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Original Article

MiR-1 Variations in Colorectal Cancer: Possible Implementation as a Potential Accessory Biomarker

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ABSTRACT

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Keywords
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Background and Aims: Colorectal cancer (CRC) is one of the most common human cancers. Currently, carcinoembryonic antigen (CEA) is used as the main standard biomarker of CRC, though this biomarker is not specifically made for CRC and, in a minority of cases, shows inadequate sensitivity. Therefore, searching for novel accessory biomarkers may fill these gaps in clinical management. miRNAs physiologically regulate various metabolic processes and are misregulated in various cancers. Therefore, the present investigation was conducted to evaluate miR-1 levels in CRC samples.

Materials and Methods: The CRC and adjacent tissue samples were obtained from 24 patients. In addition, sera were collected from the patient group and 24 healthy controls. Total RNA was extracted from tissue samples, and cDNA was synthesized. Real-time PCR determined the expression of miR-1. Serum levels of CEA were also measured using a Monobind ELISA assay kit.

Results: The level of miR-1 in CRC tumors was significantly down-regulated. Moreover, patients with metastasis showed lower expression of miR-1 compared to cases without metastasis; however, this difference was not statistically significant. The ROC curve for miR-1 showed an AUC of 0.69. In addition, ROC analysis revealed a sensitivity of 70.27% and a specificity of 62.96% for miR-1.

Conclusion: There is still a need for new upcoming markers in addition to the main CRC biomarker, CEA. The levels of miR-1 in colorectal cancer tissue samples may provide additional information for the management and follow-up of CRC patients; though, the clinical application needs further studies.

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Introduction

Colorectal cancer (CRC) is assumed to be the second most common cancer in females and the third in males. In 2020, more than 1.9 million new cases were diagnosed globally [1, 2], and more than 935,000 mortality rate due to CRC was estimated [2]. Numerous factors play key roles in the development of CRC, including family history, colon polyps, cholecystectomy, and lifestyle. In addition, intestinal microbiome, age, gender, race, and socioeconomic status have been identified as other risk factors for CRC [3-6]. Generally, the disease is diagnosed at progressed stages due to the constraints of the popular screening and diagnostic approaches applied in the clinics [7, 8].

Among available biomarkers, carcinoembryonic antigen (CEA) is one of the most commonly used indicators of CRC [9]. CEA, a critical glycoprotein, which could be found in the serum of patients with CRC, has been recommended as a standard prognostic biomarker to determine the prognosis and stage of CRC [9-11]. Despite the proper efficiency of this marker, auxiliary markers are still needed, and also the biomarker is not specific for CRC, and increased levels of CEA may be observed in gastrointestinal, lung, and breast cancers. In addition, CRC patients with normal serum CEA levels have been repeatedly reported, and inadequate sensitivity of the test remains a pitfall in CRC screening, management, and follow-up [12, 13]. Accordingly, researchers are looking for new biomarkers. Among them, miRNAs have been noticed as promising upcoming biomarkers [9, 14, 15].

MiRNAs are short, single-stranded, non-coding RNA sequences of approximately 21-23 nucleotides transcribed by RNA polymerase II. They are initially transcribed as longer capped transcripts (Pri-miRNA), which are further cleaved by Drosha ribonuclease to form about 70 nucleotide precursor miRNA (Pre-mRNA). The nucleic acid chain is finally cleaved by cellular Dicer ribonuclease to produce mature miRNA. Although discovered in 1993 [16], the regulatory role of miRNAs was identified in the early 2000s. In the past two decades, the number of recorded miRs has reached 38589 entries based on the miR database (Mirbase.org), and increasing roles have been cited.

The association of miRs with various diseases, including human cancers, has drawn great scientific attention. Notably, miRs act in a specific mode of action. Thereby, they may show diverse effects on human cancers. MiRs may have oncogenic (oncomir) or protection/ tumor-suppressing effects; interestingly, such effects may be tissue specific. Misregulated miRs have been investigated in a vast range of human cancers [17], and various mechanisms in human cancerogenic/ tumor suppressor effects have been discussed [18-20]; however, it seems reasonable that many of the underlying mechanisms have remained unexplored.

Among several miRNAs, miR-1 has excellent attention for investigation in different cancers [21]. MiR-1 has also been implicated in cancer protection, and dysregulated miR-1 has been shown in various cancers such as

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rhabdomyosarcoma [22], lung [23], ovarian [24], hepatocellular carcinoma [25] and head and neck squamous cell carcinomas [26]. The miR-1 has drawn scientific attention to colorectal carcinoma [27, 28], and some mechanisms related to the mode of action of miR during malignancies have been discussed [29, 30]. However, the topic remains controversial, and the degree of miR changes among CRC patients and the possible clinical application still needs further investigation from different clinical centers worldwide. Despite the noticeable current knowledge, the role of miR-1 on human cancer remains an ongoing topic to fill the gaps.

