

Serum miR-23 and miR-150 Profiles as Biomarkers for Predicting Recurrence following Surgical Intervention in Colorectal Cancer Patients

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Abstract

Background: MicroRNAs (miRNAs) play pivotal roles in post-transcriptional regulation of gene expression and have emerged as crucial regulators in cancer development, progression, and metastasis. This study aimed to assess the expression profiles of miR-23, miR-223, miR-1246, and miR-150 in serum samples obtained from colorectal cancer (CRC) patients before and three months after surgery, in comparison to a healthy control group, to explore their biomarker potential.

Methods: A total of 50 blood samples were collected from patients with CRC (pre- and post-surgery), along with 50 samples from healthy controls. The relative expression levels of miR-23, miR-223, miR-1246, and miR-150 in the serum were quantified using quantitative real-time PCR.

Results: Our findings revealed upregulated expression levels of miR-23, miR-1246, and miR-223, while miR-150 exhibited significant downregulation in the serum of CRC subjects compared to healthy controls. Receiver operating characteristic (ROC) analysis indicated that miR-23 and miR-150 could distinguish CRC cases from controls with relatively high accuracy. Moreover, three months post-surgery, miR-23, miR-1246, and miR-223 serum levels were downregulated, and miR-150 was significantly upregulated. However, no significant correlations were observed between serum levels of the studied genes and the clinical features of our patients.

Conclusions: The serum levels of miR-23 and miR-150 hold promise as potential biomarkers for the diagnosis and prognosis of CRC.

Keywords: Biomarker, Colorectal cancer, micro-RNAs, Tumorigenesis.

Introduction

Colorectal cancer (CRC), a gastrointestinal malignancy originating from the colon, rectum, or both, ranks among the leading causes of cancer-related deaths. In 2020, CRC accounted for 0.9 million deaths, with 5.2 million prevalence and 1.9 million incidences globally, making it the second deadliest cancer (1, 2). Contributing risk factors include physical inactivity, body mass index (BMI), cigarette smoking, red meat consumption, age, and obesity (3). CRC often metastasizes, leading to

late symptom manifestation. Early detection through screening methods is crucial for reducing mortality and new case incidence (4).

Potential CRC screening methods include colonoscopies, flexible sigmoidoscopies, fecal immunochemical tests (FIT), and guaiac-based fecal occult blood tests (FOBT) (5). Despite benefits, colonoscopy's expense and invasiveness limit its use. These methods suffer from issues like invasiveness, high cost, low sensitivity, and low specificity. Effective CRC

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prevention requires rapid and noninvasive screening methods. Current research focuses on identifying noninvasive bio-fluid markers such as blood, urine, saliva, and serum, with a spotlight on microRNA (miRNA) as a potential noninvasive cancer detector (6).

MiRNAs are small, endogenous, noncoding RNAs influencing gene expression and playing a pivotal role in cancer-related processes (7). Recent findings on miRNAs have illuminated the field of biomarkers. Altered miRNA expression in various cancers, including CRC, has been reported, and tissue miRNA profiles hold diagnostic potential. Studies suggest that cells secrete miRNAs into the extracellular environment via exosomes, serving as biomessengers in intercellular communication (7).

Several studies indicate that the miRNA profile in tumors mirrors the serum miRNA profile. MiRNA expression levels vary across cancer types (8, 9), offering a cost-effective, reliable, and efficient CRC screening method in serum. Stable miRNAs in serum and plasma, isolated from tissues and organs, can serve as noninvasive biomarkers for diagnosing cancer and other diseases. The release of exosomes containing miRNAs may also play a role in tumor progression (10). This study investigates alterations in miRNA expression levels before and after surgery, aiming to evaluate miRNAs as noninvasive biomarkers for early-stage CRC detection.

Materials and Methods

Patient selection

This study was designed as a case-control study under permission of Tabriz University of Medical Science's ethical committee that approved the procedure (IR.TBZMED.REC.1399.982). All participants, as well as their legal guardians, provided written informed consent. About 5 ml of whole blood samples was collected from 50 patients whose cancer had been diagnosed by a pathologist according to the histopathological result of their colonoscopy sample (two sample from every patient, one before surgery another three months after surgery). The control group also included 50 healthy subjects selected by

matching biological feature including sex and age. All participants were hospitalized at Imam Reza Hospital in Tabriz, Iran, during 2019-2022. Inclusion criteria for case group: having Colorectal adenocarcinoma, signing a written consent form, living in the northwest of Iran, not having a long term using of special drug, not having other genetic and chronic disorder. Exclusion criteria for case group: suffering from any type of CRC other than adenocarcinoma, not signing a written consent, not living in the northwest of Iran, having a long term using of special drug, suffering from other having other genetic and chronic disorder,

Inclusion criteria for control group: signing a written consent form, living in the northwest of Iran, not having a long-term using of special drug, not having any genetic and chronic disorder.

