR-650 and miR-663b expressions and clinico-pathological features

The association between miR-650 and miR-663b expressions and clinico-pathological characters was investigated in this study. As shown in Table 2, the expression of miR-650 is modulated in tumors with different TNM stages, lymph node involvements, metastasis, tumor size and patient' age, while the miR-663b is affected by stages, tumor size, and lymph node metastasis. Higher expression of miRNAs depicted a significant association with higher stages (III, IV) of colorectal cancer (Fig. 2A). However, this correlation

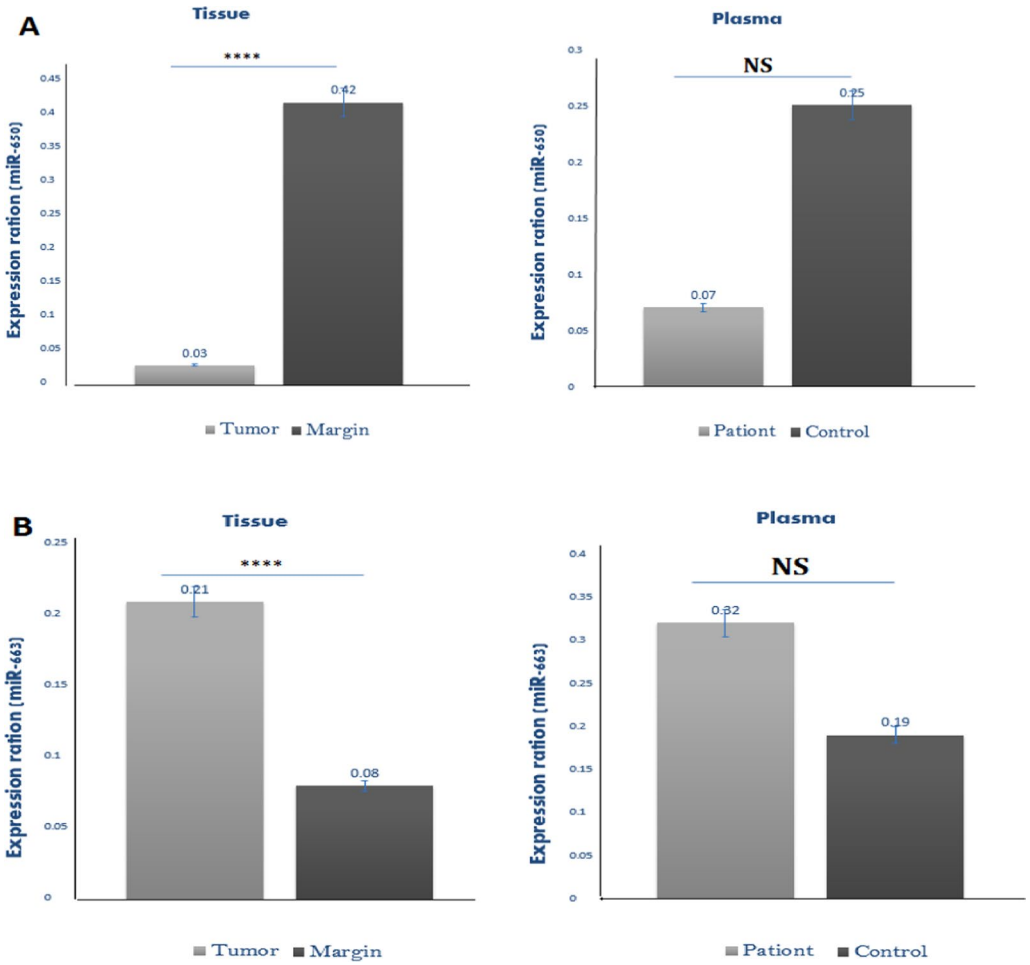

Fig. 1 Expression of miR-650 and miR-663b in tissue and plasma samples detected by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). **A** The expression level of miR-650 significantly decreased in CRC tissues ($p \leq 0.0001$) in comparison with non-tumoral adjacent tissues. **B** The expression level of miR-663b indicated increase in CRC tissues ($p \leq 0.0001$) in comparison with non-tumoral adjacent tissues. For both genes, differential expression in plasma samples were not significant compared to controls. ****: $p \leq 0.0001$