Looking for new, less invasive biomarkers, preferably providing additional data to the routinely used biomarkers is needed to improve the management of malignancies; and miRs may be a new tool to facilitate staging or determining the prognosis of cancers. In this regard, we evaluated the correlation of miR-1 levels as a biomarker of disease progression, particularly in comparison with the known CEA in a group of Iranian CRC patients in the present study.

Materials and Methods

Study groups

In this cross-sectional pilot study, freshly resected CRC and adjacent non-cancerous tissue samples from 24 patients were obtained at the general surgery department. The tissue samples were collected from Sep 2020 until May 2021. The study included all the admitted patients who were accepted to participate. The tissue samples were frozen at -70 °C until used for real-time assays. In addition, serum samples were taken from the patients before the tumor resection to measure CEA. The healthy control group for CEA assays consisted of 24 sex and gender-matched individuals who had routine biochemical serum tests at the department of clinical biochemistry of the university hospital and agreed to donate the excess remaining serum samples for CEA assay in the present investigation. All the participants filled out a questionnaire to address known underlying conditions affecting miR-1 or CEA. The characteristics of neoplasm consisted of the tumor's location, and staging data, including tumor size, lymph node involvement, and distant metastasis were recorded from patients' files. Tumor staging was done according to the Tumor-Node-Metastasis (TNM)-based staging system issued by the American Joint Committee on Cancer (AJCC)/Union for International Cancer Control (UICC).

Gene expression assessment

Total RNA was extracted from 24 CRC tissue and adjacent non-cancerous tissues sample using RNX-plus (CinnaGen. Tehran, IRAN). The concentrations and purities of RNA samples were analyzed spectrophotometrically using a NanoDrop devise. The cDNA was synthesized from equal amounts of extracted RNA from each sample using a cDNA synthesizing kit (SinaClon, Tehran, Iran). The specific primers for miR-1 and the internal control gene (SNORD 47) were designed using Allele ID version 7.7 software (Table 1).

The expression of miR-1 was quantified using LightCycler® 96 Roche real-time thermal cycler device (Roche, Switzerland) based on

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the SyberGreen method. Reactions were done as follows; denaturation at 95 °C for 10 min, 50 cycles of denaturation at 95 °C for 10 sec, and annealing/ extension at 60 °C for 45 sec. Each sample was analyzed at least twice, and ΔΔCt method was used for expression analyses [31].

Evaluation of CEA serum concentration

According to the manufacturer's guideline, CEA was measured in the healthy controls and patients' group serum using a routine enzyme-linked immunosorbent assay (ELISA) kit (Monobind Inc., CA, USA). Samples and standards were added to the Streptavidin pre-coated wells, and then a biotinylated monoclonal antibody specific for human CEA was added to the wells. After incubation and washing, the enzyme-labeled antibody was integrated with the mixture. Following the second incubation and washing steps, substrate and then stop solutions were added in order, and the intensity of color was checked spectrophotometrically at 450 nm. The study was performed according to the ethical principles mentioned in Helsinki's declaration on human research. The study proposal was reviewed and approved by the Health Research Ethics Committee of Mashhad University of Medical Sciences (Ethical approval code: IR.MUMS.MEDICAL.REC. 1400.016). Participation was voluntary and anonymous and written informed consent was obtained from all the study participants.

Statistical analysis

Statistical analyzes were performed using SPSS Statistics 22.0 software. The Kolmogorov-Smirnov test was used to check the normal distribution of data. T-test and one-way ANOVA were used for statistical significance. Tukey's method was also used for multiple mean comparisons. In all calculations, a p-value < 0.05 was considered statistically significant. The receiver operating characteristic curve (ROC) and area under the ROC curve (AUC) was created for miR-1 expression and CEA levels to assess the specificity and sensitivity of these markers in colorectal cancer.

Results

The patient group consisted of 12 men and 12 women, with a mean age of 52.4 ± 15 years (12 cases < 50 and 12 cases ≥50). The healthy individual controls for the CEA serum assay comprised 24 age and gender-matched control groups aged 51 ± 13.3 years. The clinical characteristics of the patient population are summarized in Table 2.

