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# The Role of miRNA in Colorectal Cancer for Early Detection and Potential Clinical Significances

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**Abstract:** ***Background: Colorectal cancer (CRC) is currently the third most common cancer in males and the second in females worldwide. Objective: The main objective of the current study was to investigate the possibility of using MicroRNA (miRNA) as potential biomarkers in early detection of CRC. Methodology: It was a case control study. A self-structured questionnaire was used to collect socio-demographic data, medical history and other lifestyle factors. Moreover, blood analysis was used to validate the expression and diagnostic role of miRNA when it comes to CRC. A total of 80 subjects were recruited to participate in the current study (40 cases + 40 healthy controls). For blood analysis, a total RNA was isolated from 200µl of each patient's plasma (four samples 250µl) using the QIAGEN miRNeasy serum/plasma kit. Isolated RNA was stored at -80°C. miRNAs expression was assayed by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) to generate miRNA normalized copy numbers. Relative expression of ten CRC-related miRNAs (miR-21, miR-31, miR-20a, and miR-145, miR-135b and let-7g, miR-10b-5p, miR-215-5p and miR-150-5p, and isomiRs of miR-125b-5p and miR-30a-5p and miR29a) and three CRC-related genes was detected using the SYBR Green quantitative real-time PCR technique. The correlation between gene expression levels and clinic-pathological features was evaluated. Results: Preoperative high plasma miRNA levels were associated with increased recurrence risk for miR-29a (HR ¼ 2.61 [1.34,5.07], P ¼ 0.005), and miR-31 (HR ¼ 4.03 [1.76, 9.24], P ¼ 0.001). Both plasma miR-31 (AUC: 0.717) and miR-29a (AUC: 0.703) discriminate recurrence from these patients without recurrence. In addition, high levels of miR-31 during surveillance was associated with a three-fold increased risk of recurrence across all time points. Conclusion: Pre-operative plasma miR-29a and 31 are potential CRC prognosis biomarkers. In addition, dynamic postoperative miR-31, and 145 are potential biomarkers for the early detection of recurrence during CRC surveillance.***

**Keywords:** Colorectal Cancer, miRNAs, Biomarkers

## 1. Background

Colorectal cancer (CRC) is responsible for 10% of cancer cases and deaths worldwide; therefore, it represents a significant health burden. The more we understand the fundamental and biological nature of the CRC, the better equipped we will be to design effective preventive, diagnostic and therapeutic tools to help reduce the burden of the disease.[1]Practical issues addressed include identifying effective therapeutic strategies, identifying the exact patient groups that may benefit from adjuvant chemotherapy and identifying the exact patient groups that may benefit from extensive screening for recurrence. It takes into consideration the initial radiologic imaging (rTNM), the clinical findings (cTNM), and the pathological test of resected tumour specimens.[2]

Historically, most CRC research has focused on genetic and genetic changes in protein coding genes for their role in the initiation and evolution of the CRC. Recently, emphasis has been placed on a class of unencrypted small RNAs, which are called microRNAs.[3] MicroRNAs are small, 18 to 24 nucleotide RNAs that regulate the translation and stabilization of target-specific mRNAs.[4]Ten years ago, microRNAs were implicated in the onset of chronic lymphocytic leukemia. Since this discovery, microscopic RNAs have been shown to be involved in almost every aspect of cancer biology, since tumor suppression genes or carcinogenic genes ostensibly depend on the cellular context in which they are expressed.[5,6]

In the utilitarian measure, a few miRNA inhibitors had a critical development inhibitory impact on CRC cells (for example miR-183, miR-182, miR-96, miR-31-5p and miR-18a-3p). It appeared that some miRNA imitates, at any rate in some part, caused abatement in CRC cell development, however, were not noteworthy in our test. MicroRNAs detected in blood or stool may help in early detection of colon cancer. MicroRNA expression levels in tumors may help guide therapeutic decisions and be useful as a therapeutic goal by inhibiting or replacing microRNA strategies.[7]

