



Evaluation of miR-330-3p and BMI1 Expression in Colorectal Cancer Patients, Healthy Adjacent Tissues, and Polypoid Adenomatous Lesions

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Original Article

MicroRNAs (miRNAs) have emerged as essential gene expression regulators associated with human diseases such as colorectal cancer (CRC). The purpose of this study was to evaluate the expression of miR-330-3p and its target gene BMI1 in tissue samples of patients with CRC, polyp, and healthy adjacent tissue samples and their association with clinicopathological and demographic factors such as age, tumor stage, grade, and lymph node invasion of the tumor. Following the extraction of total RNA from approximately 50 mg of colon and rectum tissue of 82 patients with CRC, 13 polypoid lesions, and 26 marginal healthy tissues using RiboEx reagent, cDNA synthesis was performed, and then quantitative real-time PCR was used to detect the expression levels of miR-330-3p and BMI1. Alterations in the gene expression were assessed using the $2^{-(\Delta\Delta CT)}$ method. The expression of miR-330-3p in all of the CRC samples was significantly lower than in adjacent healthy tissues and polyp ($P < 0.001$). BMI1 was up-regulated in 97.9% of CRC tissue compared to healthy adjacent tissues and polyps ($P < 0.001$). A negative reverse correlation between the miR-330-3p and BMI1 gene was observed in the CRC samples ($r = -0.882$, $P < 0.001$). Down-regulation of miR-330-3p and BMI1 overexpression strongly correlates with higher tumor stage and lymph node invasion. The AUC for miR-330-3p and BMI1 expression was 0.982 (sensitivity, 98.5%; specificity, 78.8%), and 0.971 (sensitivity, 97.6%; specificity, 84.6%) ($P < 0.001$), respectively. Our results indicated that miR-330-3p and BMI1 expression probably could be considered potential diagnostic or prognostic biomarkers for CRC patient.

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Introduction

Colorectal cancer (CRC) is the third most common cancer and the fourth leading cause of cancer-related deaths in the world. According to the multistage genetic model of colorectal carcinogenesis, in the process of malignancy, adenomatous polyposis coli (APC) inactivation, and Kirsten rat sarcoma virus (KRAS) oncogenic mutations occur as the initial events in the adenomatous stage, followed by, deletion of chromosome (Chr) 18q, and inactivation of the tumor suppressor gene TP53 on chromosome 17q [1]. MicroRNAs (miRNAs) are endogenous small (19–25nt) single-strand non-coding RNAs that modulate the expression of target genes through messenger RNAs (mRNAs) decay or translation repression. Mounting evidence shows miRNA dysregulation is associated with CRC carcinogenesis, consequently leading to the down-regulation of tumor suppressors or up-regulation of oncogenes [2]. A well-known oncogene involved in various types of cancer is BMI1, a member of the polycomb group (PcG) proteins. BMI1 can transcriptionally repress Ink4a/Arf (CDKN2a) locus which encodes p16 (Ink4a) and p14 (Arf) tumor suppressors. Moreover, BMI1 as a downstream mediator; participates in oncogenic signalings such as Wnt, and Akt/NF- κ B pathways [3]. BMI1 up-regulation is correlated with chemoresistance, metastasis, and cancer stem cell survival [4]. BMI1 is overexpressed in invasive ductal breast adenocarcinomas, pancreatic cancer, esophageal squamous cell carcinoma, gastric cancer, and CRC [5-9], and is reported as an independent prognostic marker with distant metastasis, tumor invasion, stage, and lymph node involvement [5]. Previously, by bioinformatics analysis, we showed that BMI1 is an optimum predicted target of miR-330-3p in CRC [10]. At the same time, Zheng *et al* by dual-luciferase reporter assay and real-time PCR showed suppression of BMI1 by miR-330-3p; furthermore, they demonstrated an increased level of miR-330-3p is related to good prognosis in osteosarcoma [11]. On the other hand, the tumor suppressive role of miR-330-3p has been revealed in osteosarcoma, prostate cancer, gastric cancer, and CRC [11-14]. In another study, miR-330-3p downregulation in CRC tumor tissues compared to the healthy tissues reflected a poor prognosis in CRC [15]. MiR-330-3p by negative regulation of cell division control protein 42 homolog (Cdc42) suppressed CRC cell proliferation, and induced cell cycle arrest in CRC cell lines [14]. Hence, this research aimed to investigate the miR-330-3p and BMI1 expression in CRC, polypoid adenomatous lesions, and normal tissues, simultaneously, and their association with clinicopathological features of CRC patients.

