BMC Cancer

RESEARCH

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Evaluation of epigenetic silencing of the *miR*-139-5p gene in the pathogenesis of colorectal cancer and its diagnostic biomarker capability in plasma samples

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Abstract

Background The pathogenesis of CRC requires primary genetic and epigenetic mechanisms including, methylation of CpG islands of the genes. In the current study, micro RNA-139-5p (*miR*-139-5p) promoter methylated DNA was evaluated in tumor tissue and plasma samples from CRC affected patients.

Methods *MiR*-139-5p promoter methylation was investigated in 80 samples of tumoral tissue and healthy marginal tissue and the same number of plasma samples, using the MethyLight method. The miR-139-5p expression was assessed using the qPCR method. BT (Bioassay Technology) Elisa kit was applied to measure RAP-1b as a target gene of miR-139-5p.

Results Median PMR values of 12.4 (95% CI, 3.23–32.25) and 0.66 (95%CI, 0.51–1.0) were obtained from plasma samples of CRC patients and controls, sequentially. In plasma samples, the sensitivity and specificity of *miR*-139-5p promoter methylated marker were 75% and 92.5%, in the same order (AUC = 0.958).

Lower expression of miR-139-5p in plasma and tumor tissue of patients ($P < 0.001$) was shown. Also, a significant rise of RAP-1b protein concentration was observed in both mentioned specimens.

Conclusion Hyper-methylation of *miR*-139-5p could be suggested as high accuracy diagnostic biomarker for the detection of CRC in plasma samples, pending further validation with large prospective studies.

Keywords Biomarkers, Colorectal Neoplasms, DNA Methylation and human MIRN139

Background

The second and third most commonly diagnosed malignancy is attributed to colorectal cancer (CRC) in women and men, respectively, and the second leading cause of cancer death globally [1, 2]. There is a noticeable difference in CRC incidence and mortality [3, 4]. It is frequently diagnosed at advanced phases due to the limitations of current clinical screening methods [5]. Relative CRC risk is specified by genetic potency and environmental influences, with age being the most imperative risk factor for sporadic CRC. Transformation of normal colonic mucosa

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into invasive cancer occurs through the accumulation of genetic and epigenetic changes [6]. Despite the identifying of several molecular pathways intervened in the development of CRC, the detailed molecular pathogenesis of this cancer has not yet been clarified [7, 8]. MicroRNAs (miRNAs) with their target genes organize a very complex network in cells, and it is expected that abnormal expression of miRNAs interrupts this RNA system. Many surveys have revealed that deregulation of miRNAs act as tumor suppressors or oncogenes in CRC cells by targeting neoplasm-related genes [8]. New advances in molecular biology have demonstrated the role of miRNAs in various biological and pathological functions including, down-regulation of tumor suppressor miRNAs in many cancers, particularly CRC [9]. Epigenetic variations, in particular DNA methylation and histone modifications, perform major pathophysiological role in the beginning and progression of CRC [10]. Covalent bonding of a methyl group to the 5-carbon status of the cytosine is widespread throughout the genome; however, when added to CpG in the promoter region, it generally results in lower expression of methylated gene. The mechanism of gene silencing by DNA methylation may be related to a barrier of the transcriptional machinery or modification in chromatin configuration or recruitment of repressors [11]. Owing to the truth that methylation in a specific region of the genome occurs in the primary steps of tumorigenesis [12], and significantly, most cancer deaths are due to metastatic spread of tumor cells not due to the primary tumor [13], early diagnosis increases the chance of recovery and prevention of subsequent complications. *MiR*−139, located on 11q13.4 and is down-regulated in several cancers, exposes anti-oncogenic and anti-metastatic activity in human tumors [14, 15]. MiR-139-5p, encoded within the second intron of the phosphodiesterase 2A gene [16], was recognized as, a tumor-suppressor miRNA, and is down regulated in a diversity of cancers, such as, gastric cancer and CRC [17, 18].

