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Dysregulation of the *miR-424-3p/5p*–lncRNA *MEG3* axis in colorectal cancer: a case-control expression study

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Abstract

Colorectal Cancer (CRC) pathogenesis is multifactorial include interactions between long non-coding RNAs (lncRNAs) and microRNAs (miRNAs). This study aimed to evaluate the expression levels of molecular parameters include *lncRNA MEG3* and *miR-424 (3p/5p)* in both blood and tissue of CRC patients. A case-control study was conducted to estimate the hematological parameters including Hemoglobin (Hb), Red Blood Cells (RBCs), White Blood Cells (WBCs) and Platelets (PLT). Expression levels of *MEG3* and *miR-424*-3p/5p were quantified using Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) in blood and tissue samples from both CRC patients and healthy controls. Fold expression was performed using $2^{-\Delta\Delta Ct}$ method, Receiver Operating Characteristic (ROC) curve analysis for diagnostic

accuracy, and Spearman's rank correlation to explore the associations between molecular parameters, age, and hematological parameters. In CRC patients, *MEG3* was significantly downregulated in blood and tissue relative to controls ($p < 0.001$). A comparison found significant upregulation of *miR-424-3p* and *miR-424-5p* ($p < 0.0001$), with *miR-424-5p* showing the largest fold change in tissue (median = 3.345). Overall, ROC analysis confirmed that *miR-424-3p* in blood represents the effective diagnostic parameter (Area under curve - AUC = 0.934, Sensitivity 92%, Specificity 96%). *MEG3*-B negatively correlated significantly with *miR-424-3p*-B (rho = −0.520, p = 0.0001). In addition, there was a significant positive correlation between *MEG3* levels and Hb and RBC counts and between *miR-424-5p* and platelet counts (p = 0.0078). Together, these findings indicate that dysregulation of the *MEG3/miR-424* axis is associated with CRC and may contribute to its underlying molecular mechanisms. Blood *MEG3* expression and tumor *miR-424-3p/miR-424-5p* expression emerged as independent variables associated with CRC in the multivariable analysis. Furthermore, circulating *miR-424-3p* demonstrated promising diagnostic performance as a potential non-invasive biomarker for CRC detection. However, these findings should be interpreted cautiously and require validation in larger independent cohorts.

Introduction

There is an increasing burden of cancer worldwide, with the populations of low- and middle-income countries experiencing higher percentages than other groups. Breast, colorectal, and cervical cancers are among the most common malignancies in the world and account for a significant proportion of all cancers worldwide contributing to cancer mortality.[1] Colorectal Cancer (CRC) makes up about 10% of cancers and is the third most common cancer and the second leading cause of cancer death globally.[2] CRC is a heterogeneous disease with different molecular pathways originating from different combinations of genetic and epigenetic alterations. Its pathogenesis is a complex interaction between genetic predisposition and environmental exposures.[3,4] Based on epidemiological data, various modifiable and non-

modifiable risk factors for CRC including diet, sedentary lifestyle, smoking, alcohol use, age, sex, family history, germline mutations, polygenic risk scores, and intestinal microbiota composition are described.[5–7] The data accumulation reveals that genetic alterations are key to the driving event of CRC by perturbing the important cellular pathways that control both initiation and progression of a tumor through molecular alterations. At the same time, dysregulation of epigenetic regulation in normal and malignant tissues also alters expression of essential genes, thereby driving tumorigenesis. These genetic and epigenetic changes regulate molecular variability between CRC subtypes and the variable transcriptional programming of tumor growth and evolution.[8,9]

According to Iraq's National Cancer Registry and the World Health Organization (WHO), cancer has become an important cause of mortality in Iraq, accounting for approximately 11% of total deaths. As of 2019–2020, CRC has emerged as the third most prevalent malignancy in the country, with an incidence of 5.5% and a mortality rate of 5.6%, following breast and bladder cancers. These rising statistics highlight a critical requirement for enhanced cancer control and targeted diagnostic strategies within the Iraqi healthcare system.

Long non-coding RNAs (lncRNAs) are RNA molecules over 200 nucleotides long that do not encode for proteins but regulate gene expression at the epigenetic, transcriptional, and post-transcriptional levels by acting as signals, miRNA sponges, or scaffolds for chromatin complexes.[10] In CRC, long non-coding RNAs participate in context-dependent oncogenic and tumor-suppressive functions where they converge with core signaling and epigenetic programs to maintain proliferation, provide apoptosis resistance and orchestrate epithelial–mesenchymal transition that promotes adenoma-to-carcinoma evolution and distant metastasis.[11]

Maternally Expressed Gene 3 (*MEG3*) is a prominent imprinted long non-coding RNA found at the DLK1-MEG3 locus on chromosome 14q32.3 and is expressed in many human tissues.[12] *MEG3* is mainly a tumor suppressor, thus exerting its effects by competitively binding with target microRNAs in oncogenic signaling activities. Importantly, *MEG3* regulates the p53-dependent and Wnt/β-catenin pathways, and its downregulation has often become linked to the progression of different cancers.[13]

