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Research article

Blood miRNAs miR-549a, miR-552, and miR-592 serve as potential disease-specific panels to diagnose colorectal cancer

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ABSTRACT

Introduction: miRNAs originating from colorectal cancer (CRC) tissue receive significant focus in the early diagnosis of CRC due to their stability in body fluids. However, if these miRNAs originated from alternative organs, their prognostic value will diminish. Thus, in this study, we aim to identify disease-specific miRNAs for colorectal cancer (CRC) by employing bioinformatics and experimental methodologies.

Method: To identify CRC-specific miRNAs, we retrieved miRNA profiles of CRC and normal tissues from the Cancer Genome Atlas (TCGA) database. Subsequently, computational strategies were utilized to select potential candidate miRNAs. Following this, the expression levels of the potent miRNAs were assessed through RT-qPCR in both CRC tissue and serum samples from patients (N = 46), as well as healthy individuals (N = 46). Additionally, the associations between clinicopathological characteristics, survival outcomes, and diagnostic accuracy were evaluated.

Results: A total of 8893 RNA-seq expression data were acquired from TCGA, comprising 8250 data from 19 distinct cancer types and 643 corresponding healthy samples. Based on the computational methodology, miR-549a, miR-552, and miR-592 were identified as the principal expressed miRNAs in colorectal cancer (CRC). Within these miRNAs, miR-552 displayed a substantial association with tumors at the N and T stages. miR-549a and miR-592 were observed to be linked exclusively to the invasion of tumor depth and tumor stage (TNM), respectively. The receiver operating characteristic (ROC) analysis conducted on the miRNA expression in serum samples revealed that all miRNAs exhibited an area under the ROC curve (AUC) of up to 0.86, thereby indicating their high diagnostic accuracy.

Conclusion: Considering the strong associations of these three identified miRNAs with CRC, they can collectively serve as a panel for specific discrimination of CRC from other types of cancer within the body. Although this study focused solely on CRC, this approach can potentially be applied to other cancer types as well.

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1. Introduction

Colorectal cancer (CRC) is the 3rd most common cancer globally, and the second principal reason of death among cancers. It is estimated that more than 1.93 million newly diagnosed CRC patients and 940,000 deaths occur in 2020 globally. Early-stage detection of CRC disease significantly decreases the death rate in patients [1–3]. However, a significant proportion of individuals with CRC receive a diagnosis at a later stage, specifically stage III or IV [4]. Presently, the gold standard methods for early detection of CRC include the serum carcinoembryonic antigen (CEA) test, fecal occult blood test (FOBT), and colonoscopy. Nonetheless, it is important to note that FOBT has a relatively high rate of false-positive results, while the CEA test exhibits low sensitivity (less than 36%) [5,6]. Furthermore, despite its high specificity, colonoscopy is an invasive, costly, time-consuming, and resource-intensive procedure [7]. Given this predicament, it is crucial to identify and validate novel, specific, and non-invasive biosignatures that can facilitate early detection of CRC.

microRNAs (miRNA), which are short noncoding RNA molecules involved in post-translational gene regulation through binding to the 3′ UTR of target mRNAs, play a role in various biological processes, including tumorigenesis and tumor progression [8]. Recent research indicates that there are distinct differences in miRNA expression profiles between cancerous tissues and adjacent normal tissues, suggesting their potential utility as biomarkers for cancer diagnosis [9]. Moreover, cancer-derived circulating miRNAs remain remarkably stable in body fluids due to their resistance to endogenous RNase activity, extreme temperatures, and pH levels [10]. However, finding miRNAs that can differentiate patients with CRC remains a challenge due to the presence of blood-borne miRNAs released from other organs or diseases, which can reduce the specificity of the proposed miRNA. For instance, miR-21 and miR-92a, which are found to be elevated in the plasma of over 90% of CRC patients, also exhibit high expression in patients with acute leukemia, cervical cancer, lung cancer, breast cancer, esophageal cancer, gastric cancer, and inflammatory bowel disease (IBD) [11]. Additionally, these miRNAs are upregulated during the process of wound healing in the skin [12]. Currently, a large amount of expression data is being generated through technologies like high-throughput sequencing, enabling researchers to explore, acquire, and extract valuable information regarding changes in miRNA expression across different parts of the body [13,14]. It is believed that the utilization of these high-throughput gene expression data could provide a significant opportunity to discover disease-specific biomarkers [15]. Considering the aforementioned discussion, this study aimed to: 1) identify potential circulating biomarkers specific to CRC using *in silico* techniques, 2) compare the miRNA profiles of CRC with those of other cancers, normal tissues, and IBD, and 3) evaluate the biological functions of prognostic miRNAs. Subsequently, the expression of candidate miRNAs was measured in both tissue and serum samples of CRC and normal individuals using Real-Time qPCR (RT-qPCR). Finally, the diagnostic capability and the correlation between candidate miRNAs and clinicopathological features of patients were assessed.

2. Materials and methods

2.1. Extraction of miRNA gene expression datasets

The miRNA gene expression datasets and clinical data of various normal and cancer tissue samples were retrieved from the Cancer Genome Atlas (TCGA) database (https://tcgadata.nci.nih.gov/tcga/dataAccessMatrix.htm). These miRNA expression data were utilized to discover the colorectal-specific miRNAs. After retrieving the raw RNA-seq datasets the read count data were combined and evaluated utilizing the DESeq package of the R software (version 4.3.1). In addition, miRNA expression profiles of inflammatory bowel disease (IBD) comprising Crohn's disease (CD) and ulcerative colitis (UC) were obtained from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). We analyzed GEO data online through the GEO2R online tool. The properties of the datasets are presented in Table 1. Then miRWalk 3.0 was applied to envisage probable miRNA target genes. The collected target genes were pooled in the EnrichR database which was used for the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment study.

