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Research Article
MicroRNA Gene Expression Profile of Colorectal Cancer in Saudi Population

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Abstract

Background and Objective: Identification of the predictive molecular markers for colorectal cancer is highly needed as most patients are diagnosed at the end stages with only a 5 year survival rate. The miRNAs play a dual role in colorectal carcinogenesis (CRC) both in tumor-suppressing and tumor-promoting actions. Deregulated miRNA genes in cancer tissues can act as early predictive molecular markers. The main objective of this study was to identify specific microRNAs associated with colorectal cancer in the Saudi population which can help to discover the novel biomarkers for the disease. **Materials and Methods:** In the present study, four miRNA genes were selected that are involved in regulating proliferation, apoptosis, invasion, migration and cancer stem cells. The expression levels of miR-21, miR-155, miR-222 and miR-23a in 20 paired samples of CRC were investigated and matched adjoining tissues through RT-PCR in the Saudi population. **Results:** The high expression levels of miR-222, miR-21 and miR-155 genes in cancer tissue as compared to adjuring normal tissue while expression levels of miR-23 levels were found in nearly the same in cancer tissues about normal adjoining tissues. Increased expression of all the genes in stages III and IV were noted whereas stages I and II showed lower levels of expression. The expression level of miR-155, miR-21 and miR-222 in the rectal was found higher as compared to the colon. Expression level miR-23 was almost the same in both sites. The expression level of miR-222 and miR-21 was significantly higher in males than in females. The miR-155 was expressed slightly higher in males while miR-23 was remarkably higher in females (4). **Conclusion:** The miRNA can be defined as molecular markers that could have crucial significance in CRC treatment in the Saudi population and also ascertain the complex alterations of gene expression underlie the development of malignant CRC.

Key words: Non-coding RNAs, microRNA, gene expression, colorectal cancer, biomarkers

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

MicroRNAs (miRNA) are small non-coding RNAs typically with 22 nucleotides and are mostly involved in many biological pathways in multicellular organisms[1]. Nearly 3000 miRNA genes have been reported in the mammalian genome, from which above 2000 belong to the human genome[2-4]. These genes are arranged in clusters in the genome and expressed independently with their promoters that share the same transcriptional regulatory units[5]. About half of the miRNAs expressed in the genome are transcribed from non-protein-coding genes and the other half are coded in the introns of coding genes[6]. The majority of miRNAs are transcribed from DNA into precursor miRNAs (pre-miRNAs), which are processed to mature miRNAs[7-9]. The RNA polymerase II produces prim-miRNAs, which are processed by Drosha ribonuclease III and a double-stranded RNA-binding protein known as DiGeorge Critical Region 8 (DGCR8) into a 70-nucleotide-long pre-miRNA. Pre-miRNAs are transported into the cell cytoplasm by the dsRNA-binding protein exportin 5. The RNase III enzyme Dicer transforms the pre-miRNAs into miRNAs in the cytoplasm[10,11]. The microRNAs play a critical role in cell proliferation, differentiation, apoptosis, cellular senescence and aging[12-15]. They can regulate telomeres function and expression levels of multiple genes. The miRNAs mainly interfere with the target mRNAs at 3 UTR to inhibit expression[16-18]. It can also interact with the 5 UTR, coding sequences and gene promoters under specific situations and trigger gene expression[19,20]. The miRNAs are also transported across several subcellular compartments to regulate the rate of transcription and even translation[21,22]. Many pathological conditions such as cancer, autoimmune disorders and cardiovascular and neurological diseases, are associated with improper expression levels of miRNA in humans[23-26]. Studies have reported both up-regulation and down-regulation of miRNAs in various types of human cancers[9,27-29]. This regulates angiogenesis, apoptosis, cell proliferation and differentiation and controls the expression of oncogenes or tumor-suppressor genes responsible for various human cancers such as lung cancer, breast cancer and colon cancer[5,8,9,20,26-29]. Many studies have confirmed aberrant levels of miRNA expression in human cancers and are currently being investigated as potential therapeutic targets and disease biomarkers[30,31].