Materials and methods

Clinical samples

The colon and rectum tissue of 82 patients with CRC, 13 polypoid lesions, and 26 marginal healthy tissues were included in the present study (Table 1). All samples were collected from Bouali Hospital (Hamadan, Iran), and Shahid Beheshti Hospital (Hamadan, Iran) from August 2018 to October 2019. Further, all tumoral, marginal healthy (distance to CRC tissues > 5 cm), and polypoid tissues were collected from CRC patients who underwent elective surgery and colonoscopy procedure of suspected patients, respectively. Patients who had not received radiotherapy, chemotherapy, and adjuvant therapy before surgery were included in the study [16]. Also, inclusion criteria for the polypoid group were no history of malignancy and inflammatory bowel disease. All samples were frozen at -80°C until subsequent tests. The relevant written informed consent signed by all participants, including CRC patients, and polypous affected individuals. The

ethical approval of the present study was obtained from the Ethics Committee of Hamadan University of Medical Sciences, Hamadan, Iran (IR.UMSHA.REC.1397.1042).

Table 1. Clinicopathological features of CRC patients and patients with the intestinal polyp.

Characteristics	Healthy adjacent	Adenomatous Polyps	CRC
Age	-	-	-
≤50	23	10	61
>50	3	3	21
Gender	-	-	-
Male	16	8	52
Female	10	5	30
Region	-	-	-
Rural	4	5	24
Urban	22	8	58
Smoking	-	-	-
No	13	10	37
Yes	13	3	45
TNM staging	-	-	-
I/II	-	-	39
III/IV	-	-	43
Grade	-	-	-
I/II	-	-	76
III/IV	-	-	6
Lymph node invasion	-	-	-
Negative	-	-	17
Positive	-	-	65
Tumor size (cm)	-	-	-
≤5	-	-	4
>5	-	-	78
OB test	-	-	-
Positive	-	6	70
Negative	-	7	12

mRNA extraction

Total RNA including mRNA and miRNAs was isolated from approximately 50mg dissected tissue samples using RiboEx reagent (Pishgam, Co, Iran) according to the manufacturer's protocol. In brief, tissue samples were homogenized in 1 mL RiboEx reagent, and then 0.2 mL of chloroform was added. The samples were centrifuged at 12,000×g for 15 min at 4 °C. RNA-containing upper aqueous phase precipitated with 0.5 mL isopropanol. The RNA pellet was washed with 75% ethanol and subsequently dissolved in RNase-free water (DEPCE water). RNA concentration and purity were determined by a Nano-Drop spectrophotometer (Bio-Tek, USA). Integrity and quality of the RNAs were assessed using %1 agarose gel electrophoresis stained with SYBR Safe dye (Invitrogen, USA). The extracted RNAs were stored at -80 °C.

cDNA synthesis

cDNA was synthesized by applying the SMOBio kit (Pishgam Co., Iran) in two main steps. In the first step, an appropriate amount of RNA was incubated with deoxyribonucleotide triphosphate (dNTP), Random Hexamer, and DEPC-treated water at 70 °C for 5 min. In the second step, RNase- free water was mixed with 5× buffer, reverse transcriptase enzyme, and RNase inhibitor. Then, the final mixture was incubated in a thermal cycler at 25 °C for 10 min, 42 °C for 50 min, followed by 85 °C for 5s. miRNA was reversely transcribed to cDNA through a specific stem-loop reverse transcription primer method using a miRNA cDNA synthesis kit (Biomir high sensitivity microRNA kit, Anacell, Tehran, Iran) according to the manufacturer's protocol.

Quantitative real-time (qRT) PCR

qRT-PCR was performed to investigate the expression alterations of the miR-330-3p using Biomir high sensitivity microRNA kit (Anacell, Tehran, Iran) according to the manufacturer's instructions with a LightCycler 480 system (Roche Applied Science). 18s rRNA and RNU6B (U6) were used to normalize the quantitative data of BMI1 and miR-330-3p, respectively. The specific primer sequences, were as follow: BMI1 forward (5' CATCCACAGTTTCCTCACATTTTC-3'); reverse (5'GAAGTTGCTGATGACCCATTTAC-3'); 18s rRNA forward (5'- GTAACCCGTTGAACCCCAT-3') and reverse 5'- CCATCCAATCGGTAGTAGCG-3') [17, 18].