Table 1
The properties of datasets used in the current Study.

Dataset	platform	No. of samples	Sample tissue (count number)	Year
TCGA	RNA seq	18	Bladder (C[a] = 420, N[b] = 19), Colon (C = 460, N = 8), Esophagus (C = 187, N = 13), Kidney (C = 1098, N = 143), Liver (C = 408, N = 60), Pancreas (C = 181, N = 4), Thyroid (C = 510, N = 61), Stomach (C = 455, N = 47), Lung (C = 1004, N = 92), Breast (C = 1100, N = 106), Uterus (C = 552, N = 22), Brain (C = 518, N = 5), Blood (C = 236, N = 0), Skin (C = 100, N = 2), Connective tissue (C = 130, N = 0), Ovary (C = 494, N = 0), Prostate (C = 365, N = 61), lymph node (C = 32, N = 0)	2020
GSE102127	miRNA profiling by array	2	Crohn's disease (35), Normal ileum (8)	2017
GSE48957	miRNA profiling by array	3	Active ulcerative colitis (10), Inactive ulcerative colitis (7), Normal colon (10)	2013

[a] C: Cancer.
[b] N: Normal.

2.2. Identification of colorectal cancer-specific miRNAs

To ascertain the miRNAs specifically expressed in colorectal cancer (CRC), the genes exhibiting a log fold change exceeding 1, as well as an adjusted P-value less than 0.05 were deemed crucial in the comparison of colorectal cancer samples with other cancer types and normal tissues using the R software. Subsequently, the expression of the obtained miRNAs was classified based on the disparity in expression between normal and cancerous tissues, employing a heatmap in the R software. Following this, the colorectal cancer-specific miRNAs were identified by selecting the miRNAs that target genes involved in regulating colorectal cancer pathways. Lastly, the alteration in expression of the nominated CRC-specific miRNAs was evaluated across various forms of Inflammatory Bowel Disease (IBD).

2.3. Participants and ethics approval

92 participants were enlisted to acquire tissue and serum samples, consisting of 46 patients and 46 healthy individuals. These participants were selected from December 2017 to March 2019 from Imam Khomeini Hospital of Tehran, Iran. Among the patients, those who had received no prior treatment such as chemotherapy or radiation therapy, and who had been clinically diagnosed with CRC through colonoscopy, abdominal radiography, and histopathological examination were chosen. The tumor staging (TNM) was classified according to the guidelines provided by the American Joint Committee on Cancer (AJCC, 8th edition). The Department of Pathology at the Cancer Institute of Imam Khomeini Hospital was responsible for the histopathological diagnosis of the collected samples. The healthy controls were individuals who exhibited no symptoms and had undergone a medical checkup that confirmed the absence of neoplasms. Furthermore, they had no gastrointestinal symptoms, cancer, or any family history of CRC. The control group was matched with the CRC patients in terms of age and sex. Supplementary Table 1provides detailed descriptions of the contributors. Before participation, all patients and healthy donors provided written informed consent in accordance with the Helsinki Declaration of 2013. The study was approved by the ethical committee of Golestan University of Medical Sciences in Gorgan, Iran under the Ethics Approval Code: IR.GOUMS.REC.1398.360.

2.4. Sample collection and RNA extraction

Peripheral blood samples were collected from CRC patients and healthy controls, with a volume of 2.5 ml. These samples were collected in EDTA anticoagulation tubes and immediately subjected to centrifugation at a speed of 3000 rpm for a duration of 10 min. This centrifugation step was performed to separate the plasma from the rest of the blood components. The plasma was then stored at a temperature of −80 °C until RNA extraction. After the surgical procedure, the primary tumors and adjacent normal tissue were gathered. These tissue specimens were immediately placed in RNase-free polypropylene tubes that contained RNAlater stabilization solution (provided by Qiagen). Subsequently, the specimens were stored in a freezer at a temperature of −80 °C until RNA extraction. The diagnosis of colorectal cancer was confirmed through histopathological examination. For the extraction of total RNA from both serum and tissue samples, the TRIzol LS isolation kit was utilized. The concentration and purity of the extracted RNA were evaluated using a UV spectrophotometer (Denovix model). To synthesize complementary DNA (cDNA), the BONmiR miRNA 1st-strand cDNA synthesis kit (developed by Bonbiotech, BONmiR) was employed. The synthesis reaction was conducted using a MiniAmp Thermal Cycler manufactured by Applied Biosystems.

2.5. Primer design and Real-time qPCR analysis

The sequence of nominated miRNAs was obtained from the miRBase database, accessible at the URL: http://www.mirbase.org. To detect miRNAs, primers were designed using Gene Runner 6.0. To ensure proper functioning, the melting temperature (Tm) of the primers and the secondary structure of all sequences were adjusted utilizing the OligoAnalyzer tool, available at: https://www.idtdna.com/pages/tools/oligoanalyzer. The designed primer sequences can be found in Supplementary Table 2. The amplification and detection of the nominated miRNAs were carried out using an ABI-Step one Real-Time PCR appliance (Applied Biosystems, USA), and the SYBR Green Master Mix (Amplicon) was employed. To normalize the expression of miRNAs in serum and tissue samples, the endogenous reference gene RUN6 was used, and the expression was then calculated using the $2^{-\Delta\Delta CT}$ formula.