Colorectal cancer (CRC) stands at third place in terms of cancer-related death worldwide with an incidence rate of about 1.4 million and a death rate of about 693,900 per annum[32,33]. Early-stage molecular biomarkers for CRC are highly needed as most patients are diagnosed at the end stages with only a 5 years survival rate[32]. Being a heterogeneous multifactorial disease, one-third of the cases are attributed to genetic factors worldwide[34]. The miRNAs play a dual role in colorectal carcinogenesis both in tumor-suppressing and tumor-promoting actions[35-37]. OncomiRs are oncogenic as they target and inhibit the expression of endogenous tumor-suppressor genes. On the other hand, tumor-suppressive miRNAs down-regulate oncogenes and retard tumor progression[35-37]. Many studies revealed elevated levels of miRNAs (including miR-21, miR-155, miR-222 and miR-23a) in cancer patients which act as oncomiR by targeting and downregulating several genes involved in regulating proliferation, apoptosis, invasion, migration and cancer stem cell.

In human colorectal cancer cells, miR-21 suppresses PTEN expression via the PTEN/PI-3K/Akt signaling pathway[38]. According to Asangani *et al.*[39], miR-21 drives invasion, intravasation and metastasis in colorectal cancer by binding to a particular region at nt228-249 of PDCD4's 3 -UTR in a negative manner. Furthermore, the resistance of 5-FU chemotherapy is linked to increased miR-21 expression. The miR-21 directly targeted the 3 -UTR of the tumor suppressor human DNA MutS homolog 2 (hMSH2) and down-regulated its expression followed by reduced 5-FU-induced G2/M damage arrest and apoptosis[40]. Overexpression of miR-21 increased cell proliferation and invasion and decreased apoptosis in response to treatment with the chemotherapy drug 5-FU in CRC cells and vice versa[41]. The miR-155 is used as a novel tumor biomarker for prognostic evaluation and clinic pathological diagnosis of CRC[42-44]. It is one of the most prominent oncogenic microRNAs, overexpressed in CRC and regulates angiogenesis, cell proliferation, invasion and migration. Its expression is associated with a poor prognosis, treatment resistance and genomic instability[44]. It suppresses the expression of Protein Tyrosine Phosphatase Receptor Type J (PTPRJ) by binding to the 3'-UTR through the miR-155/PTPRJ/AKT axis and affects the proliferation and migration of CRC cells[44]. Additionally, studies revealed up-regulation of miR-155 plays a critical role in enhancing CRC cell motility and invasion[45]. The miR-222 levels have been found to be higher in the plasma and tumor tissues of CRC patients. It promotes a positive feedback loop to raise RelA and signal transducer and activates transcription 3 expression levels, to increase CRC cell growth. Protein kinase 3 (MST3) is likely its target gene as down regulated MST3 enhances the

migration and invasion and overexpressed MST3 attenuates miR-222 overexpression in colorectal cell lines[46]. The miR-23a inhibits pyruvate dehydrogenase kinase 4 expression while activating pyruvate dehydrogenase and oxidative phosphorylation which in turn produces the necessary amount of ATP for cell proliferation[47]. The miR-23a encourages the migration and invasion of both cancer stem cells and CRC cells by targeting the Metastasis Suppressor 1 (MTSS1) gene. The miR-23a is significantly up-regulated in the serum of patients with colon cancer and enhances 5-FU resistance in microsatellite instability in cancer cells through targeting ATP binding cassette subfamily F member 1[48].

Altered expression of miR-21, miR-155, miR-222 and miR-23a in colorectal cancer has not been reported in the Saudi population. The objective of this study was to measure the expression levels of the above-mentioned miRNAs in both colorectal cancer tissue and normal adjacent tissues taken from Saudi patients.

MATERIALS AND METHODS

Study duration: The study was carried out from January, 2022 to July, 2022.

Tissue sample collection and clinical characteristics: Colorectal tumor tissues and adjacent healthy tissues were collected from twenty confirmed CRC patients in King Khalid University Hospital, Riyadh. The patients included 9 males and 11 females. Collected tissue was immediately stored in RNA later solution (Ambion) for DNA and RNA extraction. Patients had no radiotherapy, chemotherapy or other anticancer treatments before the collection of samples and were informed and signed consent.