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Table 1 contains detailed clinical features of the study subjects. For molecular studies, blood samples were sent to the laboratory in a sterile tube and serum of every sample separated using centrifugation (1000 G, 4 °C, 5 min), ensuing an immediate storage at -80 °C.

RNA Extraction

The serum samples were obtained from peripheral blood of the study subjects by centrifugation. Tripure isolation reagent (Roche, Cat No.11667165001.Germany) was used in order to isolate total RNA from samples, considering the company's manual. To determine the quality and quantity of the extracted RNAs, optical density (OD) of all the samples were detected by using Nanodrop (NanoDrop ND-2000C, Thermo Fisher Scientific, USA).

cDNA Syntheses and Real-time quantitative reverse transcription-PCR (RT-qPCR)

miRNAs were converted to cDNA using stem-loop method according to the manufacturer's procedure (TAKARA.Cat No. 6130. Japan).

Then, Real-time PCR was performed by light cycler 96 machine (Roche, Germany) using specific primers and Sybr green Master Mix (Biofact. Cat No. DQ322-25h. South Korea) (Table 2). The transcript level of U6 was used as internal control to standardize the

expression level of target genes. The relative expression level of studied genes was determined by standardization based on the transcript level of the housekeeping gene in each sample using the comparative Ct method ($2^{-\Delta\Delta CT}$) (11).

Table 1. Baseline data and clinicopathological features of the study subjects.

Characteristic	CRC patients (N=50)	Healthy controls (N=50)
Gender; Male/female; N (%)	29 (54%)/21 (46%)	29 (54%)/21 (46%)
Age (Year): Mean \pm SD	60 \pm 5.71	59, 5 \pm 5.81
Smoking; Smoker/non-smoker; N (%)	14 (28%)/ 36 (72%)	### (%)### (%)
Familial History; Yes/no; N (%)	6 (12%)/ 44 (88%)	-
Lymph node Invasion; Yes/no; N (%)	40 (80%)/ 10 (20%)	-
Distant Metastasis; Yes/no; N (%)	11 (22%)/ 39 (78%)	-
Differentiation; Poor/intermediate/good; N (%)	16 (32%)/ 20 (40%)/ 14 (28%)	-
Stage; I & II/III & IV; N (%)	32 (64%)/ 18 (36%)	-

CRC; Colorectal cancer, SD; Standard deviation.

Table 2. Primer Sequences were used in this study.

Primer name	Sequence (5'-3')
U6 stem loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTGCACTGGATACGACAAAAATAT
U6 Forward	GCTTCGGCAGCACATATACTAAAT
U6 reverse	CGCTTCACGAATTGCGTGTAT
miR-23 stem loop (NR-029495.1)	GTCGTATCCAGTGCAGGGTCCGAGGTATTGCACTGGATACGACAAATCC
miR-223 stem loop (NR-029637.1)	GTCGTATCCAGTGCAGGGTCCGAGGTATTGCACTGGATACGACAACTCA
miR-1246stem loop (NR-031648.1)	GTCGTATCCAGTGCAGGGTCCGAGGTATTGCACTGGATACGACCCCTGCT
miR-150stem loop (NR-029703.1)	GTCGTATCCAGTGCAGGGTCCGAGGTATTGCACTGGATACGACCACTGG
miR-23 Forward	GGGCCTGGGTTCCCTGG
miR-223 Forward	ATCGCTCGTGTATTGA
miR-1246 Forward	CAATTGAATGGATTTTG
miR-150 Forward	GACTAATCTCCAACCCCT
Universal reverse	CCAGTGCAGGGTCCGAGGTA

Statistical analysis

GraphPad Prism (version 6.0 for Windows) was used for statistical analysis and designing the graphs. The mean \pm standard deviation (SD) was used to represent data. The normal distribution of data was examined using Shapiro-Wilk test. The t-test was used to compare miRNA transcript levels between groups. A Receiver Operating Curve (ROC)

characteristic was employed to assess the diagnostic importance of various variables between the two groups (as a binary classifier). The area under the ROC curve (AUC) of this test represented the predictive value of the variable between two groups of patients. *P* values less than 0.05 (*p*<0.05) were considered statistically significant.