2.6. Statistical Analyses

Data from three separate experiments are presented as the mean ± standard deviation. The relative expression of target miRNAs (miR-552, miR-549a, and miR-592) was normalized to the RUN6 gene as a reference control. The statistical analysis involved the application of the Students' T-test and Kruskal-Wallis tests to assess the variations in miRNA expression between patients and healthy controls in different groups. For correlation analysis, the Spearman Rank coefficient was employed. Statistical analysis was conducted using SPSS 16.00 and GraphPad Prism 7.00 software. A significance level of $P < 0.05$ was deemed noteworthy.

3. Results

3.1. miR-549a, miR-552, and miR-592 were identified as CRC-specific biomarkers

Of 8893 miRNA genes resulting from RNA-Seq datasets of 8250 various cancer samples and 643 normal tissues, 11 miRNAs perfectly had a log (FC) greater than 1 (data are not shown). The examination of the RNA-Seq data demonstrated that miR-147b, miR-549, miR-552, miR-592, miR-4791, miR-33b, and miR-556-5p mostly expressed in colon cancer and less expressed in other cancers and normal tissue as demonstrated in Fig. 1A and B. Furthermore, two additional datasets, namely GSE102127 and GSE48957 were collected from GEO, which encompassed 52 sample data related to inflammatory bowel disease (IBD) (Table 1).

Biological pathways analysis indicated that only target genes of miR-549a-5p, miR-552-5p, and miR-592-5p are significantly involved in colorectal cancer among these miRNAs. Therefore, these three miRNAs can be considered CRC-specific miRNAs. These three miRNAs significantly regulate TGF-beta signaling, the hippo signaling pathway, endocytosis, morphine addiction, and ubiquitin-mediated proteolysis in addition to colorectal cancer (Table 2).

3.2. Expression level of miR-549a, miR-552, and miR-592 in the IBD

IBD is a chronic intestinal inflammation that may be associated with CRC [16]. Several studies indicate that most miRNAs related to CRC are highly expressed in IBD as well. With an increasing prevalence of IBD in the world, there are certain difficulties in differentiating CRC from inflammation [17,18]. To evaluate the potential miRNA targets to differentiate CRC from IBD, we assessed their expression in data derived from GEO that compromises Crohn's disease (CD), active and inactive ulcerative colitis (UC), and CRC. As can be seen in Table 3 only miR-552 is significantly downregulated in Crohn's disease.

3.3. Association analysis of clinicopathological features and miRNA expression level

As demonstrated in Supplementary Table 3, the features of patients may influence the survival rate. 170 female patients and 189 male patients were examined in this study. 267 patients (74.4%) were over 60 years old and 92 patients (25.6%) were under 60 years old. According to the statistical analysis of the tumor stage, patients with stage I accounted for 15.9% (n = 57), while 38.2% (n = 137), 32.6% (n = 117), and 13.4% (n = 48) patients were in stage II, III, and IV, respectively ($P < 0.001$). Considering the prior tumor malignancy, 45 tumor cases (12.5%) were tumor malignancies. Also, the topographical distribution included M stage ($P < 0.001$): 72.4% M0 (n = 260), 27.6% other (M1+MX + NA) (n = 99); N stage ($P < 0.001$): 58.2% N0 (n = 209), 22.3% N1 (n = 80), 19.5% other (N2+NA) (n = 70); and T stage ($P < 0.001$): 18.7% T1+T2 (n = 67), 68.5% T3 (n = 246), 12.8% T4+NA (n = 46), and 11.3% T4 (n = 68).

Besides, the association between these three miRNAs (miR-552, miR-592, and miR-549a) and clinical features were studied (Supplementary Tables 4 and 5). As demonstrated, miR-552-5p was considerably related to tumors in the N stage ($P < 0.013$) and T stage ($P < 0.013$). miR-592 expression level was prevalent with race ($P < 0.001$) and T Stage ($P < 0.043$). However, miR-549a was mainly related to the tumor stage ($P < 0.027$). No significant association between these miRNAs and other clinicopathological features was found.