The Ras family of low molecular weight GTP-binding proteins have been occupied in a numerous cellular processes, comprising differentiation and cell growth, intracellular vesicular transferring, nucleocytoplasmic transport, and cytoskeletal restructuring [19]. Ras-Related Protein Rap-1b (RAP- 1b) is a low molecular mass GTP-binding protein (22-kDa) which is both a substrate for cAMP-dependent protein kinase and a member of the Ras superfamily [20]. In previous studies, RAP-1b has been identified as one of the straight target of miR-139-5p [17, 21]. We aim to determine and evaluate the expression of miR-139-5p, RAP-1b protein, and the methylation condition of the *miR*−139-5p promoter region in plasma samples of CRC affected individuals, healthy controls and in tumor tissue and healthy near specimens. Furthermore, we examined the diagnostic value of these biomarkers and their relationship with clinical and pathological features.

Methods

Tissue and plasma samples

A total of 80 plasma samples from CRC patients and healthy matched individuals, as well as 40 paired tumor and adjacent healthy tissues were provided in the present survey. The inclusion and exclusion criteria of clinical samples have been reported in our previous study [22]. Written informed was signed by all participants. This survey authorized by the Ethics Committee of Hamadan University of medical sciences (IR.UMSHA.REC.1396.533).

Methylation analysis using MethyLight method

This section of the experiments has explained in detail in the previous article [22] and it is briefly as follows: The promoter area of the *miR*−139-5p gene was identified using the miR Start and Ensemble Databases. After that, the most specific primers and probes for fully methylated bisulfite-converted DNA (one set for *miR*−139-5p gene and one for ALU-C4)were designed for a GC-rich area inside the promoter region using Beacon designer software (version 8.13) (Table 1). The probes and the primers were synthesized by the German company Metabion.

Table 1 The primers and probes specifications

Primers and probes	Sequence	Tm^{o}_{C}	Length (bp)
ALU-C4 Forward primer	5′-GGTTAGGTATAGTGGTTTATATTTGTAATTTTAGTA-3	66	36
ALU-C4 Reverse primer	5′-ATTAACTAAACTAATCTTAAACTCCTAACCTCA-3′	65	33
ALU-C4 Probe	5′-CGCTAACCGAACGCAACGAC-3′	51	16
miR-139 Reverse Primer	5′-TTTCGTTTGAGGAATTGGATAATAGCG-3′	61.2	30
miR-139 Forward Primer	5′–6-FAM-ACCGACGAACGAACGAACGACTACGAAC-BHQ-1–3′	60	29
miR-139 Probe	5′–6-FAM-CCTAAACCACAAACAACCACGAACCTCGCC-BHQ-1–3′	68.9	30

Next, DNA of all specimens extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Germany, Cat.No:51104) [23] followed by bisulfite modification using an EpiTect Bisulfite kit (Qiagen.Cat.No.59104) in duplicate. Sodium bisulfite has no impression on methylated cytosine, but it causes deamination of non-methylated cytosine and modifies it to uracil. This difference is the basis for distinguishing methylated from non-methylated DNA [24].

Finally, the qPCR reactions were done in 96-well plates on an ABI 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) in duplicate, using the EpiTect® MethyLight PCR + ROX Vial kit (Qiagen GmbH). ALU-C4, a consensus DNA sequence, was applied as the housekeeping gene for normalization.

The percentage of methylated reference (PMR), the degree of methylation of every sample relatively to the entirely methylated control, of the samples, calculated using saleable methylated human bisulfite converted DNA (EpiTect PCR Control DNA; Qiagen GmbH), as an entirely methylated control.

PMR, at a defined locus, was assessed using the subsequent equation: $[(\text{miR/ALU-C4})^{\text{sample}}/(\text{miR/ALU-C4})^{\text{positive control}}] \times 100$ [25].