Results

Expression level of miRNAs

In serum of patient group, miR-23b expression levels were significantly higher than control group ($p=0.0074$), before surgery (Fig. 1). In addition, there was a significant decrease in the serum level of miR-23b in patients three months after surgery ($p=0.043$) in comparison to before surgery. There was also a significantly higher levels of miR-223 expression in serum samples of patients before surgery in comparison to healthy control ($p=0.023$). Furthermore, expression level of miRNA-223 was significantly downregulated

three months after surgery ($p=0.044$).

In contrast, miR-150 was significantly downregulated in CRC group ($p=0.0039$) before surgery and up regulate three months after surgery ($p=0.0078$) (Fig. 1c). Also, expression level of the miR-1246 was higher before surgery in compare control group ($p=0.045$); but after three months since surgery, expression level of the miR-1246 was downregulated ($p=0.41$). However, there was not significant correlation between expression level of miRNAs and clinical features of patients (Table 3).

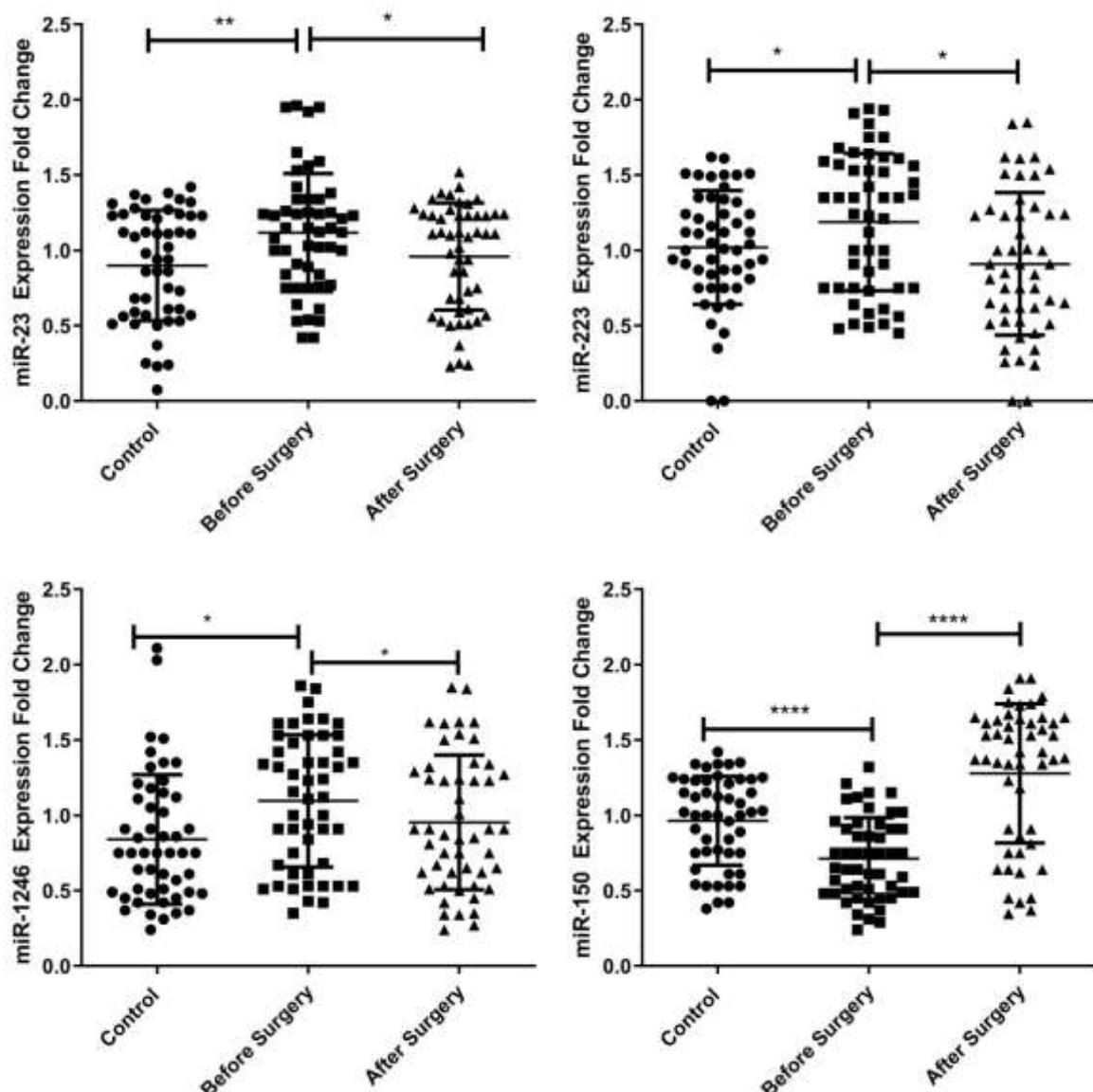


Fig. 1. The expression levels miR-23, miR-1246, miR-223 and miR-150 in patients and control group, before and after surgery groups.