Ethical consideration: The study was approved by the Ethics Committee of King Khalid University Hospital, Riyadh.

Nucleic acid isolation: The AllPrep DNA/RNA/Protein Mini Kit from Qiagen was used to extract high-puri DNA and RNA from colorectal tissues and nearby tissue. The manufacturer's instructions were followed for every extraction. The quality and quantity of nucleic acids were assessed using spectrophotometry (NanoDrop 8000, Thermo Fisher Scientific, Epsom, UK).

Expression level of selected miRNA genes

cDNA preparation: Before Real-Time PCR (q-PCR), cDNA was synthesized by reverse transcription from 2 µg of purified RNA using a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) in accordance with the manufacturer's instructions. The qPCR was used to determine the expression levels of the targeted genes (LightCycler 480 II, Roche Applied Science, Switzerland). Reactions were carried out in a total volume of 20 µL under conditions of four incubation steps. Each real-time reaction contains 2.0 µL of 10X reverse transcription buffer, 0.8 µL of 25X dNTP mix, 2.0 µL of 10X reverse transcription random primers, 1.0 µL of multiScribe reverse transcriptase, 10 µL of RNA preparation and 4.2 µL of nuclease-free water. The obtained cDNA samples were stored at -20°C until use.

Real-time PCR quantification of selective microRNA: One microgram of total RNA was reverse transcribed in a final volume of 80 µL using random primers under standard conditions using the Prime Script RT reagent Kit. The relative levels of selective miRNAs (21, 222, 23a and 155) were estimated by quantitative RT–PCR (ViiA™ 7 Real-Time PCR System-Applied Biosystems) using TaqMan Gene Expression Assay with gene-specific primers. The assay ID was shown in Table 1. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as an internal control, as its expression showed minimal variation in colorectal cancer samples (n = 20) and matched normal samples (n = 20). Data was analyzed by using the comparative threshold cycle (2-ΔCT) (Livak) relative expression method.

Assay was performed in a total volume of 10 µL, containing 5 µL of TaqMan™ 2X Universal PCR Master Mix, 0.5 µL of 20X TaqMan Assay Mix, 2 µL of cDNA and 2.5 µL of nuclease-free water.

Table 1: Assay ID for the selected genes

miRNAs	Assay ID
miR-222	Hs04415495_s1
miR-23a	Hs03659093_s1
miR-155	Hs03910067_s1
miR-21	Hs04231424_s1
GAPDH	Hs02758991_g1

RESULTS

The present study included 20 colorectal cancer patients. The average age of patients was 56 years (with the range of 23-73 years). The cancer stage was classified according to pathological tumor node metastasis classification (pTNM) by pathologists. More than half of the patients were in the late stages (III-IV) (51.8%), whereas 48% of them were in the early stages (I -II). The majority of patients (62.9%) were male and most patients (59.26%) had colon cancer. Table 2 summarized the clinical characteristics data of colorectal cancer patients.

Gene expression of miRNAs: The expression of 4 selected miRNAs in 20 colorectal cancer tissues and their adjacent normal mucosa samples was estimated quantitatively by RT-PCR, using GAPDH as a reference gene. Relative expression levels of selected miRNA genes (miR-222, miR-21, miR-23 and miR-155) were shown in Fig. 1a-d. The high expression levels of miR-222, miR-21 and miR-155 genes found in cancer tissue as compared to adjuring normal tissue. Expression levels of miR-23 levels were found nearly the same in cancer tissue and in adjuring normal tissue. To evaluate the relationship between the expression level of selected miRNA with the stages of CRC, samples were divided into two groups. The expression levels were shown in Fig. 2a-d. Increased expression levels of all the genes were found in stages III and IV whereas stages I and II showed lower levels of expression.