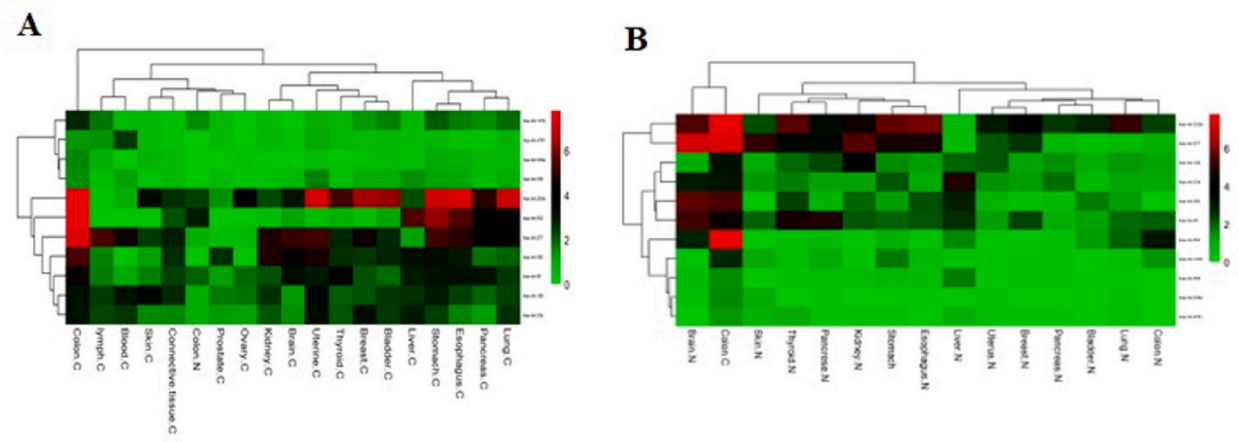

Fig. 1. Expression pattern of selected miRNAs. The result of this step identifies eleven miRNAs with specific expressions in colorectal cancer. A: Expression in different cancers, B: Expression in different normal tissues (Red: high expression; Green: low expression; Black: average expression of miRNAs in the corresponding tissue, C: Cancer, N: Normal).

Table 2
Pathways that are regulated by target genes of upregulated miRNAs (retrieved from miRWalk V3.0.).

miRNA	Related phenotypes	Overlapped genes	Adjusted P-value
hsa-miR-147b	Colorectal cancer	21/86	0.68
	Gastric acid secretion	35/76	0.001
	Axon guidance	67/182	0.0016
hsa-miR-549a-5p	Colorectal cancer	31/86	0.038
	TGF-beta signaling pathway	39/94	$2.19E^{-05}$
	Hippo signaling pathway	59/163	$3.30E^{-05}$
hsa-miR-33b-5p	Colorectal cancer	8/86	0.189
	Sphingolipid signaling pathway	22/119	0.0019
	Dopaminergic synapse	22/132	0.0035
hsa-miR-556-5p	Colorectal cancer	3/86	0.97
	Ubiquinone and other terpenoid-quinone biosynthesis	2/12	0.97
	Pantothenate and CoA biosynthesis	3/18	0.97
hsa-miR-552-5p	Colorectal cancer	24/86	0.0129
	Endocytosis	70/252	0.005
	Morphine addiction	31/91	0.009
hsa-miR-592-5p	Colorectal cancer	9/86	0.0317
	Ubiquitin mediated proteolysis	17/140	0.016
	Axon guidance	20/182	0.163
hsa-miR-4791	Colorectal cancer	4/86	0.82
	Long-term depression	6/60	0.543
	Shigellosis	15/246	0.543

Table 3
Comparative analysis of miR-549a, miR-552, and miR-592 levels between UC, CD, and CRC patients.

Disease	Expression level of		
	miR-552	miR-549a	miR-592
Crohn's disease (CD)	−0.84[a]; 0.003[b]	−0.12; 0.628	0.04; 0.92
Active ulcerative colitis (UC)	−0.93; 0.168	−0.127; 0.765	−0.055; 0.939
Inactive ulcerative colitis	0.0446; 0.991	−0.223; 0.916	−0.245; 0.917
CRC (Colorectal cancer)	4.62; $2.02E^{-17}$	2.67; 0.0016	7.33; $8.97E^{-15}$

[a] Log (FC).
[b] Adjusted P-Value.

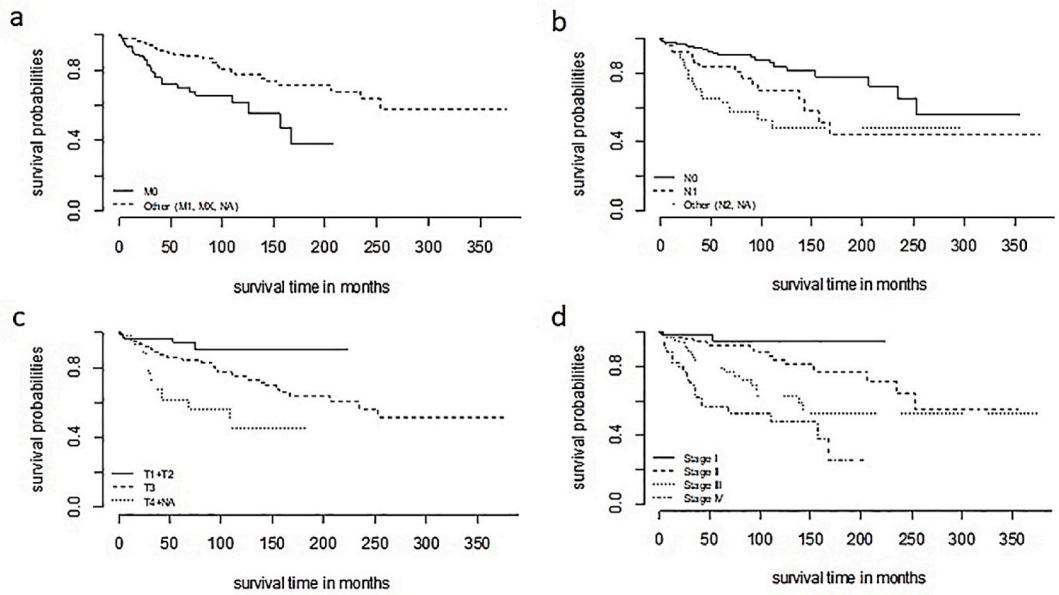

Fig. 2. Kaplan-Meier's survival curves for (a) Stage M, (b) Stage N, (c) Stage T, and (d) Tumor stage for hsa-miR-552.