The extraction of RNA from fresh tissue samples was confirmed by measuring OD 260/ 280 ratio. The RNA sample concentration was determined between 500 - 800 µg/ml. The extracted RNA was used for cDNA synthesis and applied for real-time PCR assays. Finally, 18 of 24 tissue samples showed laboratory-acceptable curves and were included in expression analyses. The sigmoidal pattern of amplification curves indicated the target sequences' amplification. The melting curve analysis of both miR-1 and *SNOR47* amplified sequences showed a single peak, indicating the specificity of the PCR reaction. The expression of miR-1 in colorectal patients was significantly down-regulated compared to adjacent tissue ($t = 2.82$, $df = 36$, $p = 0.007$), and the difference between means ± SEM was -3.029 ± 1.071.

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The amount of CEA in the healthy control and patient group was quantified. The normal data distribution was confirmed with the Kolmogorov-Smirnov test $p > 0.1$. The level of CEA (ng/ml) in the two study groups showed a significant difference between colorectal cancer patients and healthy control, $t = 6.11$, $df = 42$ ($p < 0.0001$). As shown in Figure 1, the CEA level among CRC cases was 50.76 ± 8.304 ng/ml, indicating significantly increased levels in the patient group ($p<0.0001$).

The ROC curve analysis showed that the expression of the miR-1 is above the half-line (50%) of the curve (Fig. 2). According to ROC curve analysis, miR-1 showed a sensitivity of 70.27% and specificity of 62.96% (AUC = 0.6962, $p = 0.0068$), while CEA showed a 100% sensitivity and 71.43% specificity (AUC = 0.8520, $p <0.0001$).

Regarding tumor size and metastatic condition, the ANOVA test showed a significant difference in miR-1 expression among tumor sizes ($p=0.0105$). Tukey's multiple comparison tests indicated a significant difference in miR-1 expression level in tumor sizes of T2-T4 compared to adjacent tissue expression levels ($p < 0.05$). There was also a significant difference between the T1 tumor size and T2-T4 groups ($p < 0.05$). No significant difference in miR-1 expression was observed between T1 and adjacent tissue. In addition, there was no significant difference between T2-T4 groups regarding miR-1 expression (Fig. 3).

Regarding the metastatic condition of the patients, miR-1 was relatively lower in patients with metastasis; however, the difference was not statistically significant ($p = 0.44$) (Fig. 4).

Table 1. Primers used in Real-Time PCR

Primer name	Tm (°C)	Sequence
miR-1 F	55	CAACCTGGAATGTAAAGAAGT
SNORD47 F	54	ATCACTGTAAAACCGTTCCA
Universal R	56	GAGCAGGGTCCGAGGT

Table 2. Clinical information on colorectal cancer samples

	Group	N (%)
Tumor location	Colon	8 (62.5)
	Rectum	15 (33.34)
	Polyp	1 (4.16)
Lymph node metastasis	Yes	12 (52.18)
	No	11 (47.82)
Tumor size	T1	7 (30.45)
	T2	6 (26.09)
	T3	5 (21.73)
	T4	5 (21.73)

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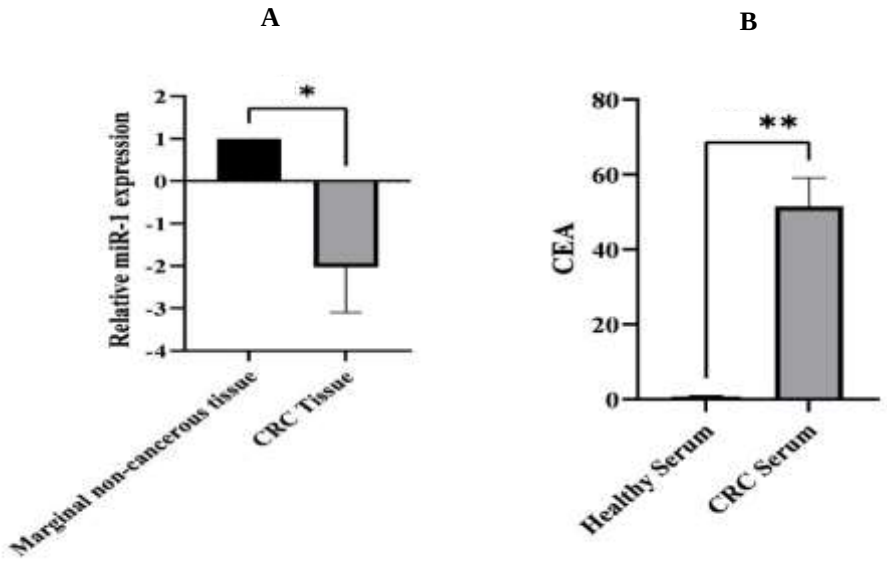