The evidence supports the role of microRNA at every stage of the CRC's initiation, progress and development. Intensive research now aims to determine whether microRNAs can be used as diagnostic biological markers and therapeutic targets for cancer (Figure 1).[8,9,10]

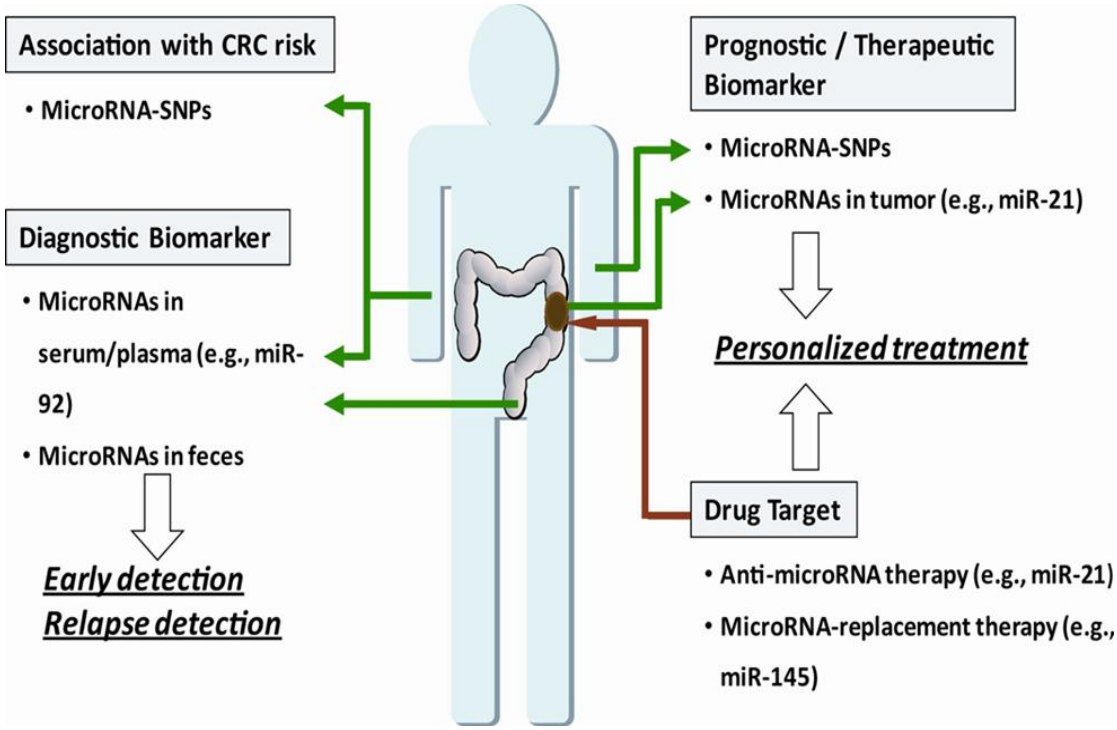


**Figure 1:** Potential diagnostic strategies and rRNAforthe detection of colon cancer

The number of RNAs showing a high expression in the CRC is consistent with the findings that microscopic RNA is overrepresented in genomic regions that show an increase in the number of copies while underrepresented in areas that show the number of copies lost in the CRC output. Regardless of these findings, functional studies show that some microRNAs have important carcinogenic functions while others have important tumor suppression functions and these functions need to be assessed for each microRNA individually in the context of the specific tissue / tumor type.[11,12]

There is evidence to support a mechanical role in the exact RNAs defined in the CRC. Many microRNAs have been shown to have roles in the CRC and their function have been verified. While we cannot discuss all these findings in this review, we will present some important findings on specific RNA, their objectives, and their role in colon cancer.[13]