MicroRNAs (miRNAs) are small, non-coding regulatory molecules that modulate protein synthesis by binding to the 3' untranslated regions (3' UTRs) of target mRNAs, leading to their degradation or translational inhibition. Their biogenesis is a complex, multi-step process involving nuclear transcription and cytoplasmic maturation, with final expression levels further influenced by decay mechanisms and interactions at gene promoters.[14]

In cancer, miRNAs are often dysregulated due to chromosomal alterations or epigenetic modifications. These molecules serve as onco-miRNAs, which are upregulated in cancer, or tumor suppressor miRNAs, which are downregulated in cancer. Thus, dysfunctional miRNAs function to promote tumor initiation, migration, and invasive potential in CRC by modulating specific oncogenic or suppressive targets.[15,16]

While molecular biology for CRC has advanced significantly, the exact role of these long non-coding RNAs and microRNAs has yet to be determined. Clinical use of the *miR-424-3p/5p-lncRNA MEG3* axis has not been addressed to date. This work has helped fulfill this gap in knowledge to study the expression of *miR-424-3p*, *miR-424-5p*, and *MEG3* in the blood and tumor tissues of CRC patients. By correlating these molecular signatures with certain hematological factors, we hope to assess whether the axis can be seen as a functional diagnostic or prognostic means. We hypothesize that low *MEG3* expression in tandem with high *miR-424-3p/5p* expression may correlate with aggressive disease activity serving as a minimally invasive biomarker for risk stratification and real-time monitoring of CRC advancement. However, the proposed a competing endogenous RNA (ceRNA) mechanism through which *MEG3* acts as a molecular sponge for *miR-424* remains a steadfast hypothesis that requires experimental validation. Therefore, this article is intended to establish to what degree this axis, the basis of any kind of regulatory interaction possible, is clinically relevant. The function of long non-coding RNAs (lncRNAs) on microRNA (miRNA) regulatory networks remains open questions. This paper bridges this gap by examining the expression profiles of *miR-424-3p, miR-424-5p*, and lncRNA *MEG3* in blood as well as tumor tissues in patients with CRC.

Materials and Methods

Ethical approval

The study proposal, with description and consent form, was reviewed and approved by the College of Science Research Ethics Committee at Mustansiriyah University (Approval ID: BCSMU/1222/00036Z on January 1, 2023), and all procedures were performed in compliance with the ethical principles of the Declaration of Helsinki. All participants provided written informed consent.

Sample collection and selection criteria

This study was designed as a targeted prospective pilot study, with the aim of identifying molecular dysregulation in the *lncRNA MEG3/miR-424* axis in an Iraqi CRC cohort. On the basis of the study cohort, from January to November 2023, 100 fresh tissue and blood samples were obtained from the Gastroenterology and Liver Diseases Teaching Hospital and Baghdad Teaching Hospital in Baghdad, Iraq. A total of 50 participants were enrolled in this study, including 25 patients with CRC and 25 apparently healthy controls. Overall, 100 biological samples were collected, comprising 25 peripheral blood and 25 tissue samples from CRC patients, and 25 peripheral blood and 25 normal colorectal tissue samples from healthy controls. Participants were men (n=27) and women (n=23), 33–61 years of age and 26–61 years of age in control and patients respectively, from different regions of Iraq. Ethical standards were met by obtaining signed consent from participants. To maintain the control group integrity, we had stringent exclusion criteria; patients were excluded if they were experiencing controlled inflammatory bowel disease, colonic diverticulosis, enteritis, benign colorectal polyps, proctitis, or rectal ulcers. All the samples were taken surgically and immediately processed for molecular preservation. Tissue samples (~ 100 mg) were immersed in 750 µL of TransZol reagent (TransGen Biotech, Beijing, China).

After the surgery, aseptic venous blood samples (5 mL) for each individual were collected by trained health care professionals. For this volume, 3 mL were transferred into Ethylenediaminetetraacetic Acid (EDTA)-filled tubes for Complete Blood Count (CBC) tests, and 2 mL were then allocated to nucleic acid purification. The blood sample collected in EDTA tube selected for molecular examination were separated into two aliquots (one for miRNA extraction and the second for lnRNA analysis). Each aliquot consisted of 250 µL of whole blood

immediately mixed with 750 µL (3:1 ratio) of TRIzol reagent to keep RNA integrity. All prepared samples were kept at −80°C until examination.

Hematological parameters

A comprehensive CBC analysis was performed on all participants by Mindray (BC-30) 3-Part Differential Hematology Analyzer (Mindray Company, Darmstadt, Germany). The CBC was performed to evaluate Hemoglobin (Hb), Red blood cells (RBCs), White Blood Cells (WBCs) and Platelets (PLT).

Molecular study

Total RNA was isolated from whole blood and tissue samples following the procedures and using the TransZol Up Plus RNA Kit (ER501-01, TransGen Biotech, Beijing, China), while miRNA extraction was performed using the EasyPure® miRNA Kit (ER601-01, TransGen Biotech, Beijing, China) according to the manufacturer's instructions.[17] A NanoDrop One^{C} spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the concentration and purity of extracted RNA. The analysis yielded RNA concentrations ranging from 48 to 62 ng/µL, while purity was determined by the A260/A280 ratio. The resulting ratios of 2.00 to 2.32 confirmed high-quality RNA purification ensuring that the RNA samples met quality requirements for downstream quantitative real-time polymerase chain reaction (RT-qPCR) analysis.