3.4. Survival analysis

Kaplan-Meier survival analysis was employed to assess the survival rate (Fig. 2A–D). The relationship between the expression of target miRNAs and the overall survival of patients with CRC was visualized using the Survminer package in R software, as demonstrated in Table 4 and Supplementary Tables 6 and 7In model 1, the influence of miRNAs on patient survival was examined. The findings revealed that solely miR-552 exhibited a relatively crucial impact on patient survival ($P = 0.053$). Thus, as the miR-552 value increases, the risk of mortality decreases. In model 2, the simultaneous examination of the effect of miRNAs and the variables in Supplementary Table 6 on patient survival was conducted. Among all these variables, only miR-552 and tumor stage variables remained in the model. The impact of miR-552 was not significant; however, the effect of tumor stage on patient survival was substantial ($P < 0.001$).

3.5. The characteristics of samples

The demographic and clinical characteristics of both colorectal cancer (CRC) patients and controls are presented in Supplementary Table 7. Based on the statistical analysis, there were no substantial correlations observed between the CRC and control groups concerning gender, age, hematochezia, past surgical history, familial CRC history, familial cancer histories, constipation, diarrhea, bloating, weight loss, cigarette smoking, alcohol consumption, diabetes, Helicobacter pylori test, tumor differentiation status, lymph vascular invasion, perineural invasion, tumor depth invasion, TNM stage, tumor size in greatest dimension, and location of the tumor.

3.6. miR-549a, miR-552, and miR-592 considerably augmented in CRC tissues and were associated with clinicopathological features of CRC

The RT-qPCR test results revealed that the levels of three miRNAs, namely miR-549a-5p, miR-552-5p, and miR-592-5p exhibited a noteworthy increase in CRC tissues in comparison to adjacent normal tissues ($P < 0.05$). Specifically, miR-549a was found to be associated with the invasion of tumor depth ($P = 0.044$). Furthermore, miR-552 and miR-592 were observed to be correlated with the TNM stage ($P = 0.026$ and $P = 0.033$, respectively). However, no significant association was identified between the expression of these miRNAs and other clinicopathological characteristics (Fig. 3A–F).

3.7. The expression of miR-549a, miR-552, and miR-592 varies in the serum of control and CRC patients

The serum of colorectal cancer (CRC) patients exhibited a significant increase in the expression of miR-549a, miR-552, and miR-592 when compared to the control group, with a statistical significance of $P < 0.05$. A thorough evaluation of the correlation between the expression of these miRNAs and clinicopathological features was performed in patients diagnosed with colorectal cancer. Notably, the level of miR-552 demonstrated a significant association with the TNM stage, with a corresponding P-value of 0.043 (refer to Fig. 4A–D and Supplementary Table 8).

Table 4
Results of comparing survival curves across demographic and pathological variables.

Variable	Category	N (%)	No. of the event (%)	Mean (S.D) of survival time	P-value[a]
Gender	Female	170(47.4)	33(19.4)	267.43(17.72)	0.663
	Male	189(52.6)	39(20.6)	216.09(18.26)	
Onset Age (years)	<60	92(25.6)	18(19.6)	261.57(57)	0.985
	≥ 60	267(74.4)	54(20.2)	238.45(15.14)	
Race	White	183(51.0)	40(22.7)	241.76(17.48)	0.887
	Others	176(49.0)	32(17.5)	208.99(12.31)	
Stage M	M0	260(72.4)	40(15.4)	130.27(10.78)	<0.001
	Other (M1, MX, NA)	99(27.6)	32(32.3)	272.05(15.77)	
Stage N	N0	209(58.2)	25(12.0)	268.41(17.64)	<0.001
	N1	80(22.3)	20(25.0)	219.62(28.41)	
	Other (N2+NA)	70(19.5)	27(38.6)	165.33(19.73)	
Stage T	T1+T2	67(18.7)	4 (6.0)	206.14(8.63)	<0.001
	T3	246(68.5)	51(20.7)	251.37(15.54)	
	T4+NA	46(12.8)	17(37.0)	108.77(13.61)	
Tumor stage	I	57(15.9)	2(3.5)	213.74(6.95)	<0.001
	II	137(38.2)	18 (13.1)	268.23(18.77)	
	III	117(32.6)	30(25.6)	232.83(23.53)	
	IV	48(13.4)	22(45.8)	107.09(14.06)	
Prior Malignancy	No	314(87.5)	60(19.1)	253.77(15.03)	0.267
	Yes	45(12.5)	12(26.7)	191.24(16.92)	

[a] Based on the Log-rank test.