MiR-139-5p expression and measurement of RAP-1b protein in clinical samples

A favorgen miRNA (Favorgen, Taiwan, Cat.No: FAMIK002) and NucleoSpin® miRNA Plasma (Macherey–Nagel, Germany) extraction kits were applied to extract miRNAs from tissue and plasma samples, respectively. The extraction of miRNA from approximately 10 mg of tissue sample consists of four following steps: 1- Cell lysis 2- Binding of DNA to the silica column membrane, 3- Column washing, and 4- Dilution of DNA in buffer which in the first step,tissue sample was ground into smaller pieces using a scalpel blade. Then the sample was transferred to a 1.5 ml microtube and the next steps were performed according to the kit instructions. The quality and quantity of RNA was evaluated using the nanodrop (ND-1000). BON miR Kit (Bon yakhteh, Inc) was applied to synthesis cDNA. Finally, qPCR was carried out to assess the expression of miR-139-5p supported by the BON miR SYBR®green PCR Kit (Bon yakhteh, Inc) in accordance with the company's instructions. U6 as a housekeeping gene used to normalize data and the expression level was computed by the $2^{(-\Delta\Delta CT)}$ manner [26]. ELISA kit (Cat.no:E2153Hu) was applied to measure RAP-1b Protein in an ELISA reader (Rayto 2100 C).

Statistical analysis

The Chi-square test was used to analyze descriptive data. The normality of data distribution evaluated by Kolmogorov–Smirnov test. Therefore, non-parametric Mann–Whitney, Kruskal–Wallis tests, Anova, and independent t-tests were used depending on the results of normality test. Using SPSS 16 software, P value <0.05 was considered as the level of statistical meaningful. The specificity and sensitivity of studied biomarkers evaluated by Receiver Operating Characteristics (ROC) analysis using PMR, ΔCT, and RAP-1b quantities. In general, the area under the ROC curve (AUC) ≥ 0.8 is assumed acceptable and AUC ≥ 0.9 is interpreted excellent [27].

Results

Forty tissue samples from CRC affected patients and 40 from marginal normal tissue (22 females and 18 males) with a mean age of (57.7 ± 17.2) and the same number of plasma samples from case group and healthy individuals (22 females and 18 males) with a matched ages as a control participants were included in the study.

Hyper methylation of *miR*−139-5p in CRC without any clinical and pathological association was observed

Median PMR values of 0.78 (95% CI, 0.12–6.58) and 0.1 (95% CI, 0.01–0.29) were obtained in tumor and adjacent normal tissues, at the same order. The uppermost PMR value got from marginal normal tissues was assumed as a threshold level; specimen with a PMR >5.1 were translated positively for methylation status (36/40, (90%)). As demonstrated in (Fig. 1), the PMR level was remarkably larger in the cancer tissues compared with that of the normal tissues ($p < 0.0001$).

The median PMR levels of 12.4 (95% CI, 3.23–32.25) and 0.66 (95%CI, 0.51–1.0) in plasma samples from the CRC and the normal groups, were obtained, sequentially. Also, the maximum PMR value from the healthy plasma specimens was assumed as the threshold of methylation condition; thus, all samples with a PMR >4.8 were supposed positively methylated (29/40, (72.5%)). The PMR values for CRC patients were noticeably greater in plasma samples in comparison with those of control subjects ($p < 0.0001$) (Fig. 1).

Results from PMR values showed no statistically meaningful association between the tissue and plasma specimens and clinicopathological characteristics of CRC patients (Table 2).