**Table 1:** MicroRNAs with roles in colon cancer and their targets

| microRNA | Tumor Suppressor or Oncogene | Examples of experimentally validated microRNA Targets |
|---|---|---|
| let-7 | Tumor Suppressor | KRAS |
| miR-17-92 cluster | Oncogene | E2F1 |
| miR-21 | Oncogene | PDCD4, PTEN, RECK, NFIB, TPM1, SPRY2, RHOB, TIMP3, maspin, CDC25a, TIAM1, MSH2 |
| miR-29 | Tumor Suppressor | MMP2, DNMT3A/B |
| miR-30a | Tumor Suppressor | DTL |
| miR-34a | Tumor Suppressor | FRA1, SIRT1, MYC, BCL2 |
| miR-95 | Oncogene | SNX1 |
| miR-101 | Tumor Suppressor | COX2 |
| miR-135a/b | Oncogene | APC |
| miR-137 | Tumor Suppressor | CDC42 |
| miR-143 | Tumor Suppressor | KRAS, DNMT3A, ERK5 |
| miR-145 | Tumor Suppressor | IRS-1, c-Myc, YES1, STAT1, OCT4, SOX2, KLF4,FLI1 |
| miR-155 | Oncogene | MLH1, MSH2, MSH6 |
| miR-200c | | ZEB1, ZEB2 |
| miR-342 | Tumor Suppressor | DNMT1 |
| miR-365 | Tumor Suppressor | CCND1, BCL-2 |
| miR-451 | Tumor Suppressor | MIF |
| miR-499 | Oncogene | FOXO4, PDCD4 |
| miR-675 | Tumor Suppressor | RB |

The first study identified a microRNA expression in miR-143 and miR-145 colon. Most of the expression of miR-499-5p in CRC cell lines is targeted at FOXO4 and PDCD4 to promote cell migration and invasion in in vitro, lung and liver metastases in mouse models. MiR-675 can target a retinoblastoma inhibitor gene (Jones et al.) to increase tumor growth.[10]

MiR-365 acts as a tumor suppression to prevent cell cycle progression and promotes programmed cell death of colon

cancer cells by targeting Cyclin D1 (CCND1) and Bcl-2. The loss of mi-29 leads to increased expression of MMP2 to promote metastases in rat models of colon cancer. The miR-95 carcinogen promotes tumors by targeting nexin 1 (SNX1) screening. Because variable microRNA expression can affect colon cancer initiationand development, it suggests that microRNAs have potential goals as therapeutic goals for the CRC.[14,15,16]

## 2. Methodology

***Study Population***

The population of the current study will include patients (cases) of the oncology center of King Abdullah Medical City in Makkah and Jeddah.The King Abdullah Medical City is considered as one of K.S.A. premier cancer treatment centres,out of the population, a sample of 40 patients plus 40 controls had been set. Relevant data were gathered from the patients' past medical records.

***Blood sample collection***

One to three milliliters of blood were collected in EDTA (Ethylene-diamine tetra acetic acid) tube, after overnight fast. EDTA functions by binding calcium in the blood and keeping the blood from clotting. The blood samples were then centrifuged at (3500 rpm for 15 min) and the separated plasma was transferred to appropriately labelled 1.5 ml Eppendorf tubes and stored at -80$^0$ C until use in experiments. All plasma samples were spectrophoto-metrically analysed to be free from haemoglobin. Haemolysed plasma samples were excluded from further analysis.

***Instruments and Software's***

***Nanodrop (QIAGEN miRNeasy Mini Kit) (MS2 RNA, Roche)***

- This protocol is for purifying total RNA, including small RNAs.
- Microcentrifuge(s) (with rotor for 2 ml tubes) for centrifugation at 4°C and at room.
- After addition of chloroform, the homogenate is separated into aqueous and organic phases by centrifugation. RNA partitions to the upper, aqueous phase, while DNA partitions to the inter-phase and proteins to the lower, organic phase or the inter-phase.
- Chloroform, Ethanol, RNase-free pipet tips, RNeas free water, RNeasy Mini column, Elution Volume, buffer RW, and buffer RPE.