Table 3. Association of miRNA with clinicopathological features of the study subjects.

Clinicopathological Characteristics	Subgroup	P value			
		miR-23	miR-223	miR-150	miR-1246
Age	>60				
	<60	0.124	0.098	0.158	0.085
Sex	Female				
	Male	0.089	0.24	0.095	0.141
Familial History	Positive				
	Negative	0.081	0.086	0.104	0.098
Smoking	Positive				
	Negative	0.098	0.45	0.117	0.245
Lymph node Invasion	Positive				
	Negative	0.052	0.086	0.053	0.075
Distant Metastasis	Positive				
	Negative	0.152	0.067	0.096	0.059
Differentiation	Poor				
	Intermediate	0.0583	0.091	0.073	0.057
	Good				
Stage	I & II	0.067	0.084	0.053	0.096

Potential of miRNAs as diagnostic biomarkers for CRC

ROC curves were employed to evaluate the specificity and sensitivity of the identified miRNAs as potential biomarkers for CRC. Among the patients, miR-23b ($p=0.0055$) and miR-150 ($p<0.0001$) exhibited ROC areas (AROC) of 0.63 and 0.73, respectively. Conversely, although miR-223 showed upregulation in CRC samples before surgery,

it demonstrated a less favorable AROC of 0.60 ($p=0.07$), suggesting its unsuitability as a CRC biomarker. Similarly, miR-1246, with an AROC of 0.57 ($p=0.116$), was not a suitable candidate for CRC biomarker. Therefore, based on our data, only miR-23 and miR-150 emerge as potential biomarkers for CRC screening (Fig. 2).