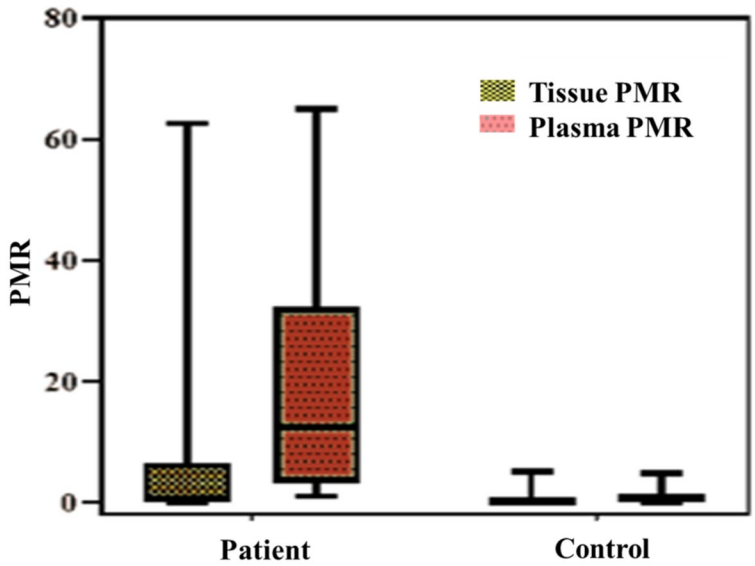

Fig. 1 Box plot of PMR level of the *miR*−139-5p gene in clinical samples. The median PMR values for CRC patients were higher in plasma samples in comparison with those of control persons (12.4 vs. 0.66, $p < 0.0001$) as well as in tissue specimens (0.78 vs. 0.1, p (< 0.0001)

Table 2 Relationship between *miR*−139-5p methylation and clinicopathological specifications in clinical samples of case group

Clinicopathological characteristic	No. of patients	Tissue PMR Mean ± SD*	*P*-value	Plasma PMR Mean ± SD	*P*-value
Tumor type					
Adenocarcinoma	29	4.19 ± 7.98	0.353[a]	17.22 ± 17.13	0.338[a]
Mucinous	11	12.89 ± 19.41		21.14 ± 18.22	
Histological grading					
I	15	5.96 ± 10.07	0.490[c]	21.74 ± 19.45	0.583[c]
II	21	7.98 ± 15.12		15.15 ± 13.99	
III	4	1.61 ± 2.19		21.91 ± 25.87	
Tumor location					
Distal	13	8.41 ± 17.99	0.732[a]	13.03 ± 14.55	0.168[a]
Proximal	27	5.71 ± 9.21		20.83 ± 18.17	
Tumor size					
< 50 mm	21	7.89 ± 14.85	0.390[a]	19.70 ± 19.33	0.452[a]
≥ 50 *mm*	19	5.14 ± 9.62		16.75 ± 15.09	
TNM Stage					
I	13	4.83 ± 3.69	0.422[b]	22.55 ± 16.46	0.348[c]
II	19	5.13 ± 9.01		14.47 ± 16.50	
III	8	12.91 ± 21.99		20.49 ± 20.52	
Lymph node involvement					
Yes	8	12.91 ± 21.99	0.209[a]	20.49 ± 20.52	0.209[a]
No	32	5.01 ± 8.74		17.75 ± 16.71	

*Standard deviation

[a] Mann–Whitney U two-tailed tests

[b] ANOVA

[c] Kruskal–Wallis

Down-regulation of miR-139-5p and greater quantity of RAP-1b protein in both plasma and tissue samples without any association with clinicopathological parameters

The expression of miR-139-5p in tumor tissue ($p < 0.0001$) and plasma of patients ($p < 0.001$) had a significant decrease, in comparison with the healthy participants (Fig. 2).

There was no remarkable association between down regulation of miR-139-5p and any clinical and pathological characteristics (supplementary Table 1).

The concentration of RAP-1b, a valid target of miR-139-5p, was higher in CRC patients than in control groups. (Table 3 and supplementary Fig. 1). Also, the results revealed that there is no statistical meaningful association amongst RAP-1b concentration and clinicopathological characteristics (supplementary Table 2).

The results of diagnostic value of studied biomarkers using ROC curve analysis

A high level of carcinoembryonic antigen (CEA) in plasma specimens of CRC subjects was observed in our previous work [22]. As observed in Fig. 3, the specificity and sensitivity of the *miR*−139-5p methylated biomarker in plasma is higher than CEA and FOBT tests.