***RNA Isolation***

Total RNA was isolated from 200µl patient plasma (four samples 250µl) using the QIAGEN miRNeasy serum/plasma kit. In brief, QIAzol lysis buffer (1000µl) were added to the sample to stabilize the RNA byeliminating ribonucleases, cellular DNA and proteins released by cell lysis. Addition of chloroform (200µl) and subsequent centrifugation allowed phase separation of the lysate, and the upper aqueous supernatant was separated and mixed with ethanol (2:1 ratio to volume of supernatant) before loaded onto the membrane in the spin columns provided in the kit. RNA bound to the column and contaminants were washed away before RNA was eluted using RNase-free water. Isolated RNA was stored at 80°C.

**Table 2:** Reaction Setup

| | Volume/reaction | | |
|---|---|---|---|
| Component | 96-well block | Capillary cycler | Final concentration |
| 2x QuantiFast SYBR Green PCR Master Mix | 12.5 µl | 10 µl | 1x |
| Primer A* | Variable | Variable | 1 µM |
| Primer B* | Variable | Variable | 1 µM |
| Template DNA or cDNA (added at step 4) | Variable | Variable | ≤100 ng/ reaction |
| RNase-free water | Variable | Variable | |

- The reaction had been mixed thoroughly and dispensed appropriate volumes into PCR vessels or plates.
- Template cDNA (≤100 ng/reaction) had been added to the individual PCR vessels or wells containing the reaction mix.
- For two-step RT-PCR, the volume of the cDNA added (from the undiluted RT reaction) should not exceed 10% of the final PCR volume.

**Table 3:** Real-Time Cycler Conditions

| Step | Time | Temperature | Ramp rate | Additional comments |
|---|---|---|---|---|
| **PCR initial activation step** | 5 min | 95ºC | Maximal/ fast mode | HotStarTaq Plus DNA Polymerase is activated by this heating step |
| **Two-step cycling** | | | | |
| Denaturation | 10 s | 95ºC | Maximal/ fast mode | |
| Combined annealing/ extension | 30 s | 58ºC | Maximal/ fast mode | Performfluorescence datacollection |
| Number of cycles | 35–40 | | | The number ofcycles depend onthe amount oftemplate DNA |

- Place the PCR vessels or plates in the real-time cycler and start the cycling program.
- Perform melting curve analysis of the PCR products to verify the specificity and identity.
- Melting curve analysis is an analysis step built into the software of real-time cyclers.

***Statistical Analysis***

Kaplan-Meier and Cox proportional hazard regression models were used to assess the influence of miRNA levels on overall survival, measured from the date of diagnosis. The Cox regression model was run with coxph package and p-values were adjusted for multiple testing using Benjamin Hochberg correction. Student's t-test and linear regression were used to identify miRNAs associated with clinicopathological parameters. When linear regression was 21 used, continuous clinical parameters were converted to log2. In the transfection experiment, P-values were calculated using a two-tailed t-test by comparing the replicates measured at time point 96h. All statistical analyses

were performed using the R software (v3.2.2). Pvalues<0.05 were considered statistically significant.

**Ethics and consent statements**

Ethical approval was obtained from ethical committee King Abdullah Medical City in Makkah and patients were recruited with their informed consent.

***KAMC IRB***

A. Written informed consent has been obtained from all subjects.

B. Privacy and confidentiality have been assured for the participating persons.

C. All tests were done in the center of excellence of Genomic Medicine Research, King Abdul-Aziz University**.**

## 3. Results

**Patient Demographics**

As shown in table (4) males are the dominant gender (62.5%). 29% of the participants there ages ranged from 31 – 40 years old.

* N-stage, two patients could not be assessed.

** Different KRAS mutations were tested in the patients; G12D (n=16), G13D (n=6),G12A. (n=3) and G125 (n=1).

*** For 10 patients, tumor differentiation grade was not listed in the medical records.

**** Results from CEA measurement was either unavailable from medical records or analysis was not conducted before preoperative treatment was given, for 15 patients.