Complementary DNA (cDNA) synthesis was performed using the EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TransGen Biotech, Beijing, China). The reaction mixture contained the provided reaction mix, random primers, anchored oligo(dT) primers, genomic DNA remover, RNase-free water, reverse transcriptase, and the extracted total RNA as the template. The synthesis process was carried out in a thermal cycler (QIAamplifier 96, Qiagen, Hilden, Germany) under the following conditions: initial incubation at 25°C for 10 min, reverse transcription at 42°C for 15 min, followed by enzyme inactivation at 85°C for 5 s.[18] The primers used in this study were custom-designed using Primer 3 Plus and synthesized by

Macrogen Company (Seoul, Korea). Sequences were delivered in lyophilized form and reconstituted in nuclease-free water to a working concentration of 10 pmol/µL as described by Shanyoor *et al.,* 2026.[19] The primer sequences and their details are described in Table 1.

To ensure the reliability of the quantitative real-time PCR (RT-qPCR) results, rigorous quality control measures were implemented. The stability of the selected reference genes, *GAPDH* for *MEG3* and *snRNA-U6* for *miR-424* isoforms, was validated by assessing their consistent threshold cycle (Ct) values across all patient and control samples, demonstrating minimal inter-group variation. Genomic DNA contamination was eliminated using the integrated gDNA remover during the cDNA synthesis step. Amplification specificity was verified through melting curve analysis, ensuring single-product amplification for each target.

Quantitative PCR amplification was carried out using the QIAGEN Rotor-Gene Q Real-Time PCR System (Qiagen, Hilden, Germany) with the TransStart® Top Green qPCR SuperMix kit (TransGen Biotech, Beijing, China).[20] Relative fold changes of *MEG3* were normalized using *GAPDH*, while *miR-424-3p* and *miR-424-3p* were normalized by snRNA-U6. PCR cycling conditions were adapted from a previously published protocol,[16] with an annealing temperature of 62°C for *MEG3*, 60°C *GAPDH*, and 58°C for each *miR-424-3p*, *miR-424-5p* and *snRNA-U6*. Gene expression fold change was calculated by the 2^-ΔΔCt method.[21]

Statistical analysis

Statistical Package for the Social Sciences (SPSS) software version 26 (SPSS Inc., Chicago, Illinois, USA) and MedCalc® Statistical Software version 23.5.5 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2026) were used to analyze data. Fold change was calculated by the $2^{-\Delta\Delta Ct}$ method. Categoric variables were presented as number and percentage. Non-normally distributed data are presented as the median and Interquartile Range (IQR) Differences between groups were evaluated using the Mann-Whitney test, with p values < 0.05 considered statistically significant. Receiver Operating Characteristic (ROC) curve analysis was performed to assess diagnostic accuracy, with area under the curve (AUC) values reported. Spearman rank correlation coefficient among study parameters was used to analyze associations

between variables, including corresponding p values. Multivariable Linear Regression Analysis was evaluated.

Results

Clinicopathological characteristics of the study groups

Median age was 45.0 (IQR 40.0–55.5) years in controls and 54.0 (48.0–55.8) in patients; a non-significant difference was shown (p=0.254). The demographic and clinical analysis revealed significant associations between lifestyle factors and CRC incidence within the Iraqi people. As shown in Table 2, smoking was significantly prevalent among patients (48%) compared to the control group (16%), yielding a statistically significant difference (p = 0.032). Furthermore, 32% of the patient group had undergone prior chemotherapy, a factor that showed a highly significant distribution (p = 0.004) when compared to the control group. These findings suggest that environmental factors, particularly smoking, may play a contributory role in the pathogenesis of CRC in this specific population.

Hematological profile variations

Comparative analysis of CBC parameters between CRC patients and healthy controls demonstrated a state of systemic physiological impairment in the patient group (Table 2). A significant reduction in Hemoglobin (Hb) levels was observed in patients (median: 12.000 g/dL) compared to controls (median: 13.300 g/dL, p = 0.02*). Similarly, RBCs and WBCs counts were significantly lower in the patient group (p = 0.02* and p = 0.004**, respectively). In contrast, PLT counts exhibited a significant elevation in CRC patients (median: 336.000 $\times 10^3/\mu L$) relative to the control group (median: 249.000 $\times 10^3/\mu L$, p = 0.008**).

Differential expression of lncRNA **MEG3** *in blood and tissue*

Differential expression of lncRNA *MEG3* in blood and tissue samples validated by Quantitative real-time polymerase chain reaction (qRT-PCR). lncRNA *MEG3* showed significantly downregulated expression in both peripheral blood and tumor tissue from patients compared to healthy controls. Figure 1 a, b explains the qRT-PCR output where Panel A displays the amplification curves from SYBR Green-based qRT-PCR, demonstrating efficient exponential amplification while Panel B shows the corresponding melt curves, confirming primer specificity with single sharp peaks at ~79–84°C, indicating a unique amplicon without primer-dimers or non-specific products.