PCR amplification reactions for BMI1 were consequently applied under the following conditions: 15 min at 95 °C, 20 sec at 95 °C, 30 sec at annealing temperatures, and 30 sec at 72 °C. For quantitative miRNA expression analysis, after cDNA synthesis, PCR was run under the following conditions: 95 °C for 5 min, 40 cycles of 95 °C for 10 sec, 60 °C for 45 sec, and 70°C. All no template controls were negative. The $2^{-\Delta\Delta CT}$ method was applied to analyze qRT-PCR data [19]. Two replicates of each biologic sample were included in the qRT-PCR.

Statistical analysis

Data analysis was done using the SPSS16.0 software. All data were expressed as mean±SD. Kolmogorov–Smirnov test was used to normality of data before the parametric test. Data were compared by one-way ANOVA, t-test, and Mann-Whitney. A p- value less than 0.05 ($P < 0.05$) was considered statistically significant. The Pearson correlation coefficient was applied to investigate the relationship between miR-330-3p and its target.

Results

miR-330-3p and BMI1 expression in CRC, Adenomatous Polyps, and adjacent normal non-neoplastic tissues

There was significantly higher BMI1 expression in 97.9% of the CRC samples compared to adenomatous polyps and normal marginal tissues ($P < 0.001$). In 100% of the CRC samples, there was decreased expression of the miR-330-3p gene in comparison to adenomatous polyps and normal marginal tissues ($P < 0.001$). MiR-330-3p and BMI1 expression were not significantly different in adenomatous polyps and normal marginal samples ($P > 0.05$). In comparison to the healthy adjacent group, the miR-330-3p expression in CRC and polyp groups were down regulated 0.32 and 0.28 fold, respectively. Compared to the

healthy control group, the BMI1 expression in CRC and polyp samples was upregulated 3.92 and 2.62 fold, respectively. (Tables 2-3, Figure 1)

Table 2. Δ CT values (Mean \pm SD*) in different colorectal tissue samples.

Variables	CRC			Adenomatous Polyps			Healthy adjacent			P-Value (ANOVA)
	Number	Mean	SD	Number	Mean	SD	Number	Mean	SD	P-Value
BMI1	82	6.85	2.69	13	8.25	0.14	26	8.67	0.98	<0.001
miR-330	82	12.01	0.22	13	10.21	0.06	26	9.95	0.09	<0.001

*SD: standard deviation

Table 3. $\Delta\Delta$ CT difference (Mean \pm SD*) and relative quantification (fold change) between studied groups.

Gene	BMI1			miR-330-3p		
	$\Delta\Delta$ CT difference	Fold change	p. value	$\Delta\Delta$ CT difference	Fold change	p. value
CRC vs. Healthy adjacent	-1.82 \pm 0.23	3.92	<0.001	2.05 \pm 0.24	0.32	<0.001
CRC vs. Polyps	-1.39 \pm 0.31	2.62	<0.001	1.79 \pm 0.32	0.28	<0.001
Polyp vs. adjacent	-0.42 \pm 0.35	1.21	0.965	0.25 \pm 0.36	0.86	0.052

*SD: standard deviation

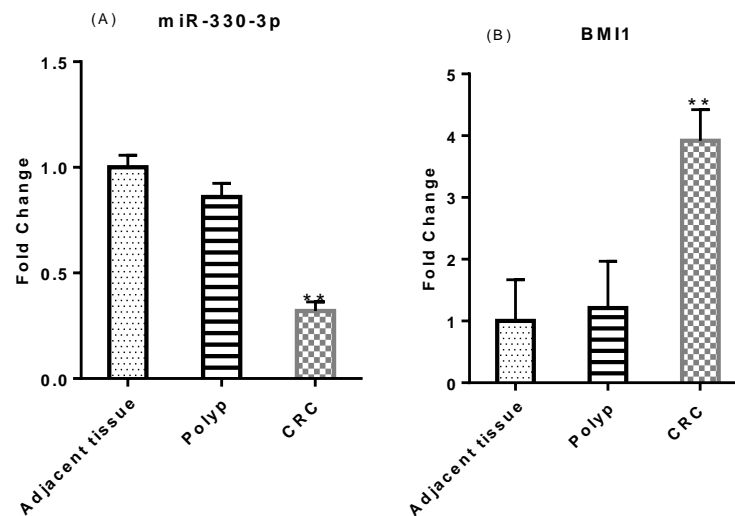


Fig.1. The relative expression of BMI1 and miR-330-3p in different colorectal tissue samples. miR-330-3p was significantly decreased in CRC tissues compared with adjacent normal tissues as well as adenomatous Polyps (**P<0.001), (A). BMI1 was significantly overexpressed in CRC tissues compared with adjacent normal and adenomatous Polyps tissues (**P<0.001) (B). (Data are illustrated as mean \pm SD).