Discussion

MiRNAs have essential effects on gene expression and signaling pathways so that deregulated miRNAs could be related to different diseases, including CRC [28]. In previous studies, miR-139-5p, as a tumor suppressor, has been identified as an influential factor in cancer diagnosis, prognosis, and treatment in various types of human cancers [29, 30]. Many molecular mechanisms lead to miRNA deregulation [31], including transcriptional tumor suppressor miRNAs silencing by special

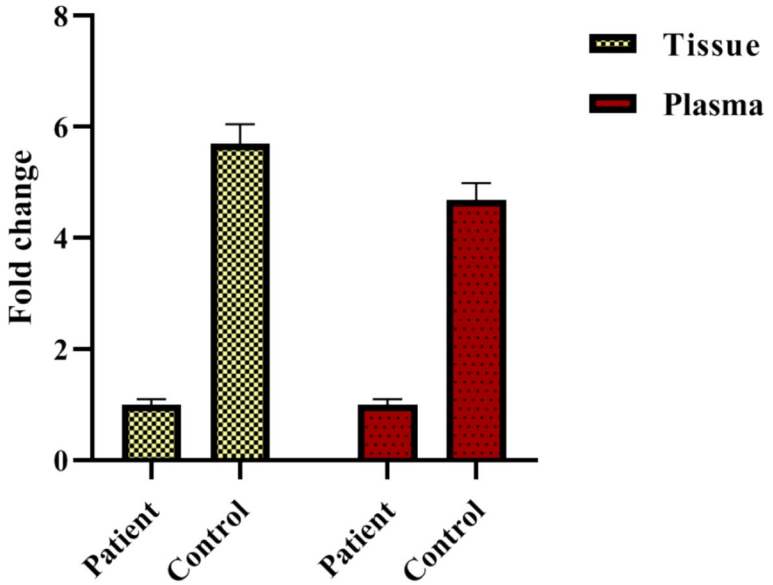

Fig. 2 Overall 5.7 and 4.69 fold ($2^{-\Delta\Delta CT}$) lower expression of miR-139-5p in tissue and plasma specimens of CRC affected patients, respectively (Mean ± SEM, $n = 40$, $p < 0.0001$ and $p < 0.001$, respectively.)

Table 3 The greater quantity of RAP-1b protein in clinical samples of CRC patients

Parameter	No. of patients	Minimum	Maximum	Percentiles			*P*-value
				25th	50th	75th	
Tissue RAP-1b(ng/l)							
Patient	15	29.00	85.00	41.00	51.00	64.30	< 0.0001
Control	15	9.50	36.80	11.80	21.00	29.00	
Plasma RAP-1b(ng/l)							
Patient	30	3.10	93.00	14.53	32.67	40.25	0.004
Control	29	0.10	36.3	9.82	15.70	28.60	