The expression levels of the long non-coding RNA *MEG3* were evaluated across blood and tissue samples (Table 3). Our results indicated a significant down-regulation of *MEG3* in CRC patients. In blood samples (*MEG3*-B), the median expression decreased significantly to 0.748 in patients compared to 1.008 in controls ($p < 0.0001$). A consistent and significant decrease was also observed in tissue samples (*MEG3*-T), where the median expression in cancerous tissue was 0.811 versus 1.107 in healthy controls ($p = 0.0039$).

***Up-regulation of* miR-424-3p *and* miR-424-5p**

Figure 1 demonstrates the qRT-PCR output of *miR-424-3p* where panel c shows the amplification curves while panel d displays melt curve. The amplification curves exhibit the usual sigmoid profile with baseline, exponential increase, and plateau, confirming the effective PCR on samples. The melt curve has one bright peak at 74~80°C demonstrating the true amplifications, without primer-dimers. Amplification curves and melt curve for *miR-424-5p* are displayed in panels e and f, respectively, which indicates a strongly amplified amplification and a pure single melt peak at ~72–81°C suggesting high specificity for the target miRNA amplicon.

The *miR-424* (*3p* and *5p*) expression was significantly upregulated in CRC patients when compared with *MEG3*; *miR-424-3p* expression showed significant increases in blood and tissue ($p < 0.0001$), the median tissue expression was higher in patients (1.761) than in controls (1.025). Additionally, *miR-424-5p* was also strongly upregulated in blood patients (median: 1.376, $p = 0.0001$) and tissue (median 3.345 versus 1.174 in the controls; $p < 0.0001$; Table 3).

The quantitative real-time PCR analysis in *Supplementary materials, Table 1* showed significantly different expression of lncRNA *MEG3* and *miR-424* isoforms in control blood (Control-B), control tissue (Control-T), patient blood (Patients-B), and patient tissue (Patients-T). The expression of *MEG3* in the patient samples was significantly downregulated with median 0.748 (IQR: 0.595–0.843) in Patients-B and 0.811 (0.726–0.941) in Patients-T versus 1.008 (0.912–1.120) in Control-B and 1.107 (0.884–1.207) in Control-T, whereas *miR-424-3p* and *miR-424-5p* were significantly upregulated in patients with *miR-424-3p* medians of 1.691 (1.402–2.141) in Patients-B and 1.761 (1.476–2.191) in Patients-T and ~1.02 in controls, respectively, and *miR-424-5p* displayed the largest median value increases to 3.345 (2.201–6.385) in Patients-T versus 1.376 (1.165–1.549) in Patients-B and ~1.00–1.17 in controls.
These differences in *MEG3/miR-424* expression (which are seen as superscripts, a, b & c) were found in the post-hoc analysis (Conover), and the samples of patients were consistently different than those of the controls demonstrating a clear and inverse *MEG3/miR-424* expression pattern, which was associated with CRC.

Receiver Operating Characteristic (ROC) curve analysis

ROC curves for the different study parameters (Supplementary materials, Table 2; Figure 2) indicated highest sensitivity and specificity with high value of AUC as *miR-424-3p* (Blood) had a 0.934 AUC (95% CI: 0.827–0.985), a 92% sensitivity, and a 96% specificity for cut-off point >1.179 (Youden index 0.88). Blood *MEG3* demonstrated very good diagnostic performance, indicating that it is a reliable biomarker (AUC = 0.898, sensitivity = 88%, specificity = 76%). Regarding diagnostic performance, *miR-424-5p* (Tissue) demonstrated high accuracy with an AUC of 0.930 (95% CI: 0.821–0.983), achieving 96% sensitivity and 80% specificity at a cutoff value of >1.649.

Overall, *miR-424-3p* (Blood), *miR-424-5p* (Tissue), and *MEG3* (Blood) exhibited robust diagnostic potential, each yielding an AUC >0.89. Notably, *miR-424-3p* (Blood) provided the most optimal diagnostic profile for non-invasive CRC screening, with an AUC of 0.934, 92% sensitivity, and 96% specificity.

DeLong test results

To compare the diagnostic performance of the molecular indicators, we employed the DeLong test to evaluate the differences in the AUC (ΔAUC) as presented in Table III in supplement file. Our analysis indicated that Maternally Expressed Gene 3 in tissue sample (*MEG3*_T) demonstrated distinct diagnostic characteristics when compared with *miR-424-5p*_T (ΔAUC = 0.193, p = 0.021) and *miR-424-3p*_B (Δ AUC = 0.197, p = 0.033). These results reflect statistically significant differences in diagnostic accuracy between these specific pairs. While *miR-424-3p*_B and *miR-424-5p*_T exhibited the highest absolute AUC values (Table III in supplement file), the DeLong test provided insights into the comparative utility of these markers. In contrast, other comparisons showed no significant differences in diagnostic performance; for instance, the comparison between *miR-424-3p*_B and *miR-424-5p*_T yielded a p value of 0.9393, suggesting that these two parameters offer nearly identical diagnostic utility. Furthermore, the comparison between Maternally Expressed Gene 3 in blood sample (*MEG3*_B) and *MEG3*_T resulted in a p value of 0.0788, indicating a non-significant trend in their performance differentiation.