Samples were classified into two groups according to gene expression levels in the tumor site, in the colon, or the rectum. A significantly higher expression level of miR-155 (p-value = 0.031), miR-21 (p-value = 0.036) and miR-222 was found in the rectal as compared to the colon. Expression level miR-23 was almost the same in both sites as shown in Fig. 3. The expression level of all test miRNA genes according to gender as shown in Fig. 4. The expression pattern of all the genes was found different in males and females. The expression level of miR-222 and miR-21 was significantly (p-value = 0.04) higher in males than in females. The miR-155 was expressed slightly higher in males while miR-23 was remarkably higher in females.

Table 2: Demographic characteristics of the study subjects

Characteristics	CRC (n = 20)
Age in years (Mean±SD)	
≤56	8 (40%)
>56	12 (60%)
Sex	
Males	9 (45%)
Females	11 (55%)
Tumor location	
Colon	11(55%)
Rectum	9 (45%)
Tumor node metastasis	
Stage I-II	6 (30%)
Stage III-IV	14 (70%)

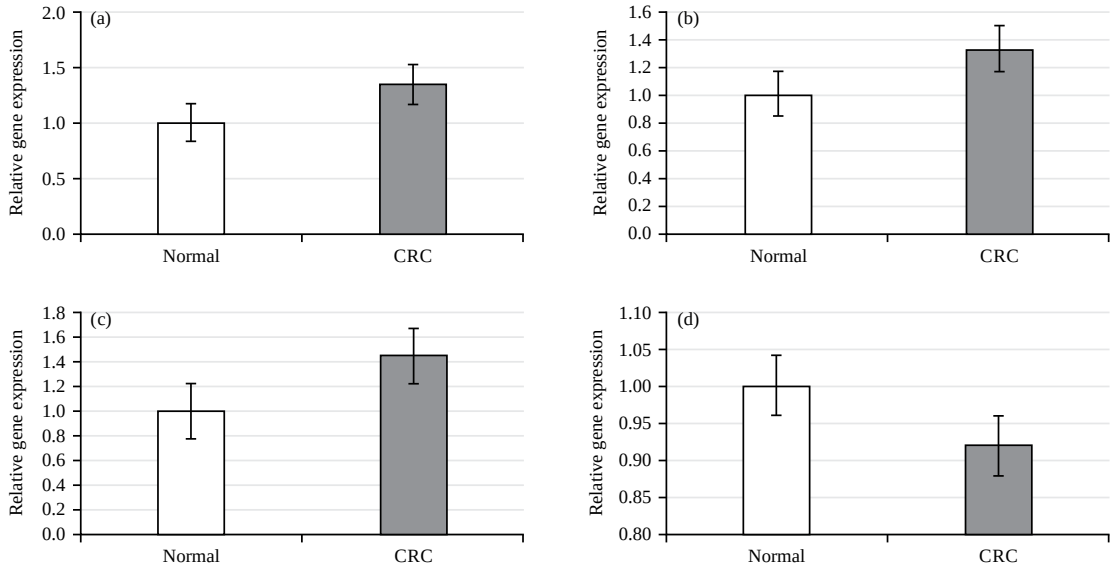

Fig. 1(a-d): Relative expression level of selected miRNA genes, (a) miR-222, (b) miR-21, (c) miR-155 and (d) miR-23 in cancer and adjuring normal tissue