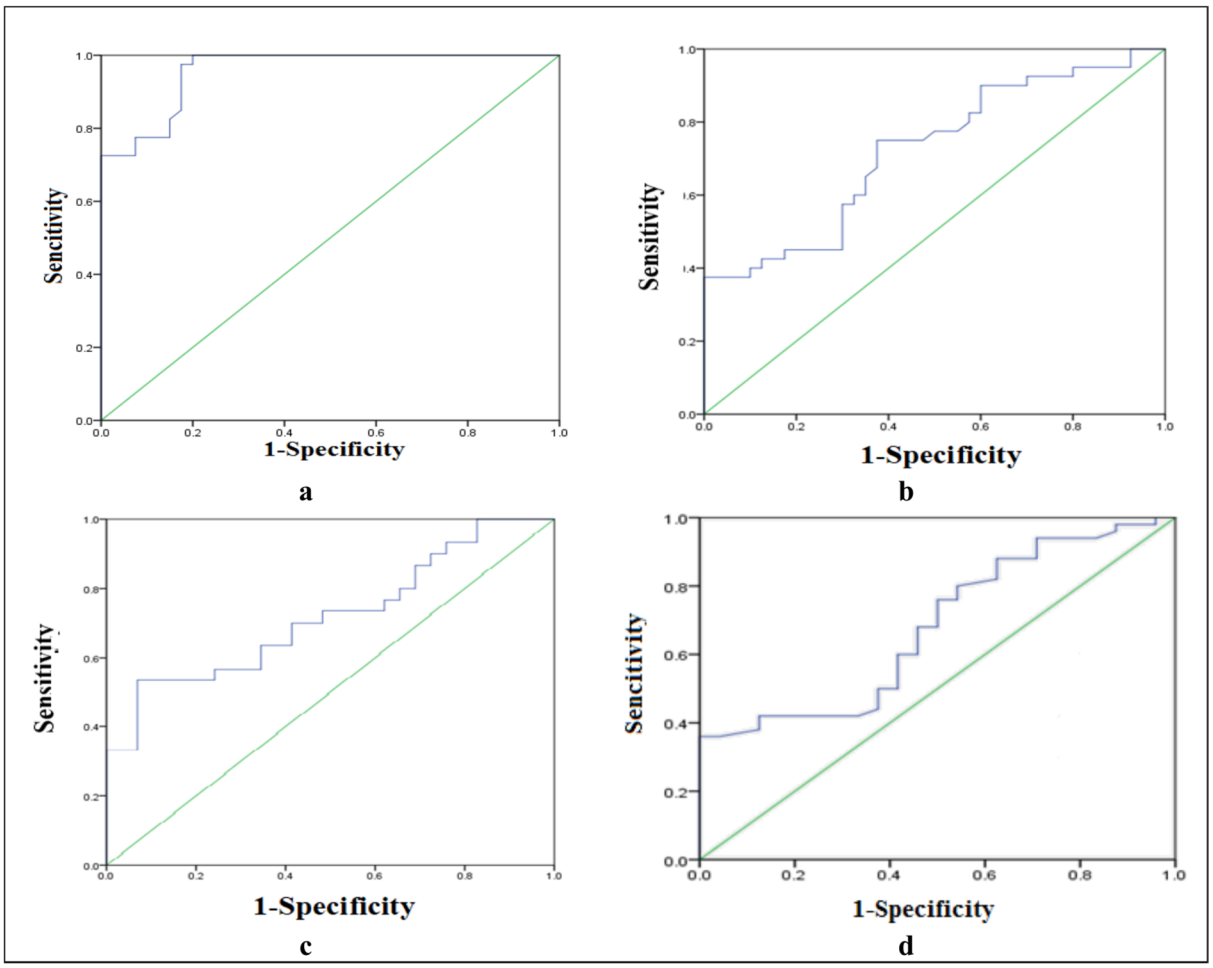

Fig. 3 Diagnostic value of studied plasma biomarkers using ROC curve analysis shows an excellent AUC, and higher specificity and sensitivity for the plasma *miR*−139-5p methylated biomarker which is able to detect 75%(sensitivity) of CRC patients and distinguishes 92.5% (specificity) of individuals without CRC a; PMR *miR*−139-5p (AUC = 0.958, the sensitivity and specificity were 75% and 92.5%, respectively), b; miR-139-5p expression (AUC = 0.767, the sensitivity and specificity were 70% and 65%, in the same order), c; RAP-1b (AUC = 0.718, sensitivity = 73.3%,and specificity = 73.3%), d; CEA (AUC = 0.708, sensitivity = 46.8%,and specificity = 98%)

DNA methylation in cancer cells [32]. Haiyan Guo et al. demonstrated that miR-139 decreases proliferation in CRC through targeting RAP-1b directly [33]. The current research examined methylation pattern of *miR*−139-5p, its expression and target (RAP-1b) in both cancer and healthy groups to acquire more vision about the miR-139-5p function in CRC disease. This study showed that the methylation level of the promoter area of the *miR*−139-5p gene was significantly higher in the plasma and tissue specimens of CRC patients. On the other hand, the level of miR-139-5p significantly decreased in the tumoral group compared with the healthy group in both tissue and plasma samples.