Logistic regression analysis

Based on the analysis in Table IV in supplement file, univariate logistic regression analysis was conducted to detect factors associated with CRC status. Smoking was significantly associated with an increased likelihood of CRC (OR=4.85, p=0.0197). Similarly, elevated expression levels of *miR-424* were significant predictors of CRC. Specifically, *miR-424-5P_T* (OR=8.40, p=0.0021), *miR-424-5p_B* (OR=215.77, p=0.0013), *miR-424-3*_T (OR=64.21, p=0.0003), and *miR-424-3P_B* (OR=12755.05, p=0.0014) were all positively correlated with CRC occurrence.

In contrast, *MEG3* expression demonstrated a protective association with CRC, as both *MEG3*_T (OR=0.032, p=0.0092) and *MEG3*_B (OR=0.000, p=0.0004) showed inverse relationships with disease status. Hb and WBCs count also exhibited significant inverse associations with CRC (Hb: OR=0.614, p=0.0228; WBC: OR=0.750, p=0.0456), whereas platelet count was positively associated with CRC risk (OR=1.01, p=0.0047). Age and RBC did not demonstrate significant associations with CRC status (p>0.05). Overall, the findings suggest that *miR-424* parameters,

particularly blood-derived *miR-424-3p* and *miR-424-5p*, together with *MEG3* expression levels, may serve as important predictors of CRC status.

Multivariate logistic regression analysis using a forward stepwise approach identified PLT, *miR-424-3p_T*, and *miR-424-5p_B* as independent predictors of CRC status. Increased PLT levels were associated with a higher probability of CRC (OR=1.018, p=0.0309). Likewise, elevated *miR-424-3p_T* and *miR-424-5p_B* levels significantly increased CRC risk (OR=561.11, p=0.0062; and OR=34276.58, p=0.0210, respectively). The overall model was statistically significant (χ^2=52.418, p<0.0001), with excellent predictive performance (Nagelkerke R^2=0.866, AUC=0.966, classification accuracy=94%). The Hosmer–Lemeshow test indicated good model calibration (p=0.544).

Spearman rank correlation coefficient

Spearman correlation analysis revealed several significant associations among hematological and molecular variables (Supplementary materials, Table 5). Hb levels were positively correlated with RBC ($\rho = 0.574, p < 0.0001$) and *MEG3-B* expression ($\rho = 0.288, p = 0.0428$), while RBC was also positively correlated with *MEG3*-B ($\rho = 0.327, p = 0.0207$).

WBC showed a positive correlation with *MEG3*-T ($\rho = 0.391, p = 0.0049$). PLT exhibited a significant negative correlation with *MEG3*-B ($\rho = -0.529, p = 0.0001$) but a positive correlation with *miR-424-5p*-T ($\rho = 0.372, p = 0.0078$). *MEG3*-B and *MEG3*-T were moderately correlated ($\rho = 0.295, p = 0.0374$).

Regarding miRNA interactions, strong positive correlations were observed between *miR-424-3p*-B and *miR-424-5p*-T ($\rho = 0.673, p < 0.0001$) as well as between *miR-424-3p*-T and *miR-424-5p*-T ($\rho = 0.651, p < 0.0001$).

Discussion

The current study highlights a significant dysregulation of the *MEG3/miR-424* axis in Iraqi patients with CRC. Our data demonstrate a significant down-regulation of lncRNA *MEG3* in

both blood and tissue samples, alongside a significant up-regulation of *miR-424* (3p and 5p strands), supporting their opposing tumor-suppressive and oncogenic roles, respectively, in CRC pathogenesis**.**

In our cohort, *MEG3* expression was significantly reduced in CRC patients compared with healthy controls in both blood and tissue compartments, supporting its characterization as an established tumor-suppressor lncRNA**.** Mechanistically, *MEG3* has been reported to inhibit proliferation and promote apoptosis through stabilization of p53,[12] modulation of *TGF-β* signaling, and interference with oncogenic pathways that regulate cell-cycle progression and genomic integrity. As a previous study demonstrated, downregulation of the tumor-suppressive lncRNA *MEG3* in colorectal adenocarcinoma enhances tumor cell proliferation and attenuates apoptosis by upregulating *TGF-β1* and its downstream effector sphingosine kinase 1.[22]

Moreover, the demonstrated expression pattern of *MEG3* in this study corroborates with the recognized role of *MEG3* as a tumor suppressor in CRC. Frequent downregulation of the DLK1-MEG3 locus at chromosome 14q32.3 is a hallmark characteristic of numerous malignancies. Functionally, *MEG3* serves to inhibit the progression of tumors (e.g. proliferation, migration, invasion, angiogenesis, and metabolic reprogramming) with apoptosis promoted. These tumor-suppression effects are largely mediated by ceRNA mechanisms that sequester oncogenic miRNAs to restore essential regulatory pathways.[12,23] The observed downregulation of *MEG3* is likely to remove critical transcriptional and post-transcriptional constraints on tumor growth, consistent with reports demonstrating that reduced *MEG3* expression is associated with advanced Tumor, Node, Metastasis (TNM) stage and poor prognosis in gastrointestinal malignancies.[24,25]