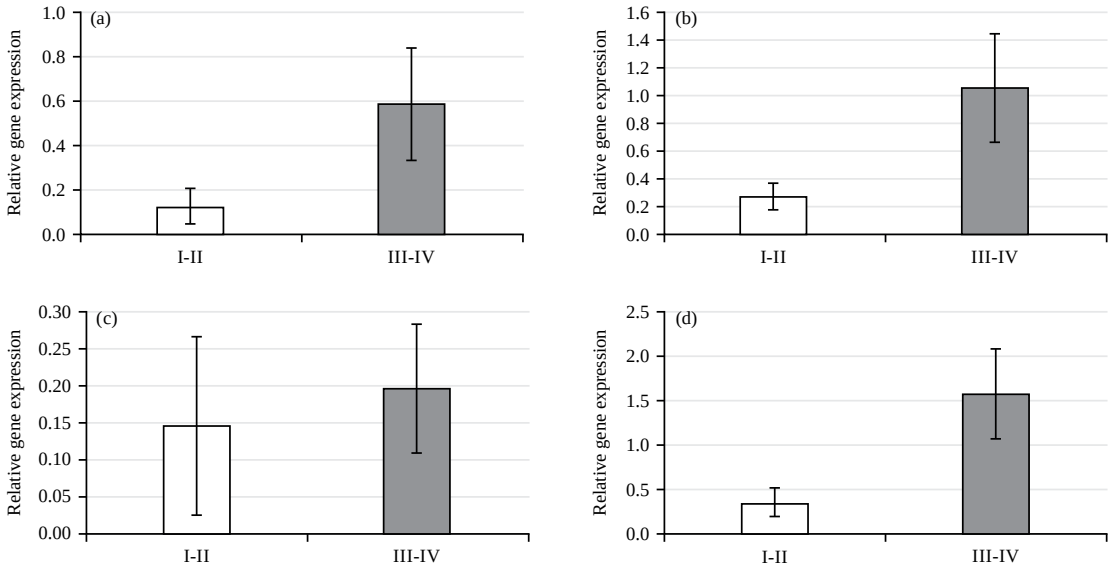

Fig. 2(a-d): Relation between relative expression level of selected miRNA genes, (a) miR-222, (b) miR-21, (c) miR-155 and (d) miR-23 in colorectal cancer tissue with tumor stages

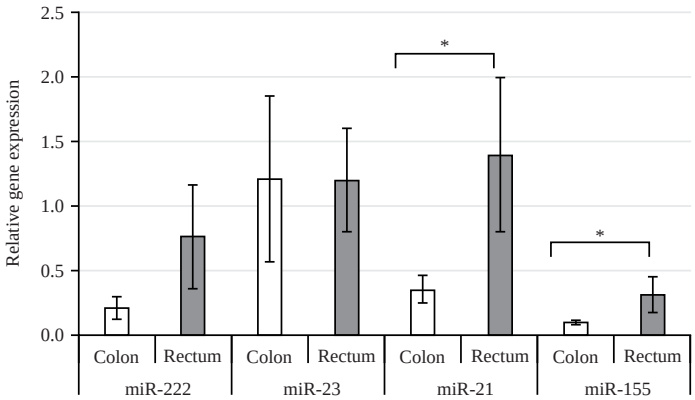

Fig. 3: Expression pattern of the different gene for tumour site
*Statistically significant ($p<0.05$)

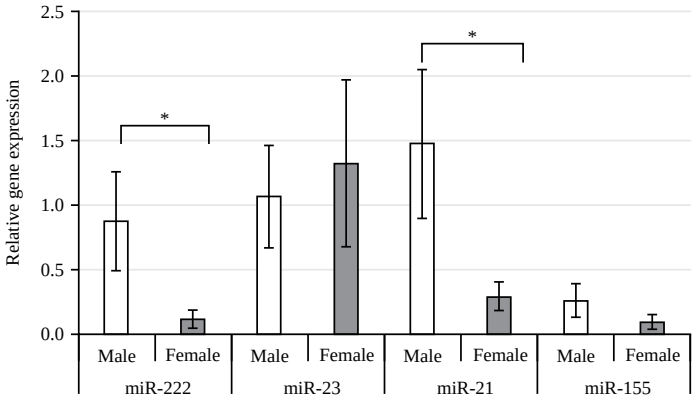

Fig. 4: Expression pattern of different genes with respect to gender
*Statistically significant ($p<0.05$)

DISCUSSION

Colorectal cancer is a typical gastrointestinal tumor with the primary clinical characteristics of high mortality and morbidity rate. Currently, there are many issues with its diagnosis like low time efficiency and poor specificity. Previous research has demonstrated a tight link between specific genetic alterations and the development of colorectal cancer in many populations. The miRNA has been investigated as a possible biomarker for CRC diagnosis, prognosis and therapy prediction with the main focus as a biomarker for early-stage CRC detection. To the best of our knowledge, this is the first study reporting the expression levels of miRNA in CRC patients in the Saudi population.