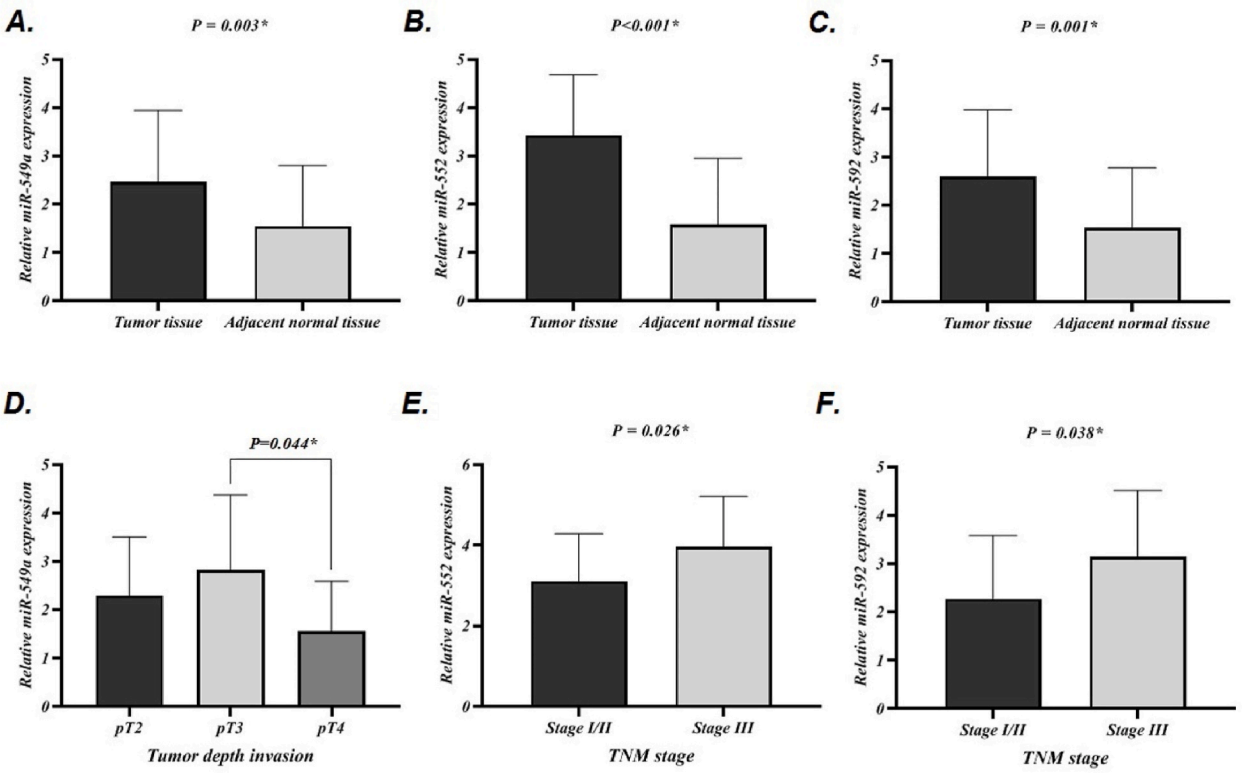

Fig. 3. Expression levels of miR-549a-5p, miR-552-5p, and miR-592-5p in CRC tissues and flanking normal tissues. The comparisons of the (a) miR-549a-5p, (b) miR-552-5p, and (c) miR-592-5p expression levels in CRC tissues and flanking normal tissues were statistically significant; $P = 0.003$, $P < 0.001$, and $P < 0.001$, respectively. The relation of (d) hsa-miR-549a-5p expression with tumor depth invasion, the relation of (e) hsa-miR-592-5p expression with TNM stage, and the relation of (f) miR-552-5p expression with TNM stage were statistically significant ($P = 0.044$, $P = 0.026$, and $P = 0.033$, respectively).

3.8. Diagnostic value of miR-549a, miR-592, and miR-552 in CRC

According to the results of the area under the ROC curve (AUC) analysis (Fig. 5A–C), AUC for miR-549a, miR-552, and miR-592 were 0.86, 0.94, and 0.88 respectively, suggesting their high diagnostic accuracy in the recognition of patients with CRC.

4. Discussion

Colorectal cancer (CRC) is an insidious neoplasm with escalating prevalence in developed nations. In instances where this ailment is detected during its incipient phases and polyps are present, a straightforward surgical intervention can be employed for its management. However, the dissemination and metastasis of malignant cells engender deleterious outcomes for the afflicted individual. Consequently, the prompt identification of infected individuals and the early initiation of treatment for this condition is of utmost significance [19,20]. In recent years, the attention of numerous researchers has been captivated by miRNAs as a means of diagnosing various cancers, including CRC [21]. This is due to their ability to not only demonstrate a significant function in the initiation, growth, and metastasis of cancer but also their stability and detectability in other bodily fluids. Conventional approaches for identifying biomarkers primarily rely on the use of single-tissue DEGs (differentially expressed genes). However, miRNAs discovered in this manner may exhibit high expression in other tissues, cancers, or diseases, which can compromise the specificity of detection [22,23]. miRNAs have been found to serve a dual function in colorectal cancer (CRC) as both oncogenes and suppressors. Previous studies have indicated that miR-21 influences the proliferation, apoptosis, invasion, migration, and chemo-resistance of CRC by targeting genes such as *PDCD4*, *TIAM1*, *SPRY2*, *PTEN*, *TGFBR2*, *CDC25A*, and *hMSH2* [24]. Additionally, miR-96 and miR-92a have been shown to promote the metastasis and proliferation of CRC cells [25,26]. Furthermore, miR-31 and miR-200c have also been identified as contributors to CRC proliferation [27,28]. Several other miRNAs, including miR-135a/b, miR-155, miR-224, miR-214, miR-182/503, and miR-301a, have been classified as oncogenes in the pathogenesis of CRC [29,30].