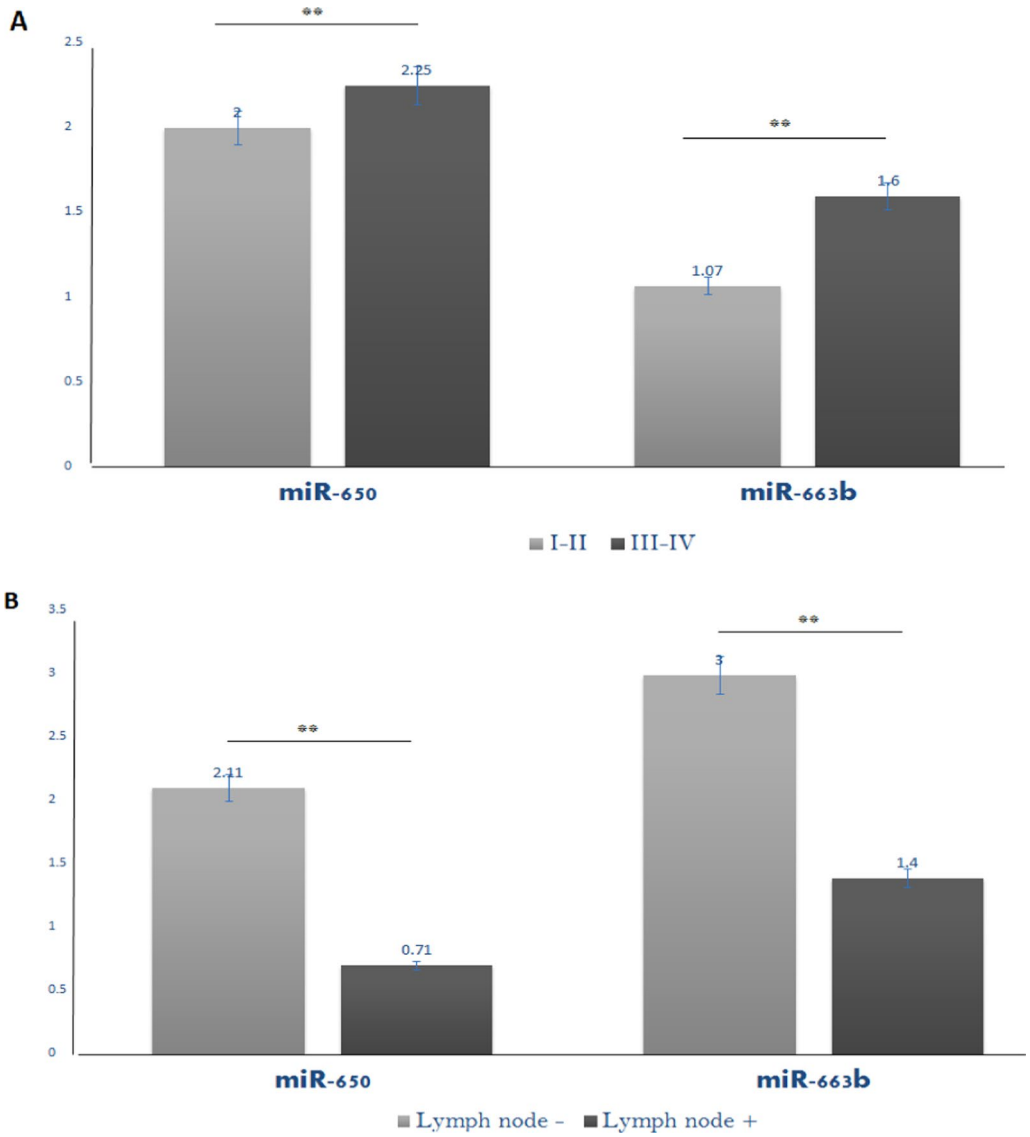

Fig. 2 Correlation of miR-650 and miR-663b expression with **A** TNM Staging **B** Lymph node metastasis. **: $p < 0.01$

was reversely detected for lymph node involvements. The analyses revealed statistically that these two miRNAs had decreasing trends in metastatic patients (Fig. 2B, p value < 0.05).