MiR-330-3p significantly decreased in CRC tissues compared with adjacent normal tissues as well as adenomatous Polyps (**P<0.001), (A). BMI1 was significantly overexpressed in CRC tissues compared with adjacent normal and adenomatous Polyps tissues (**P<0.001) (B). (Data are illustrated as mean \pm SD.)

Relationship between expression of miR-330-3p/BMI1 and clinicopathological features

According to the results, a significant higher expression of BMI1 was observed in patients at high stages compared with low stages samples (P<0.001). There was no obvious correlation between miR-330-3p /BMI1 expression and age, gender, tumor grade, and tumor size (P>0.05). Although there was no relationship between smoking and BMI1 expression, significant down regulation of miR-330-3p was obtained in smoker patients (P= 0.04) (Table 4).

Table 4. Associations between age, gender, smoking, TNM staging, tumor grade, lymph node invasion and tumor size, and fold change of BMI1 and miR-330-3p in CRC patients.

Variables	Number	BMI1 Mean \pm SD	P. value	miR-330-3p Mean \pm SD*	P. value
Age	-	-	-	-	-
≤ 50	61	4.39 \pm 2.93	0.12	0.31 \pm 0.24	0.238
>50	21	2.78 \pm 1.17		0.33 \pm 0.12	
Gender	-	-	-	-	-
Male	52	3.90 \pm 2.55	0.62	0.31 \pm 0.22	0.61
Female	30	4.11 \pm 2.93		0.33 \pm 0.21	
Smoking	-	-	-	-	-
No	37	3.51 \pm 2.51	0.19	0.37 \pm 0.23	0.04
Yes	45	4.36 \pm 2.78		0.27 \pm 0.20	
TNM staging	-	-	-	-	-
Low(I/II)	39	1.84 \pm 0.47	0.001	0.52 \pm 0.15	0.001
High(III/IV)	43	5.92 \pm 2.35		0.13 \pm 0.04	
Grade	-	-	-	-	-
I/II	76	3.82 \pm 2.52	0.08	0.33 \pm 0.22	0.09
III/IV	6	6.01 \pm 3.91		0.15 \pm 0.06	
Lymph node invasion	-	-	-	-	-
Negative	17	1.81 \pm 0.27	0.001	0.59 \pm 0.17	0.001
Positive	65	4.54 \pm 2.74		0.25 \pm 0.17	
Tumor size (cm)	-	-	-	-	-
≤ 5	4	2.39 \pm 1.34	0.16	0.46 \pm 0.17	-
>5	78	4.06 \pm 2.71		0.31 \pm 0.22	0.11
OB** test	-	-	-	-	-
Positive	70	4.23 \pm 2.76	-	0.30 \pm 0.21	-
Negative	12	2.52 \pm 1.49	0.080	0.42 \pm 0.22	0.018

*SD: standard deviation; **OB: Occult Blood

Correlation between expressions of the miR-330-3p and BMI1

A negative reverse correlation between the miR-330-3p and BMI1 gene was indicated in the CRC samples ($r:-0.882$, $P<0.001$) (Figure 2) in Pearson correlation coefficient analysis (Figure 2).

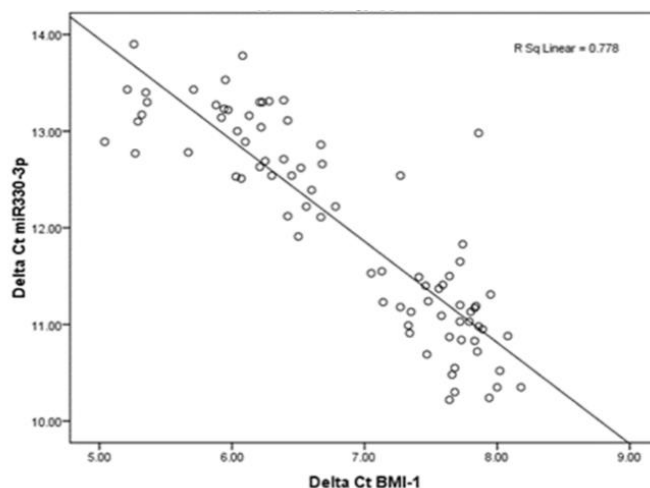


Fig.2. Pearson correlation scatter plot of miR-330-3p and BMI1 expression in CRC.