***** Serum was collected before treatment or after treatment. In the after-treatment group (n=29), serum was either collected after preoperative treatment alone (n=23), after preoperative treatment and surgery (n=5), or after surgery alone (n=3).

**Table 4:** Clinical and histopathological characteristics of the investigated patient cohort

| | | |
|---|---|---|
| **Gender** | Male | 62.5% |
| | Female | 37.5% |
| **Age** | <25 | 3% |
| | 25-30 | 5% |
| | 31-40 | 29% |
| | 41-55 | 17% |
| | <55 | 46.00% |
| **T** | Tis | 1 |
| | T1 | 5 |
| | T2 | 11 |
| | T3 | 13 |
| | T4 | 6 |
| **N*** | N0 | 7 |
| | N1 | 14 |
| | N2 | 13 |
| **M** | M0 | 5 |
| | M1 | 21 |
| **Stage Grouping** | 0 | 1 |
| | I 6 | 13 |
| | II 24 | 38 |
| | III 16 | 23 |
| | IV 13 | 21 |
| **KRAS mutation**** | Wild type | (73%) |
| | Mutation | (27%) |
| **Tumor differentiation grade***** | High | 3 |
| | Moderate | 9 |
| | Moderate - low | 3 |
| | Low | 6 |
| **CEA******** | <5 | 10 |
| | 5 - 10 | 11 |
| | 10 - 100 | 17 |
| | >100 | 3 |

***Association between microRNA expression and patient survival***

Survival analysis was performed to investigate the potential prognostic value of circulating miRNA levels in CRC patients. Overall survival across all patients relative to TNM stage (I- II, III and IV) is illustrated as a Kaplan-Meier survival curve in (Figure. 3), showing that increasing TNM stage confers worse survival. High expression levels of four mature miRNAs (MIR-133B, MZX, miR-320) was found to be significantly associated with poor overall survival ($p< 0.05$) compared to low levels of these miRNAs. In addition, high levels of 15 isomiRs and low levels of four isomiRs had a significant negative influence on OS ($p< 0.05$).

**Table 5:** Multivariate analysis for overall survival (mature miRNA)

| MicroRNA | HR* | 95% CI | P-value | Adjusted p- value |
|---|---|---|---|---|
| MIR-133B | 1.61 | 1.34 - 1.95 | 6.71e-07 | 0.0001 |
| MIR-20A | 1.64 | 1.33 - 2.02 | 3.89e-06 | 0.0004 |
| MIR-145 | 1.63 | 1.29 - 2.04 | 2.31e-05 | 0.0017 |
| MIR-31 | 1.73 | 1.30 - 2.34 | 0.0002 | 0.0099 |

## 4. Discussion

In the current study, small RNA sequencing was used to identify miRNA isolated from plasma of 60 rectal cancer patients to search for potential non-invasive biomarkers in CRC. A total of 498 mature miRNAs and 3758 isomiRs were detected across all patient samples. Investigation of the relationship between miRNA expression levels and clinicopathological characteristics showed that several mature miRNAs and isomiRs were significantly associated with metastasis (stage IV), CEA levels and overall survival. High levels of the miR-320 family (miR-320a-e), miR-10a-5pand low levels of let-7b- 5p were associated with poor overall survival and metastasis at diagnosis. High levels of miR-320 and miR-10a-5p also showed positive correlation with CEA levels. High levels of miR-200c-3p and miR-29a-3p were associated with metastasis and increasing levels correlated with increasing CEA levels. Not surprising, we found that CEA levels were significantly higher in patients diagnosed with metastatic (stage IV) CRC compared to non-metastatic patients (stage I-III).