In contrast to *MEG3*, we observed an up-regulation of *miR-424-3p* and *miR-424-5p* among patient samples (blood and tissue), with the most obvious increase for *miR-424-5p* in tumor tissue. This inverse association between *MEG3* and *miR-424-3p/5p* strongly suggests a functional regulatory axis in which *MEG3* acts as a molecular "sponge" for miR-424, and its down-regulation leads to de-repression of miR-424 activity, When MEG3 expression is attenuated, miR-424 is liberated to target multiple tumor-suppressive mRNAs, thereby promoting oncogenesis.[26] Unlike Nouri Ghonbalani *et al*., who reported hypermethylation-induced downregulation of miR-424 driving VEGF upregulation and CRC angiogenesis, our

Iraqi CRC cohort shows upregulated *miR-424-3p/5p* (especially miR-424-5p in tumor tissue) alongside *MEG3* downregulation.[27]

Although miR-424 has been described as a context-dependent "double-edged sword," acting as either a tumor suppressor or an oncogenic microRNA depending on cellular context, accumulating evidence in gastrointestinal cancers including CRC supports its oncogenic role.[28] Specifically, *miR-424-5p* has been reported to drive proliferation, migration, invasion, and metastasis by targeting Hippo pathway effectors like *LATS2* thus inactivating tumor-suppressive signaling as seen in nasopharyngeal carcinoma and melanoma angiogenesis, where its upregulation links to advanced disease while being counteracted by competing RNA sponges such as *circLARP4*.[29]

Xu *et al.* demonstrated that *miR-424* acts as an immune-regulatory and chemo sensitizing miRNA in ovarian cancer by targeting PD-L1/CD80 3′-UTRs, suppressing checkpoint signaling; low *miR-424* promotes immune evasion and platinum resistance. Additionally, *miR-424* family members contribute to chemoresistance and Epithelial-Mesenchymal Transition (EMT) via *E2F1* modulation and immune checkpoint pathways.[30] The highly significant elevation of *miR-424* in our tissue samples, particularly *miR-424-5p*, and its strong inverse correlation with *MEG3* emphasize the concept of a *MEG3/miR-424* oncogenic axis that may be especially relevant to CRC in the Iraqi population.[28]

The clinicopathological analysis revealed a significant association between smoking and CRC incidence in our cohort, with smokers being substantially more frequent among CRC cases than controls. This is consistent with previous work highlighting tobacco smoking as a major environmental risk factor that promotes oxidative stress, chronic inflammation, and widespread epigenetic alterations in colonic epithelium.[31] Cigarette smoke condensate has been shown to induce promoter hypermethylation and transcriptional silencing of tumor-suppressive lncRNAs, including *MEG3*, in gastrointestinal cells.[32] Our observation of pronounced *MEG3* down-regulation in smoking CRC patients supports the hypothesis that smoking-driven epigenetic silencing contributes directly to disruption of lncRNA tumor-suppressor networks.

Within this regulatory framework, *MEG3* downregulation may liberate the oncogenic potential of *miR-424* isoforms, thereby integrating environmental exposures, epigenetic reprogramming,

and miRNA-driven oncogenesis within a cohesive *MEG3/miR-424* regulatory axis.[26,32] This mechanism aligns with accumulating evidence that tobacco-induced epigenetic alterations including DNA hypermethylation and ncRNA dysregulation that serve as pivotal mediators of gastrointestinal carcinogenesis.[33]

Beyond local tumor biology, our patients exhibited significant hematological imbalances, including reduced Hb, RBC and WBC counts while elevated PLT counts, consistent with cancer-associated anemia and reactive secondary thrombocytosis commonly reported in CRC.[34,35] The positive correlation between *MEG3* expression and Hb/RBCs levels in our cohort suggests that preserved *MEG3* may be associated with less severe anemia, in agreement with recent studies proposing that tumor-derived ncRNAs modulate systemic hematopoiesis and anemia in cancer.[36,37]

In parallel, the strong positive association of tissue *miR-424-5p* and PLT counts is notable. PLTs are recognized to sequester, protect, and transport tumor-derived microRNAs enabling systemic communication between tumor and distant organs.[38] Elevated PLT counts and activation have been linked to EMT, metastasis, and poor prognosis in CRC, partly through Transforming Growth Factor beta (TGF-β) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-dependent signaling pathways.[39] Our results indicate increased *miR-424* levels associated with thrombocytosis can lead to PLT-driven spread of oncogenic agents, driving metastasis and physiological deterioration of CRC sufferers more profoundly.[40]

For translational reasons, ROC-based data indicated excellent diagnostic utility of both *MEG3* and *miR-424* in our group, with circulating *miR-424-3p* showing outstanding sensitivity and specificity, further establishing it as a promising liquid biopsy tool for early detection of CRC. Our findings are consistent with growing evidence that circulating lncRNAs/miRNAs are non-invasive prognostic tools in CRC.[41,42] Furthermore, DeLong test comparisons confirmed enhanced diagnostic precision with a combined *MEG3*/*miR-424* panel versus individual signature, suggesting a multi-analyte strategy for clinical implementation.[43]