Receiver operating characteristic (ROC) curve analysis

The ROC curve analysis was used to assess the biomarker potential of miR-650 and miR-663b in discriminating tumor and non-tumor samples. This analysis demonstrates the specificity and sensitivity of expression levels for both genes. The ROC curve data for miR-650 and miR-663b revealed an area of 0.81 and 0.91 in tissue samples, respectively (Fig. 3, $p < 0.01$).

Discussion

Colorectal cancer is the most common malignancy of the gastrointestinal tract and the third leading cause of cancer-related mortalities in the world [24]. Compared to the developed countries, the incidence of CRC is lower in Iran. However, its rate in the country's younger generation is growing, which may significantly increase the burden of disease in the future [25].

The diagnosis of patients in higher stages are associated with a poor prognosis, while early diagnosis and appropriate therapy are associated with a better chance of cure, and caused to a reduction in mortality from CRC [26, 27].

Despite recent advances in diagnosis, there is an urgent need to develop novel diagnostic biomarkers in cancer.

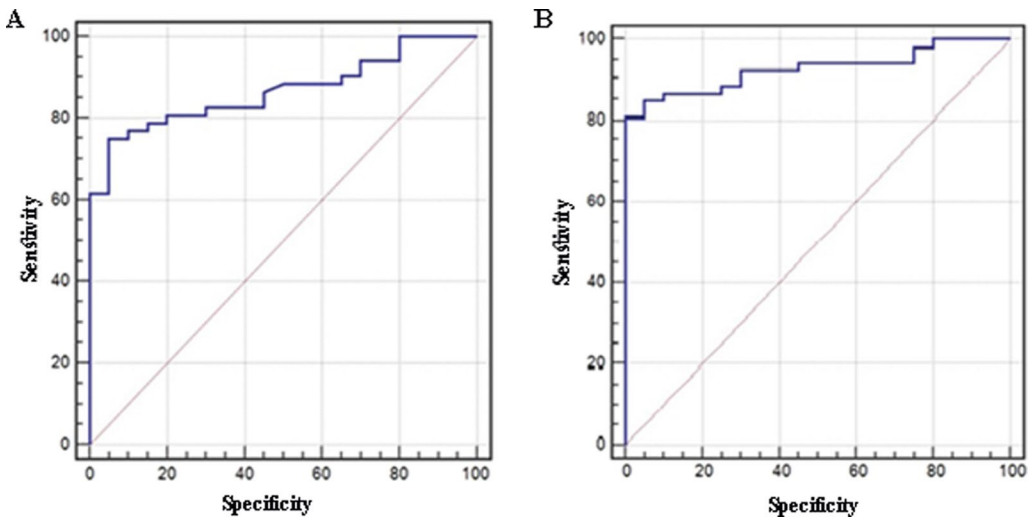

Fig. 3 Receiver operating characteristic (ROC) curve analysis to evaluate tissue and plasma microRNAs expression levels for the detection of CRC patients. ROC curve analysis **A** miR-650 and **B** miR-663b in tissue samples was plotted for CRC from non-tumoral adjacent tissues

The study of the potential roles of microRNAs will lead to better understand the development, progression, earlier detection and appropriate treatment of colon cancer. Studies have shown that miRNAs could be employed as novel potential biomarkers in CRC.

MicroRNAs are released to the extracellular media from the tumor cells in a highly stable form that enables scientists to track and detect them in body fluids. Remarkable stability, detection feasibility (fast and accurate quantification), and the direct effect of microRNAs in cancer pathogenesis make them as ideally noninvasive biomarkers for early cancer diagnosis [28, 29].