Fig. 1. A) Expression of miR-1 in CRC and marginal tissues of colorectal tumor samples, **B)** CEA level in serum of CRC patients and healthy controls, t-test was used to determine significant differences among groups, and a P-value less than 0.05 was considered as statistically significant.
CRC = Colorectal carcinoma; CEA = Carcinoembryonic antigen; * p<0.01; ** p < 0.0001

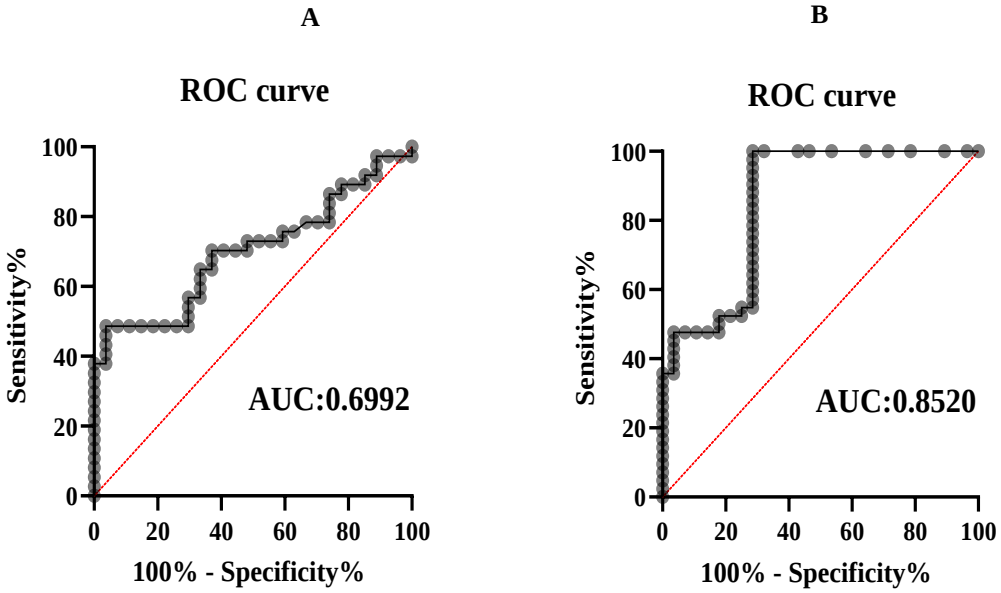

Fig. 2. A) ROC curve for expression of miR-1 in CRC and adjacent tissue samples. **B)** ROC curve for CEA in the serum of CRC patients and control group. ROC= Receiver operating characteristic curve; AUC= Area under the ROC Curve; CRC= Colorectal cancer; CEA= Carcinoembryonic antigen

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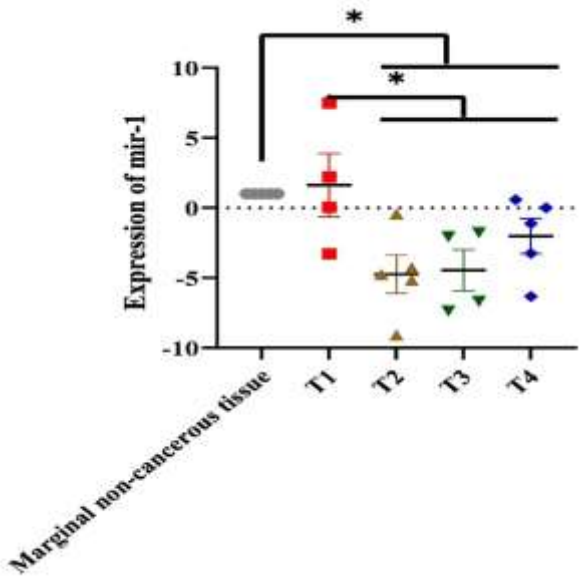

Fig. 3. Analysis of miR-1 expression in marginal tissue and different tumor size in patients with colorectal cancer, T1-T4 represents the tumor size based on the TNM (tumor, node, metastases staging) system. One-way ANOVA test was used to determine differences between T1-T4 groups, and Tukey's multiple comparisons test was used to compare expression levels in tumor samples with healthy marginal tissues while a p-value less than 0.05 was considered statistically significant.