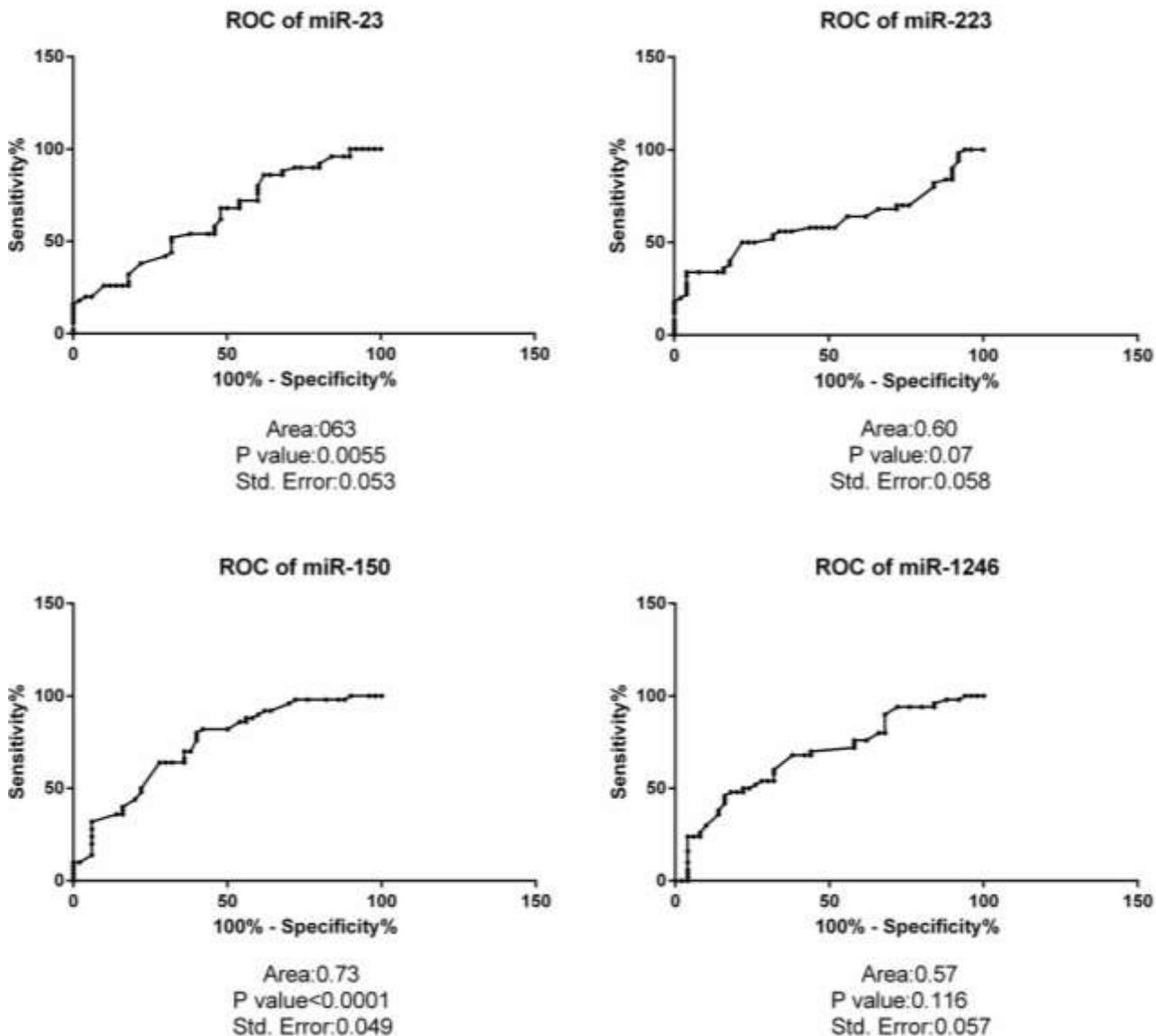


Fig. 2. Results of ROC curve analysis of miR-23, miR-1246, miR-223 and miR-150 in CRC patient compression to healthy controls.

Discussion

As identifying specific molecular markers capable of detecting CRC in early stages is imperative, our study explored the role of several candidate miRNAs as biomarkers for CRC and their association with clinicopathological characteristics of the patients. We detected that miR-223, miR-23, miR-1246 were overexpressed, while miR-150 was downregulated in the serum of CRC subjects before and after surgery compared to

controls. However, none of them were involved in determining the clinicopathological picture of CRC. The serum levels of miR-23 and miR-150 hold promise as potential biomarkers for the diagnosis and prognosis of CRC.

Numerous reports have demonstrated miRNA deregulation in human disorders, particularly in cancers (12). Some studies designate this class of RNAs as cancer-specific

biomarkers (13). Circulating miRNAs, influenced by tumor presence, have potential as cancer detection biomarkers (14). Previous studies have reported the deregulation of miR-23 and miR-223 in various cancers (15, 16). Our study aligns with these findings, indicating that up-regulation of miR-23 and miR-223 is linked to clinical features in malignancies (16). Additionally, miRNA upregulation in the serum correlates with pathological features like metastasis and lymph node invasions (17). MiR-1246, a recently highlighted miRNA in cancer research, may act as a prognostic marker in some cancers (18). Previous research identifies miR-150 as a tumor suppressor miRNA, capable of reducing cancer cell metastasis and proliferation rates (19).

While earlier data suggest the potential of these miRNAs as diagnostic and prognostic markers in cancer, their exact roles and values remain unclear, posing unanswered questions in cancer biology (16-18, 20). This paper evaluated circulating levels of miR-150, miR-23, miR-223, and miR-1246 in CRC patients, comparing results with healthy controls to demonstrate their potential in differentiating study groups. MiR-1246 exhibits alterations between patients and controls both before and after surgery. MiR-23 and miR-223 are significantly up-regulated in patients before surgery but down-regulated after surgery. In contrast, miR-150 is significantly down-regulated in patients before surgery but up-regulated after surgery. According to ROC analysis, only miR-23 and miR-150 may serve as prognostic markers for CRC. However, no significant correlation with clinical features is found for the evaluated miRNAs. The expression levels of these miRNAs are also measured three months after surgery to confirm the relationship between tumor presence and miRNA expression.