Univariate and multivariate binary logistic regression analyses further underscored the clinical utility of the *MEG3/miR-424* axis as an integrated biomarker system, capturing both tumor-

suppressive loss and oncomiR activation. Univariate analysis identified elevated *miR-424* isoforms as positive predictors of CRC status, while *MEG3* expression in both blood and tissue served as a strong independent inverse predictor. Furthermore, multivariate binary logistic regression confirmed that PLT count, *miR-424-3p_T*, and *miR-424-5p_B* function as independent predictors of CRC, with the resulting model demonstrating exceptional diagnostic performance (AUC=0.966, 94% accuracy). These findings align with established literature on competing ceRNA networks such as those involving *miR-150-5p/miR-10b-5p* which have demonstrated diagnostic utility in CRC patient serum.[44] By integrating molecular parameters with hematological parameters like Hb and WBCs, this research provides critical insights into the immunogenetic architecture of CRC within the unique genetic and epidemiological context of the Iraqi population, supporting the potential incorporation of *MEG3/miR-424* profiling into future clinical and screening strategies.

The study's limitation is the relatively small sample size (N = 50 blood sample/50 tissue sample), which may limit the generalizability of the findings. However, the paired-sample design, where matched tumor tissue and peripheral blood from each patient was used, minimized inter-individual variations and enhanced the accuracy of the procedures. Moreover, the bicompartmental molecular approach and recruitment of a relatively genetically homogeneous population assisted in reducing biological confounding. Nevertheless, larger multicenter studies involving more diverse populations are needed to validate these findings and confirm the clinical utility of the investigated biomarkers for the non-invasive detection and monitoring of colorectal cancer. In addition, the multivariable logistic regression model may be susceptible to overfitting because of the relatively small sample size. Therefore, these findings should be interpreted cautiously and validated in larger independent cohorts.

Conclusions

This investigation suggests the *MEG3/miR-424* axis as a potential regulator associated with CRC pathogenesis. *MEG3* downregulation in tumor tissue and peripheral blood may be linked to epigenetic silencing and could serve as a potential prognostic indicator for CRC. Furthermore, the inverse correlation between *MEG3* and the upregulated *miR-424-3p/5p* isoforms supports the

hypothesis of a competing endogenous RNA (ceRNA) mechanism, wherein *MEG3* depletion may facilitate *miR-424* oncogenic activity. Circulating *miR-424-3p* demonstrated high diagnostic accuracy (AUC = 0.934), highlighting its potential as a non-invasive biomarker for CRC. Associations between this axis and hematological parameters, such as low Hb, low RBC, and thrombocytosis, suggest that these molecular alterations may be associated with systemic responses accompanying CRC. Together, the *MEG3/miR-424* pair develops as a candidate molecular signature that warrants further functional validation to integrate its epigenetic, transcriptomic, and clinical dimensions in CRC.

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Table 1. Primer sequences and their details for *MEG3*, *miR-424-3p*, *miR-424-5p* and housekeeping genes.

Gene	Direction	Sequence	Tm (°C)	Ta (°C)
MEG3	Forward	CTGCCCATCTACACCTCACG	63.2	62
	Reverse	CTCTCCGCCGTCTGCGCTAGGGGCT	75.7	
GAPDH	Forward	GGCCTCCAAGGAGTAAGACC	59.46	60
	Reverse	AGGGGTCTACATGGCAACTG	59.38	
MiR-424-3p	Forward	CAAAACGTGAGGCGCTGCTAT	61.4	58
MiR-424-5p	Forward	CAGCAGCAATTCATGTTTTGAA	55.5	58

SnRNA-U6	Forward	AGAGAAGATTAGCATGGCCCCT	60.0	58
MiR-R.P*	Reverse	GCGAGCACAGAATTAATACGAC	62.0	-
Universal miR	-	CAGGTCCAGTTTTTTTTTTTTTTVN	-	-

***** MiR-R.P: MiRNA-universal Reverse primer. Tm: melting temperature. Ta: annealing temperature.

Table 2. Clinicopathological characteristics and hematological parameters among study groups.

Variable	Category	Control (n=25)	Patients (n=25)	Total (N=50)	p value
Age #	Median (IQR)	45.000 40.000 to 55.500	54.000 48.000 to 55.750		0.254 NS
@Smoking	Smoker	4 (16%)	12 (48%)	16 (32%)	0.032 *
	Non-smoker	21 (84%)	13 (52%)	34 (68%)	
@Therapy	Yes	0 (0%)	8 (32%)	8 (16%)	0.004**
	No	25 (100%)	17 (68%)	42 (84%)	
# Hb (g/dL)		13.300 12.725 to 14.300	12.000 11.600 to 13.325		0.0201*
# RBC ($\times 10^6/\mu L$)		5.050 4.480 to 5.347	4.470 4.135 to 5.130		0.0182 *
# WBC ($\times 10^3/\mu L$)		8.200 7.800 to 10.000	7.200 5.597 to 8.825		0.0039**
# PLT		249.000	336.000		0.0080**

(×10^3/µL)		164.000 to 263.000	186.500 to 416.000		

Hb, hemoglobin; IQR, interquartile range; NS, not significant; PLT, platelets; RBC, red blood cells; WBC, white blood cells.