In this study, our objective was to identify potential miRNAs that can be used for the diagnosis and differentiation of colorectal cancer (CRC) from other types of cancers and intestinal diseases such as inflammatory bowel disease (IBD). To achieve this, we analyzed 8250 gene expression data from cancer patients using *in silico* high-throughput gene expression analysis techniques. As a result, we have discovered three miRNAs, namely miR-549a-5p, miR-552-5p, and miR-592-5p which demonstrate a higher specificity

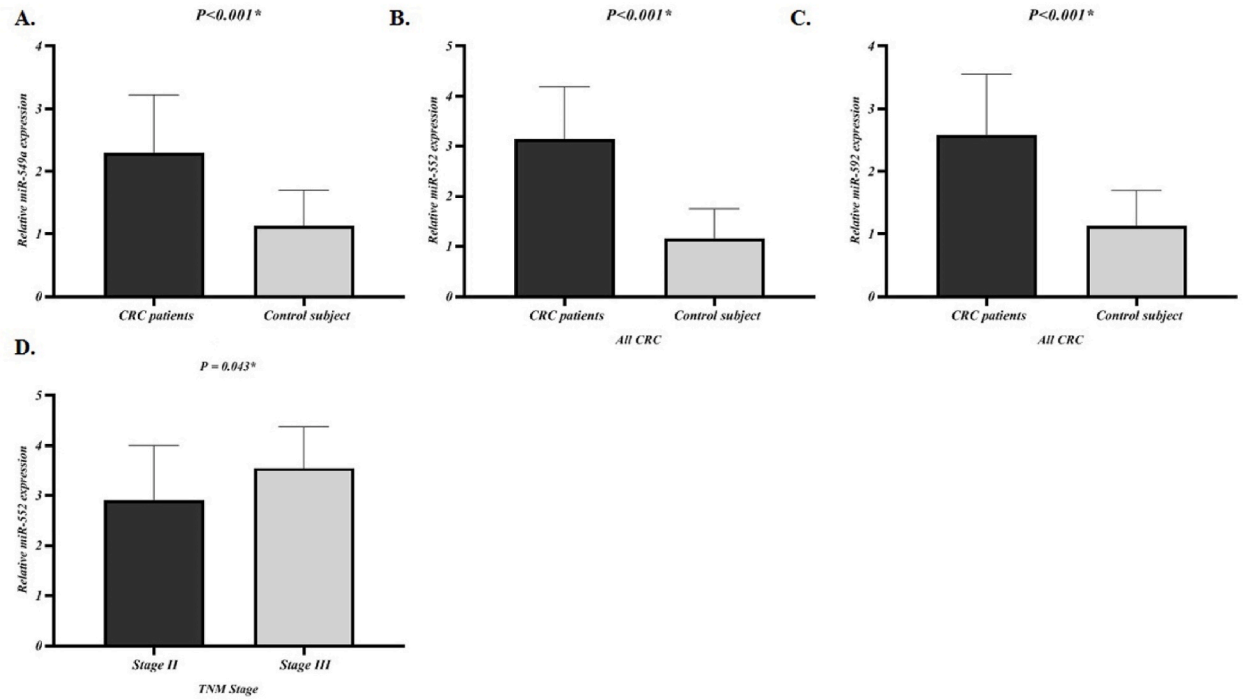

Fig. 4. Different expression levels of miR-549a, miR-552, and miR-592 were observed in the blood serum of CRC patients and the control group. The expression level of (A) miR-549a, (B) miR-552, and (C) miR-592 in the serum of CRC patients compared to the control group. (D) The relative expression of miR-552 in the TNM stage was statistically significant ($P < 0.05$).

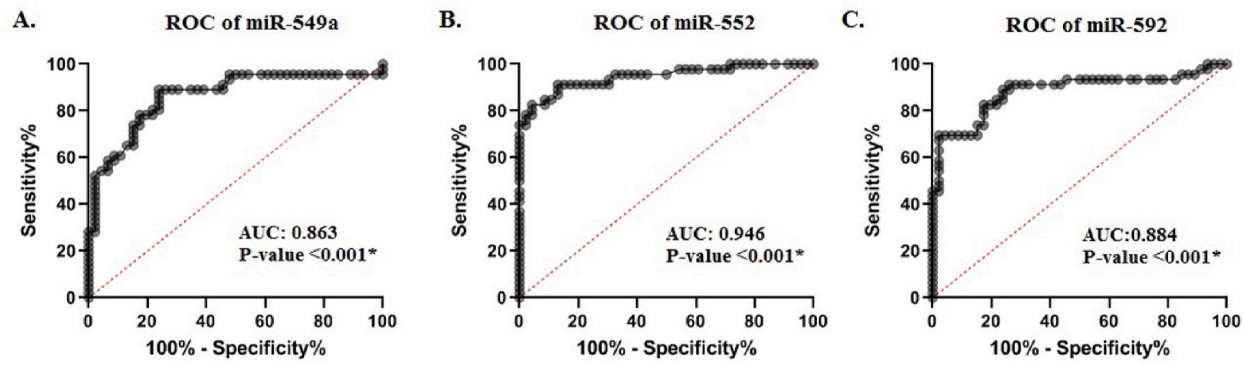

Fig. 5. ROC curve made based on the expression levels of miR-549a-5p, miR-552-5p, and miR-592-5p in the serum of CRC patients compared to healthy samples. Results show that all three miRNAs could differentiate CRC patients from healthy samples with an AUC of more than 0.85.