MiR-330-3p and BMI1 as potential biomarkers for CRC diagnosis

Receiver operating characteristic (ROC) curves and area under the curve (AUC) were developed to evaluate the specificity and sensitivity of miR-330-3p and BMI1 expression for CRC diagnosis. The AUC for miR-330-3p expression was 0.982 (Youden index: 0.774; sensitivity, 98.5%; specificity, 78.8%), and that for BMI1 was 0.971 (Youden index: 0.821; sensitivity, 97.6%; specificity, 84.6%) which indicated excellent diagnostic accuracy to differentiate CRC patients from healthy controls (Figure 3).

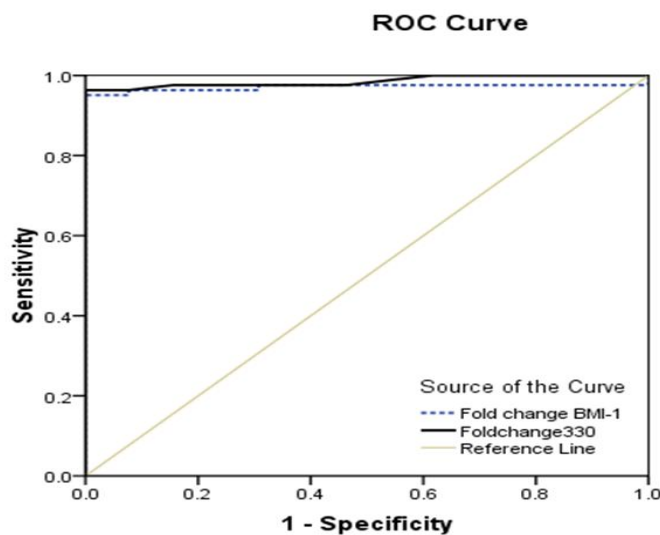


Fig.3. ROC curve analysis of miR-330-3p and BMI1 levels in CRC.

Discussion

CRC is the fourth leading cause of cancer mortality in the world. Identification of dysregulated signaling pathways mediators could be useful in CRC management. Colorectal tumorigenesis initiates with the transformation of the normal colorectal epithelium into a benign adenoma, and then develops with the gradual accumulation of multiple genetic and epigenetic abnormalities, leading to cancer invasion and metastasis [20]. Hence, it is imperative to explore novel biomarkers associated with early diagnosis of CRC. MiRNAs have emerged as crucial epigenetic regulators in the proliferation of cancer cells, migration, invasion, angiogenesis, maintenance of tissue homeostasis, and resistance to chemo or radiotherapy in various types of human cancers, including CRC [21, 22]. In addition, many studies have shown that miRNA dysregulation in the tissues and biological fluids of cancer patients has remarkably associated with CRC diagnosis, prognosis, and response to therapy [23, 24]. This study evaluated the association between tissue expression of miR-330-3p and BMI1 and clinic-pathological characteristics of CRC including tumor stage, size, grade, and lymph node invasiveness. In the present study, it has been demonstrated that miR-330-3p is abnormally down-regulated in CRC, which may be noticed as a potential biomarker in CRC detection. Although miR-330-3p exerts an oncogenic role depending on the type of cancer [25], the tumor suppressive role of miR-330-3p has been revealed in various types of cancer. It has been shown that decreased expression of miR-330-3p in glioma tissues and cell lines compared to normal. miR-330-3p by targeting CELF1 could suppress the proliferation and migration of glioma cells [26]. MiR-330-3p induction could negatively regulate HMG2 expression and induced apoptosis in HCT116 and SW480 cells. Furthermore, miR-330-3p suppressed CRC cells viability, migration and EMT via reducing EMT-related proteins (Snail-1 and VEGFR), and increasing E-cadherin expression [27]. Shirjang et al. have shown miR-330-3p reduction in CRC tissue compared to healthy colorectal tissue, which was associated with poor survival. Moreover, they indicated that miR-330-3p inhibited CRC cell lines proliferation by targeting BACH1 which led to the inhibition of epithelial-mesenchymal transition (EMT) [15]. In another research, Huang et al. showed down-regulation of miR-330-3p in CRC tissue [28]. They demonstrated a negative correlation between miR-330-3p and Profilin 1 (PFN1) as a target gene in CRC. They showed that miR-330-3p suppression negatively regulated CRC cells' apoptosis, proliferation, migration, and invasion. In our study, we showed that miR-330-3p down-regulated significantly in CRC tissue compared to normal marginal tissue, which confirmed the expression pattern of this miRNA in CRC according to other studies. Although miR-330-3p expression was reduced in polypoid adenomatous lesions compared to corresponding healthy tissue, this reduction was not significant. It might indicate the possible role of this tumor suppressor gene in the initiation of the tumorigenesis process. It may be attributed to a stepwise transformation from normal to carcinoma. Also, healthy cells surrounding the tumor mass may show molecular changes such as dysregulation of oncogenes and tumor suppressor genes, so it is better to consider the tissue biopsy of suspected patients who are reported healthy pathologically in the design of such studies.