In this work, the expression levels of miR-21, miR-222, miR-155 and, miR-23a were examined in CRC patients which were found overexpressed in cancer tissue in comparison to adjacent normal tissue. Higher expression levels of miR-222 levels in colorectal tumor tissue were reported earlier in the Chinese population[49]. The miR-222 is encoded on the X chromosome as part of the miR-221/222 cluster which is overexpressed in different kinds of cancer, including CRC. Many previous studies have reported the association of miR-222 expression in cancer tissue or blood of patients with the metastatic potential of CRC. Iida *et al.*[50] reported the over-expression of miR-222 and its association with metastatic activity in CRC patients in the Japanese population. Microenvironments of the tumor and extracellular miR-222 can impact tumor progression through bidirectional communication between stroma and tumor suggesting crosstalk miR-222 between cancer cells and stromal cells as one possible mechanism involved in cancer progression[51]. Overexpression of miR-222 is also reported in lung, gastric and bladder cancers. Studies have reported that overexpressed miR-222 down regulates target p27 resulting in increased proliferation in many cancer tissues and targeting the expression of the p53 upregulated modulator of apoptosis[52-54]. Higher expression levels of miR-155 have previously been observed in lung, cervical, colorectal and thyroid carcinomas[55,56]. It has been suggested that over-expression of miR-155 promotes colon cancer growth and increases chemo-resistance to cisplatin by directly targeting Forkhead Box O3 (FOXO3)[57-59]. The expression levels of miR-21 reported in the present study are supported by You *et al.*[60] reported on the Chinese population. They reported higher expression levels of miR-21 in colon cancer tissues which was associated with the degree of differentiation, lymph node metastasis, distant metastasis and TNM stage. Studies have also reported that miR-21c promotes cell proliferation in different tumor tissue and cancer cell lines considering miR-21 as a potential marker for the diagnosis and prognosis of colon cancer[61,62]. The expressions of miR-23a were found lower in colorectal cancer tissue and higher in corresponding adjacent tissues, suggesting its crucial role in colorectal cancer. The expression of miR-23a is downregulated by oncogenic promyelocytic leukemia/retinoic acid receptor alpha and c-Myc in cancer tissue[63]. However, higher expression levels of miR-23a are reported in several human malignancies, such as pancreatic cancer, glioblastoma, hepatocellular carcinoma, bladder cancer and gastric cancer[63]. Chhabra *et al.*[64] reported the expression level of miR-23a in colorectal cancer clinical tissue samples at different malignant stages which was found restricted to invasive colorectal cancers. The miR-23a expression levels are increased with intestinal invasive adenocarcinomas as colon cancer stem cells play a critical role in the invasion and metastasis processes[64].

The expression levels of all four miRNA genes were high in rectal cancer as compared to colon cancer. To our knowledge, there was no previous literature elucidating the difference of miR-21, miR-222, miR-155 and miR-23a expression between colon and rectal cancer. Also, the level of miRNA expression at TNM stages III and IV was higher than that at stages I and II which is supported by previous reports[65-67]. *In vivo*, studies also reported the role of miRNA expression in promoting tumor progression and their up-upregulation in a metastatic group compared to that in the non-metastatic group[68].

The expression levels of miRNA genes as a potential biomarker for early colorectal cancer (CRC) diagnosis are promising; however, further validation in a larger sample size is imperative to enhance scientific rigor.

CONCLUSION

The microRNA genes might support tumor growth by promoting cancer cell spreading and migration as the expression levels of the tested genes were different in cancer tissues as compared to normal tissue. Current findings also revealed stage-specific and sight-specific expression profiles as stage I and II expression patterns were different from stage III and IV, the colon and rectum showed different patterns, respectively. Thus, the result of the current study reveals an obvious relation between miRNA expression and tumor migration, invasion and metastasis of CRC in the Saudi population.

SIGNIFICANCE STATEMENT

The microRNAs play a pivotal role in the regulation of gene expression and have been implicated in various cellular processes, including those associated with cancer. Examining the microRNA gene expression profile in colorectal cancer can potentially lead to the identification of specific microRNA signatures that serve as diagnostic and prognostic markers. This information could enhance our ability to detect colorectal cancer at early stages and predict its progression, aiding in timely and targeted interventions.

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