Consistent with our results, high levels of miR-200c-3p were previously found in plasma of metastatic CRC patients.[17,18,19] observed that stage IV CRC patients had significantly higher levels of miR-200c compared to stage I-III patients and high levels were associated with lymph node metastasis, distant disease, liver metastasis, and poor disease-free and overall survival. Our results showed that other members of the miR-200 family (miR-200b-3p/5p and miR-200a-3p) were associated with metastasis. In a recent study on colorectal cancer, plasma levels of miR-200a, miR-200b and miR-200c were significantly elevated in patients with advanced disease. MiR-29a has previously been detected in plasma of

both early (stage I-II) and late stage (III-IV) CRC patients, and higher levels was association between miR-29a and stage I-III were found in our analysis. In addition, Huang et al. compared expression of miR-29a with healthy subjects. Wang et al. found significantly higher levels of miR-29a in plasma of metastatic CRC patients and were able to differentiate between metastatic patients and non- metastatic patients with a 75% sensitivity and specificity.[20]

Circulating levels of the miR- 320 family have previously been detected in various cancers, including CRC. Fang et al. analyzed miR-24, miR-320 and miR-423-5p in plasma of CRC patients and showed that this three-miRNA signature could distinguish cases from controls with a sensitivity and specificity of 92,79% and 70.77%, respectively. They also found that miR-24, miR-320 and miR-423-5p could predict development of metastasis in CRC patients after surgery.High levels of miR-1246 were associated with poor overall survival and metastasis in recent study which have investigated the expression of circulating miR-1246 in CRC.[21,22,23]

Wu et al. found significantly elevated levels of miR-1246 in CRC patients compared to healthy subjects and the positive rate of miR-1246 for identification of CRC was 95,5 % compared to 30,7% and 16,0% of CEA and CA 19-9, respectively.[24] Two miRNAs, miR-877-5p and miR-451a, were correlated with Hb levels. MiR-451a expression have previously been linked to hemoglobin content in blood-derived products. In a study by Shkurnikov et al., they analyzed the effect of hemolysis on miRNA expression in plasma andfound that hemolysis of red blood cells leads to a significant increase in the levels of several miRNAs, including miR-451a.[25]

MiRNA mimics, on the other hand, are designed to increaserepression of target genes by increasing the pool of active miRNAs. A publication by Jin et al. discusses the challenges by using miRNA mimics and points at the potential off-target effects when transfected at high concentrations and lack of target repression when transfected at low concentrations. Moreover, the mimics were often mutated or trimmed/tailed causing off-target effects and unwanted mRNA degradation. Since the mimic transfection in the current project were repeated three times with similar results, it is plausible to think that the lack of consistent growth repression could be, at least partly, explained by technical issues with the miRNA mimics.[26]

A potential source of error in our study could have been introduced during RNA isolation. We chose not to use the internal spike-in (C.elegans miR-39 miRNA mimic) provided in the QIAGEN kit, because we planned to add calibrator sequences in the library preparation step. In general, variability in recovery of miRNA from serum and plasma can contribute to quantification errors, but comparison of commonly used commercial kits for RNA isolation has shown little variation in RNA recovery. Additionally, Bioanalyzer results showed that miRNA was present at acceptable concentrations in our samples.[27]

## 5. Conclusion

Generally, metastasis is highly correlated with survival and it is likely that some of the survival-associated miRNAs and isomiRs are significant because of differences in disease stage. However, several miRNAs and isomiRs were found to be associated with survival without being related to metastasis, indicating that other mechanisms may be involved. MiR-10b-5p, miR-215-5p and miR-150-5p, and isomiRs of miR-125b-5p are potential biomarkers for estimating treatment response in CRC patients.

Our results demonstrated that isomiR expression largely corresponded with mature miRNAs. However, some isoforms were associated with clinicopathological parameters independently of the canonical sequence, suggesting that isomiRs may provide an additional layer to CRC biomarkers beyond mature miRNAs and that isomiR and miRNA expression should be investigated simultaneously in future experiments. Validation in large-scale studies and specificity and sensitivity of such biomarkers needs to be investigated in order to determine their clinical application.

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