It seems that due to the intercellular communication between the tumor mass and its surrounding microenvironment, the adjacent tissue was also affected by genetic and epigenetic alterations within the tumor or polyp cells, and this phenomenon may have led to the lack of significant changes between the polyp and the adjacent tissue [29].

miRNAs act on many genes and regulate their target genes at the post-transcriptional level [30]. BMI1 is one of the well-known oncogenes in cancer, especially CRC, which could negatively repress the Ink4a/Arf gene locus. It has been reported that BMI1 inhibition could induce cancer cell apoptosis and decreased the number of cancer stem cells in human renal and cervical carcinoma [31]. Elevated BMI1 expression is robustly associated with cancer stem cell maintenance and self-renewal, which lead to tumor recurrence, metastasis, and chemoresistance [32-34]. An experimental study revealed the regulation of BMI1 expression by miR-330-3p in osteosarcoma. The authors showed miR-330-3p decreased in osteosarcoma tissue and cell lines and, by targeting BMI1, declined tumor progression [11]. We identified for the first time the correlation between miR-330-3p and BMI1 in CRC at the level of gene expression, too. Overexpression of BMI1 was correlated to lower survival rates of CRC patients and clinicopathological characteristics such as TNM stage and tumor grade [35]. In our study, we showed that BMI1 expression increased significantly in CRC tissue compared to healthy adjacent tissue which was consistent with previous studies [36, 37]. On the other hand, increased BMI1 in intestinal polyps was not meaningful compared to healthy tissue. It could indicate the gradual development of the adenomatous polyp as the premalignant lesion towards malignancy. The main limitation of our study is that we could not assess protein and mRNA expression simultaneously therefore, we cannot express higher expression of BMI1 protein exactly.

We found higher BMI1 and lower miR-330-3p expression were correlated with advanced stage and lymph node metastasis. Down-regulation of the tumor suppressor genes and overexpression of oncogenes in higher tumor stages compared to lower stages have been shown in various studies. Liu et al. showed that miR-937 as an oncomiRNA increased in the higher stage of colon cancer tissue and promoted tumor cell proliferation and invasion [38]. Lymph node involvement is strongly related to tumor recurrence and poor prognosis [39]; therefore, evaluation of BMI1 and miR-330-3p expression would be valuable in predicting recurrence, patients' survival, and determining suitable antisense treatment options. MiR-330-3p and BMI1 did not show significant changes between tumor margin and polyp. To the best of our knowledge, no study has been performed to evaluate miR-330-3p and BMI1 expression in the intestinal polyps and CRC. Simultaneously, the statistical significance of the results between polyp and healthy tissues may influence by the increase in the number of samples.

The expression of miR-330-3p in smokers showed a notably lower expression than in non-smokers. Many studies have reported changes in the expression of key genes, including miRNAs, due to cigarette smoke carcinogens [40, 41].

Although the fecal occult blood (FOBT) test was reported positive in most cancer patients (85.4%), FOBT and serum carcinoembryonic antigen (CEA) markers have limited sensitivity and specificity [42]. Due to high sensitivity and specificity in CRC diagnosis, the excellent ability of miR-330-3p/BMI1 dual markers (sensitivity, 98.5%, and 97.6%; specificity, 78.8%, 84.6%, respectively) to discriminate between tumoral and healthy samples could improve cancer management. It seems that at least by combining the studied biomarker panel along with FOBT and CEA, it may be possible to increase the sensitivity and specificity in distinguishing between CRC patients and healthy individuals. In conclusion, our study represents a negative correlation between miR-330-3p and BMI1, which might suggest a novel dual panel diagnostic biomarker with high specificity and sensitivity in CRC.

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