Also, a significant rise of RAP-1b protein was observed in mentioned clinical specimens of the patients group compared to those in the healthy participants (Table 3). Therefore, negative regulation of RAP-1b by underexpressed miR-138-5p occurs through epigenetic changes and RAP-1b is able to induce MAPK-dependent signaling cascade and cause cell growth and proliferation of CRC cells [33, 34]. Consequently, hyper methylation of the promoter area of the miR-139-5p exerts its effects in the pathogenesis of CRC through modulation of MAPK signaling pathway. The potential of miRNAs as a therapeutic target is being considered [35] to control this cascade such as miRNA mimics or demethylating agents like 5-azacytidine which can reactivate miRNA expression [36, 37]. For example, systemic or local application of intact miRNA molecules, miR-145, into mouse xenograft colon carcinoma reduces tumor growth, increases apoptosis, and simultaneously suppresses the c-Myc and ERK5 oncogenes [38].

In line with our study, several studies had reported that RAP-1b is overexpressed in various cancers, such

as thyroid cancer, hepatocellular carcinoma, and CRC, which in turn leading to proliferation and invasion of tumoral cells [39–41].

Agree with our study, it was revealed that miR-139 is under expressed in CRC tissues. The outcomes verified that miR-139-5p reduces proliferation, invasion and migration by straightly targeting RAP-1b and tumor protein D52, manifesting miR-139 as a putative tumor suppressor miRNA in patients with CRC [33, 42]. Also, miR-139-5p might be suggested as a biomarker to predict the transformation of inflammatory bowel disease to CRC [43].

Furthermore, we realized that methylation and expression levels of miR-139-5p as well as its target, RAP-1b protein, are not correlated with clinicopathological parameters, including histological grade, tumor stage, tumor size, lymph node metastasis, tumor type, age, gender, and distant metastasis, because on one hand, all eligible untreated patients, pathologically confirmed CRC, irrespective of sex, age, tumor type and location, histological grading, TNM staging, etc. were included in the study and the control group was matched with the patient group by age and sex [22]; therefore, the number of patients was divided between the mentioned subgroups, including different grades and different stages, etc., and on the other hand we were not able to increase the number of samples in the study subgroups because of limitation of financial resources. In our opinion, it is better to calculate the sample size for subgroups to assess the relationship between the level of gene expression as well as gene methylation with clinical and pathological features of patients. Another suggestion in future surveys is that considering the larger sample size to provide a more appropriate distribution of cases between subgroups.

To the best of our knowledge, although the methylation of *miR*−139-5p in CRC disease has not been yet evaluated, a decrease in methylation-based expression has been reported for other miRNAs such as miR-148a, is hypermethylated in tissue of 65% of CRC patients with no meaningful relationship with clinicopathological specifications [44]; and other researches have illustrated that DNA methylation has an essential impression on deregulation of some miRNAs, consist of miR-125 and 34 in CRC, and accelerating the tumor development [45, 46].

The use of diagnostic tests to determine the presence or absence of a suspected disease is critical in clinical situations. The ideal test is defined by a sensitivity and specificity of 100% which is rare in the clinical setting, because sensitivity and specificity decrease as each of them increases. Also, an ideal test has a ROC curve with AUC = 1.0, but in general, AUC ≥ 0.8 is assumed suitable and AUC ≥ 0.9 is interpreted excellent [27]. Graphical plot of diagnostic classification, ROC curve, presented a great AUC of 0.767, 0.958, and 0.718 of miR-139-5p, PMR value, and RAP-1b protein for plasma specimens, in the same order (Fig. 3). These results suggest the ability of these biomarkers to recognize CRC disease from healthful persons. To our knowledge, there are no surveys have previously assessed the diagnostic value and methylation pattern of *miR*−139-5p and its expression in plasma specimens. Therefore, our results compared to other miRNA,s methylation modes and other classes of molecular biomarkers.