towards CRC compared to other cancer tissues and IBD. In the subsequent, these three miRNAs were experimentally confirmed in the specimens of both tissues and serum from patients diagnosed with CRC. In a conducted investigation, the observation was made that miR-549a initiates the process of renal cancer metastasis via the VEGFR2-ERK-XPO5 pathway [31]. Also, Drusco et al., report the expression of miR-549a in glial tumors [32]. Another study revealed that miR-592 plays a significant role in the proliferation, migration, and invasion of CRC through its targeting of secreted protein acidic and rich in cysteine (SPARC) protein and Forkhead Box O3A genes (FoxO3A) [33,34]. Pan Z. et al. have proposed that a high level of miR-592 serves as an indicator of a poorer prognosis in CRC patients [33]. However, it is worth noting that miR-592 expression has also been observed in other types of cancer, including renal cell carcinoma, prostate cancer, gastric cancer, and retinoblastoma cancer [35–37]. In 2017, Wang W. discussed the inhibitory effects of miR-592 on the proliferation, migration, and invasion of hepatocellular carcinoma through its targeting of IGF-1R [38]. These studies collectively suggest that miR-592 exhibits a dual role in the context of cancer. Our analysis in the field of bioinformatics, along with other studies, has provided evidence that miR-552 is not only expressed in colorectal cancer (CRC), but also in hepatocellular carcinoma (HCC), gastric cancer (GC), and ampulla adenocarcinoma [39]. This was demonstrated in a study conducted by Kim J. et al., where they found that miR-592 and miR-552 could potentially distinguish between colorectal cancer metastasized to the lung and

primary lung adenocarcinoma [40,41]. The human miR-549a-5p with 22 nucleotides is located on chromosome 15. According to miRWalk and EnrichR databases, this miRNA has 10,981 predicted target genes mainly regulating focal adhesion, Neurotrophin signaling pathway, ECM-receptor interaction, axon guidance, cell adhesion molecules, choline metabolism in cancer, Arrhythmogenic right ventricular cardiomyopathy, phospholipase D signaling pathway, and PI3K-Akt signaling pathways ($P < 0.05$). miR-552-5p has 21 nucleotides located on chromosome 1 and targets 7271 genes involving focal adhesion, growth hormone synthesis and action, Relaxin signaling pathway, axon guidance, spinocerebellar ataxia, thyroid hormone signaling pathway, ErbB signaling pathway, and cGMP-PKG signaling pathways. miR-592 with 22 nucleotides suited on chromosome 7 and is predicted to target 2315 genes regulating glutamatergic synapse, MAPK signaling pathway, arrhythmogenic right ventricular cardiomyopathy, axon guidance, calcium signaling pathway, long-term depression, and Rap1 signaling pathway [42].

Statistical analysis of gene expression data showed that the levels of miR-552 and miR-592 decreased from stage T1 to T4. However, experimental data showed that these miRNAs were significantly upregulated in stage III. Similar findings were reported by Pan Z., who demonstrated that miR-552 expression was higher in stage III + IV compared to stages I + II [33]. Similarly, in serum samples, miR-552 was predominantly associated with stage III, like colorectal tissue samples. As the tumor stage increases, the size of the tumor becomes bigger, suggesting that the higher expressions of miR-552 and miR-592 in stage III + IV are closely linked to tumor size. On the contrary, our examination of tissue miRNA sequencing data revealed that an augmentation in the expression of miR-552 is correlated with a diminished probability of mortality. Perhaps this occurrence is linked to easy diagnosis and removal of bigger tumors. Additionally, *in silico* analysis showed that expression of miR-549a was related to the TNM stage, while experimental data indicated a correlation with tumor depth invasion. Notably, pathways regulated by miR-549a, such as TGF-β signaling and the Hippo pathway were found to be dysregulated in CRC lymph node metastasis and the TNM stage [43,44]. The ROC curve demonstrated that all these miRNAs have the potential to accurately differentiate between healthy individuals and CRC patients with high sensitivity and specificity. While we believe that these miRNAs will not possess any diagnostic or prognostic value in other forms of cancer due to their primary control over CRC, the outcomes of the ongoing study could be influenced by limited access to a larger number of other types of cancers and diseases as well as technical issues. Additionally, the application of these miRNAs within the wider context of cancer may present difficulties due to the small size and limited abundance of miRNAs, the substantial sequence similarity among different miRNA members, and the specific expressions associated with particular stages. Therefore, it is necessary to conduct further examinations in a large population and utilize a more precise miRNA detection approach, such as probe-based Real Time qPCR, to determine the application value of these miRNAs in the diagnosis of CRC.

5. Conclusion

Our study revealed that the upregulation of miR-549a, miR-552, and miR-592 was observed in both the blood and tissue samples of individuals diagnosed with colorectal cancer (CRC). Given their remarkable correlation with CRC pathology, these miRNAs can collectively serve as a diagnostic panel for the precise differentiation of CRC from various other malignancies and diseases within the human body. While our investigation is solely focused on CRC, the methodology employed can readily be extended to encompass a wide array of diseases.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Golestan University of Medical Sciences, Gorgan, Iran (No: IR.GOUMS.REC.1398.360).

Data availability statement

Data used in this study will be made available upon request.

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Consent to participate

All study participants or their legal guardians provided informed written consent following the Helsinki Declaration of 2013.

CRediT authorship contribution statement

Soroush Akbar: Investigation. **Samaneh Mashreghi:** Investigation. **Mohammad Reza Kalani:** Supervision, Conceptualization. **Akram Valanik:** Formal analysis. **Farzaneh Ahmadi:** Investigation. **Mahdi Aalikhani:** Writing – review & editing, Writing – original draft. **Zahra Bazi:** Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Zahra Bazi reports equipment, drugs, or supplies was provided by Golestan University of Medical Sciences and Health Services. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e28492.

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