Shang et al. demonstrated that 5-Fu treatment induces miR-23a expression in colon cancer cells, contributing to chemoresistance by targeting *apoptotic peptidase activating factor 1 (APAF-1)* (21). Other studies indicate

that miR-23a promotes proliferation and migration of colorectal cancer cells by targeting *microtubule affinity regulating kinase 1 (MARK1)* (22). Deng et al. highlighted the role of miR-23a in CRC cell proliferation through direct repression of *pyruvate dehydrogenase kinase 4 (PDK4)* (23). Liu L et al. suggested that miR-223 promotes colon cancer cell invasion and metastasis by downregulating p120, reducing intercellular adhesion, promoting Ras homolog family member A (RhoA) activity, and activating β -catenin signaling (24). Zhang et al. revealed that reducing miR-223 expression decreases CRC cell proliferation, migration, and invasion (25). Fan et al. proposed that down-regulated miR-150, in conjunction with elevated GLI family zinc finger 1 (Gli1), contributes to epithelial-mesenchymal transition, promoting invasion and metastasis in colorectal cancer cells (26). High expression of miR-155 is correlated with poor prognosis, drug resistance, and genome instability in CRC patients (27). He et al. indicated that low expression of miR-150 triggers cancer cell development and progression in colorectal cancer via suppressing β -catenin (28). Zhao et al. found that serum exosomal miR-99b-5p and miR-150-5p levels are significantly downregulated in CRC patients, suggesting their potential as non-invasive diagnostic biomarkers for CRC (29).

MiR-1246 is implicated in differentiation, invasion, metastasis, and chemoresistance of certain tumor cells. Studies demonstrate increased expression of miR-1246 in CRC tissues and cell lines, inducing cell proliferation, migration, and invasion (30). Chen et al. propose that serum levels of miR-1246 can predict lymph node metastasis in cervical squamous cell carcinoma, inducing cell proliferation, invasion, and migration by targeting *thrombospondin 2 (THBS2)* (31). Wang et al. suggest that miR-1246 is upregulated in colorectal cancer and negatively regulates the expression of *cyclin G2 (CCNG2)*, facilitating tumor progression (32). However, our results did not support the

involvement of miR-1246 in clinicopathological features of the CRC patients.

The results of our research suggest that miRNAs play a dynamic role in the pathogenesis of CRC. The study specifically investigated the circulating levels of miR-150, miR-23, miR-223, and miR-1246 in CRC patients, comparing these levels with those in healthy controls to assess their potential in distinguishing between the two groups. The findings indicated that miR-1246 exhibits alterations between CRC patients and controls both before and after surgery. This suggests that miR-1246 may be actively involved in CRC progression and could potentially serve as a biomarker for detecting and monitoring the disease. MiR-23 and miR-223 show interesting patterns, being significantly upregulated in CRC patients before surgery but downregulated after surgery. This suggests a potential association between these miRNAs and the presence of the tumor, with their levels responding to surgical intervention. The dynamic changes in miR-23 and miR-223 levels may reflect their involvement in the CRC pathogenesis and response to treatment. In contrast, miR-150 displays a different behavior, being significantly downregulated in CRC patients before surgery but upregulated after surgery. This contrasting pattern suggests that miR-150 might play a role in the early stages of CRC and could potentially be associated with the response of body to surgical intervention. The ROC analysis indicated that only miR-23 and miR-150 may serve as prognostic markers for CRC. This suggests that these two miRNAs have the potential to be valuable indicators of disease prognosis and could be considered as part of diagnostic and monitoring strategies. Interestingly, despite the associations observed with miRNA expression levels and their potential prognostic value, no significant correlation was found between these miRNAs and clinical features. This implies that the

identified miRNAs may have a more generalized role in CRC, possibly influencing the overall disease state rather than specific clinical characteristics.

In summary, our research indicates that miR-223, miR-23, miR-1246 are overexpressed, and miR-150 is under expressed in the serum of CRC before and after surgery compared to controls, suggesting their potential as prognostic or diagnostic factors in CRC. Future studies are essential to confirm these findings. The serum levels of miR-23 and miR-150 hold promise as potential biomarkers for the diagnosis and prognosis of CRC.

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Conflict of Interest Statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics Approval

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions. Also, this study was approved by the Ethics Committee of Tabriz University of Medical Sciences (Ethical code: IR.TBZMED.REC.1399.982).

Informed Consent

All participants signed the informed consent. All the procedures were performed as a part of the routine care.

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