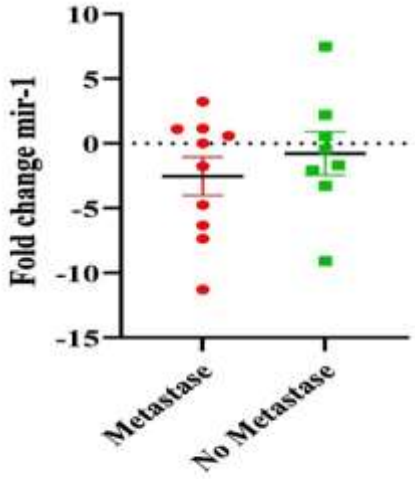

Fig. 4. Comparison of miR-1 expression in colorectal cancer patients with metastasis and non-metastasis. The t-test was used to determine significant differences among study groups.

Discussion

In this study, we investigated the expression of miR-1 in CRC patients and adjacent tissues. In addition, CEA was measured as the routine biomarker of CRC. The results showed that miR-1 expression in tissue samples was significantly reduced in CRC, consistent with previous studies based on microarray evaluations [32, 33]. Based on high-throughput evaluations, the level of miR-1 in all tumor sizes is diminished [34, 35]. The current study also showed a considerable difference between the miR-1 expressions based on tumor sizes. It has been proposed that miR-1

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has a role in cell proliferation and migration of malignant cells in different tumors [36, 37]. Accordingly, a reduction of miR-1 in patients with metastasis compared to non-metastatic cases was observed. Although the difference was not statistically significant, it seems logical that a larger sample size might reveal a significant difference. The role of miR-1 in CRC as a common fatal cancer is still an interesting topic under investigation. Different mechanisms have been proposed; for example, miR-1 is down-regulated in colorectal cancer tissues by modulating NLR family, apoptosis inhibitory protein (NAIP) expression, which affects cellular apoptosis and is involved in the multistage process of CRC development. In addition, miR-1 causes cell death in CRC cells by targeting the main anti-apoptotic protein, NAIP, and is involved in colon cancer [38]. Also, it has been proposed that miR-1 inhibits tumor proliferation, cell cycle transmission, migration, and motility via modulation of vascular endothelial growth factor expression in CRC [33]. Likewise, modulation of miR-1 and increased metastasis-associated in colon cancer-1 may contribute to the overexpression of MET and metastatic behavior of colon cancer cells [39]. Proteomic analysis showed that miR-1 interferes in epithelial-mesenchymal transmission (EMT), which has a pivotal role in the onset of metastasis [40]. In 2017 Wu et al. reported that miR-1 suppresses aerobic glycolysis and tumor cell proliferation by inactivating Smad3 and targeting hypoxia inducible factor-1α, reducing hexokinase-2 and monocarboxylate transporter expression [29]. In another study, while reducing the expression of miR-1 referred to in colorectal cancer, they also stated that miR-1, by direct binding to 3'-UTR, inhibits the expression of the NOTCH3 and assists in managing colorectal cancer [33]. MiRs are promising factors in evaluating, staging, managing, and following up on malignant patients. For example, miRs may help to predict therapeutic response [29, 41, 42]. Circulating miRNAs are stable due to encapsulation in extracellular vesicles [43]; therefore, they might serve as a rather cheap, fast, informative, and noninvasive predictor in addition to currently available tools, and they are considered as a future biomarker [42]. Presently, the main biomarker of CRC is CEA, which is helpful, but it also has some limitations, and these limitations could be improved with the implication of additional parallel biomarkers. In the present study, ROC curve analysis showed that the level of expression of miR-1 among diagnosed CRC tissue is quite promising in terms of specificity and sensitivity. Additionally, we observed that expression of miR-1 in grads T2, T3, and T4 was significantly reduced compared to adjacent healthy tissue. In a study by Li et al., rather, higher specificity and sensitivity of miR in CRC compared to our results were reported, which might be owing to included sample size; in addition, they used a panel of miRs using Universal RT microRNA PCR system [44]. Some limitations of the study should be noted, the first small sample size. Undoubtedly higher sample size would increase the strength of the study. Second, miR-1 evaluation in serum samples is highly recommendable to provide a more convenient approach for monitoring the patients. Applying more specific molecular approaches such as TaqMan advanced miRNA or probe-based assays could also be investigated. In

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addition, miRNA expression profiling assays to evaluate a series of miRs could be informative. Despite the mentioned limitations, miR-1 showed a noticeable potential marker for CRC, which is consistent with parallel studies published in the course of experimental works of the present study [36, 42, 44].

Conclusion

Identifying a sensitive and specific biomarker that can provide data in managing colorectal cancer can be very beneficial and may reduce invasive and costly procedures. The present study showed a change in the expression of miR-1 in patients with colorectal cancer. Reducing miR-1 in different cancer stages can be considered a prognostic factor in CRC patients. However, additional studies with larger sample sizes are still needed to establish miR-1 as a CRC biomarker.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

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