Studies have shown that miR-663 can operate as an oncogene or tumor suppressor in malignant progression. This microRNA behave as an oncogene in many cancers such as non-small cell lung cancer [30], lung cancer [31], prostate cancer [32], and nasopharyngeal carcinoma [33], but also as a suppressor against gastric cancer [34], pancreatic cancer [35], glioblastoma [36] and thyroid carcinoma [37]. The functional study documented the relation between miR-663b expression and proliferation, metastasis, invasion, cell growth, angiogenesis and apoptosis of cancer cells. Pellatt et al. [38] applied microarray analysis to show that miR-663b is significantly over-expressed in CRC tissues relative to the normal counterparts. In another study by Wang et al. [28], the expression of miR-663 was meaningfully up-regulated in colon cancer patients compared to the benign forms and healthy controls.

Xiao et al. [39] presented that miR-663b expression was significantly higher in CRC tissues than adjacent non-tumoral specimens. They also reported that this microRNA promotes CRC cell proliferation, migration and invasion by regulating the Wnt/β-catenin pathway through targeting APC2. In line with the previous studies, our results show that miR-663b is significantly up-regulated in tumor tissues, though it was not significantly detected in plasma samples. The analysis by ROC curve suggested that miR-663b could be potential biomarker for differentiating CRC patients from non-tumoral margins, with an area under the curve (AUC) of 0.91 for tissue samples (Fig. 3B).

Our investigation revealed that miR-650 and miR-663b expression increases with advanced TNM staging in CRC patients but these two miRNAs had decreasing trends in tumors with lymph node metastasis (Fig. 2). This potential contrast could be due to the complexity of miRNA involvement in cancers and different mechanisms for the aberrant expression of miRNA including genetic alterations and single nucleotide polymorphism (SNP), (ii) epigenetic silencing and (iii) defects in the miRNA biogenesis pathway [40]. Here, we report that miR-650 is down-regulated in tumor samples including both tissue and plasmas. However, this down-regulation was not significant in plasma samples. The effect of miR-650 seems to vary by type of cancer [41]. MiR-650 appeared as an oncoMIR in in vitro experiments. Rearrangement of the immunoglobulin gene has also been shown to up-regulate miR-650 expression in chronic lymphocytic leukemia [17]. Inhibitor of Growth 4 (ING4) is a tumor suppressor that prevents the cell growth in gastric cancer and hepatocellular carcinoma where that this gene is considered as miR-650 target [19]. In a study by Zuo et al. [20], suppression of CSR1 mediated oncogenic activity of miR-650 in prostate cancer. MiR-650 targets N-MYC downstream-regulated gene 2 (NDRG2) in colorectal cancer cells [42].

MiR-650 has also been reported to promote hepatocellular carcinoma metastasis and EMT by directly inhibiting the expression of LATS2 gene [41]. The data show that miR-650 could be considered as a potential biomarker for differentiating patients with CRC from healthy controls with AUC of 0.81 for tissue samples (Fig. 3A). More researches are needed to uncover the molecular mechanisms of miR-650 and miR-663 genes in colon cancer.

Conclusion

Our findings revealed the clinical significance and potential of miR-650 and miR-663b as biomarkers in the diagnosis and prognosis of colorectal cancer. However, further studies are required to confirm the detailed molecular mechanisms of miR-650 and miR-663b in the development and progression of colorectal malignancies.

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Author contributions
EB designed the study; MV and AY collected data and performed analyses; MV, EB and JKMA discussed the results and strategy; EB supervised, directed and managed the study; MV, EB, AY and JKMA drafted the manuscript and approved the final version to be published.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
This study was approved by the Biomedical Research Ethics Committee (IR.IAU.TABRIZ.REC.1400.009).

Consent for publication
Informed written consent was provided for each patient and health volunteer.

Competing interest
The authors declare that they have no competing interests.

Author details
[1]Department of Molecular Genetics, Faculty of Basic Sciences, Ahar Branch, Islamic Azad University, Ahar, Iran. [2]Department of Biology, School of Natural Sciences, University of Tabriz, Tabriz, Iran. [3]Department of Internal Medicine, School of Medicine and Allied Medical Sciences, Digestive Diseases Research Center, Ardabil University of Medical Sciences, Ardabil, Iran.

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