Using conventional methylation-specific PCR (MSP), the sensitivity of 76.8% and a specificity of 93.6% for stool *miR*−34a methylation test were reported [47], although compared to quantitative MSP, the mentioned technique is not quantitative and has lower accuracy for detection of CRC. Also, compared to the sensitivity (55%) and the specificity (82.5%) of plasma level of methylated *miR*−138-5p [22], the present study has higher diagnostic value with an excellent AUC.

There are different classes of molecular biomarkers by source or by type including traditional or new kind. Measurement of gene expression at mRNA level and DNA methylation in plasma belongs to genomic new biomarkers while protein detection is traditional [48]. Jelski Wojciech et al. reported diagnostic value of aldehyde dehydrogenase isoenzyme I (ADH I) in the serum of patients with CRC with the sensitivity and specificity of 76% and 82%, respectively [49]. Thus, compared to traditional type of biomarkers, the diagnostic value of *miR*−139-5p promoter methylated marker is more favorable (the sensitivity was 75%, and specificity was 92.5% with AUC = 0.958).

Because methylation changes befalls in the primitive stages of tumor formation [12], perhaps it is possible to get an admissible clinical sensitivity by evaluating this methylation marker in the biological samples including, plasma and serum. Comparing with the CEA (46.8%) and FOBT (43.5%) results, the sensitivity and AUC of *miR*−139-5p PMR was more in plasma, which shows potential as a diagnostic biomarker for the CRC, pending further validation.

Conclusion

In this investigation, we assessed the methylation pattern of the *miR*−139-5p gene in liquid and soft samples of CRC patients compared to the healthful individuals for the first experience. We illustrated that miR-139-5p is significantly under expressed in the case group compared to the healthy group. Also, compared to the normal group, the rate of gene methylation in the case group was meaningfully greater, indicating the role of epigenetic factors, especially gene methylation, in the miR-139-5p down-regulation. The superiority of

current survey was the concurrent assessment of miR-139-5p expression and RAP-1b protein and admeasuring the methylation mode of promoter region of the gene in liquid and soft tissue specimens, which prepared the feasibility of further comparison of the results. Finally, it could be supposed that hyper methylation inside the promoter area of the *miR*−139-5p gene is one of the critical epigenetic agents implicated in reducing miR-139-5p expression. Due to the occurrence of methylation alterations in primary steps of carcinogenesis, it can be proposed as a miRNA-based therapeutic target and an early detection biomarker for CRC diagnosis in plasma samples. However, further study on the more number of clinical samples is necessary to confirm it as a diagnostic biomarker in patients'plasma.

Abbreviations

CRC	Colorectal Cancer
miRNAs	MicroRNAs
RAP- 1b	Ras-Related Protein RAP-1b
PMR	Percentage of Methylated Reference
ROC analysis	Receiver Operating Characteristics analysis
AUC	The Area Under the ROC curve
CEA	Carcino Embryonic Antigen
MSP	Methylation-Specific PCR

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12885-025-14290-x.

Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

Acknowledgements
The current study, supported by Vice Chancellery for Research and Technology, Hamadan University of Medical Sciences (No. 9609286007). The authors would like to thank the staff of the Medical Genetics Laboratory, Reference Laboratory, and Kermanshah University of Medical Sciences for their help during the current research.

Authors' contributions
AM and MA designed the research. AM, MS, ARK, and NAR supervised primary data collection and cleaning. ARS and MA analyzed the data under the supervision of AM and MS. ALS performed the statistical analysis. AM and MA wrote the draft. , and all authors reviewed and approved the final manuscripts.

Funding
This study was funded by Vice Chancellery for Research and Technology, Hamadan University of Medical Sciences (No. 9609286007).

Data availability
Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate
Informed consent was obtained from all participants. The Ethics Committee of Hamadan University of medical sciences approved this survey (IR.UMSHA.REC.1396.533).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 26 November 2024 Accepted: 8 May 2025
Published online: 15 May 2025

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