Data are: Median, 25-75 percentiles. # Mann-Whitney test

Data are: Number (%). @ Fisher's Exact Test Used

NS: Non-significant. *: Significant p value. **: Highly significant p value

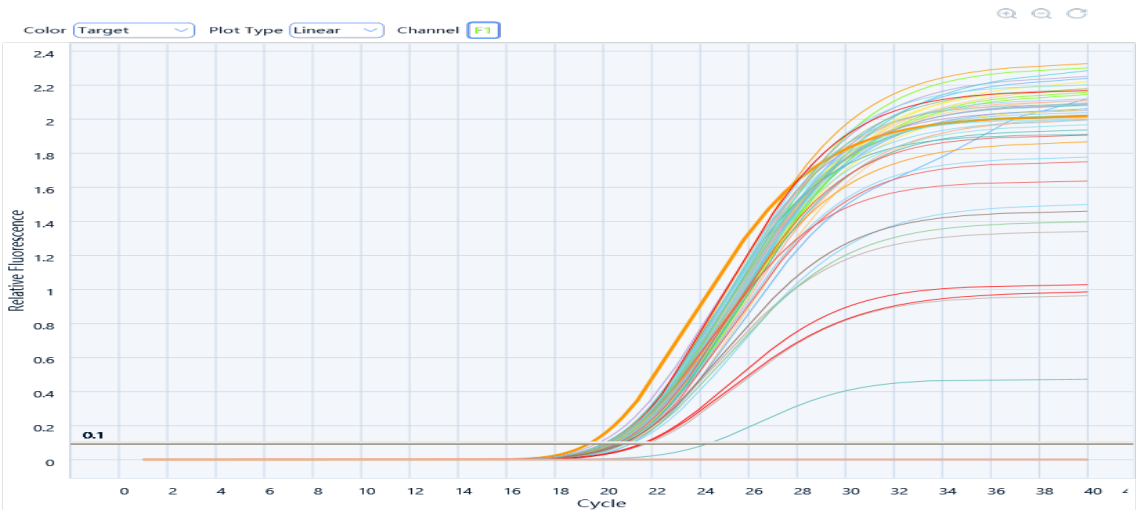
Table 3. Differential expression of molecular parameters in blood and tissue.

Parameters	Control	Patients	p value #
***MEG3*_B**	1.008 0.912 to 1.120	0.748 0.595 to 0.843	<0.0001
***MEG3*_T**	1.107 0.884 to 1.207	0.811 0.726 to 0.941	0.0039
***miR-424-3p* - B**	1.020 0.955 to 1.085	1.691 1.402 to 2.141	<0.0001
***miR-424-3p* - T**	1.025 0.932 to 1.164	1.761 1.476 to 2.191	<0.0001
***miR-424-5p* - B**	1.001 0.902 to 1.209	1.376 1.165 to 1.549	0.0001
***miR-424-5p* -T**	1.174 0.556 to 1.633	3.345 2.201 to 6.385	<0.0001

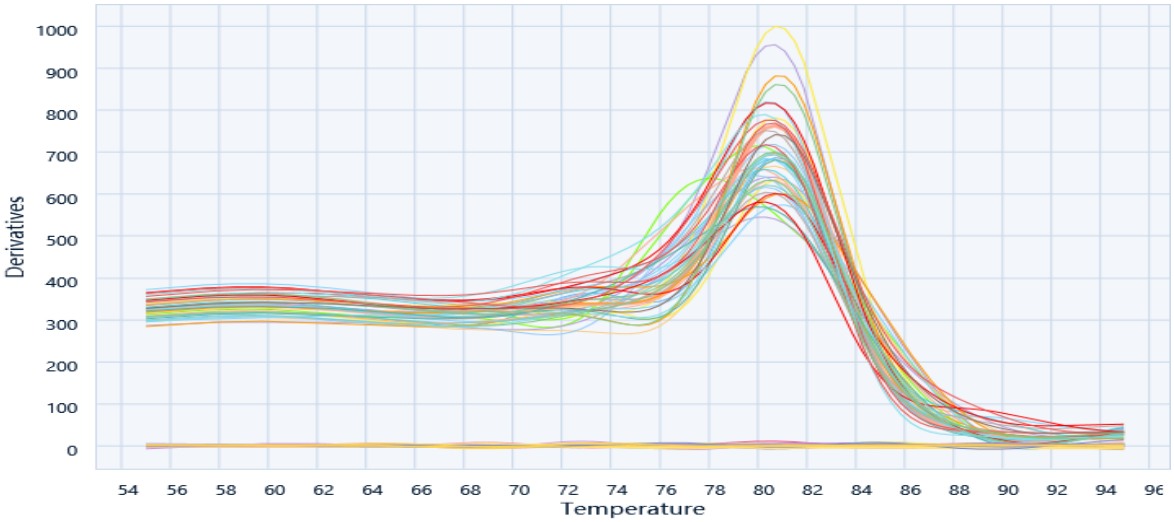
MEG3: Maternally Expressed Gene 3 (a long non-coding RNA); miR-424-3p: microRNA-424-3p;miR-424-5p: microRNA-424-5p;B: Blood (referring to the sample type); T: Tissue (referring to the sample type)

Data are: Median, 25-75 percentiles. # Mann-Whitney test

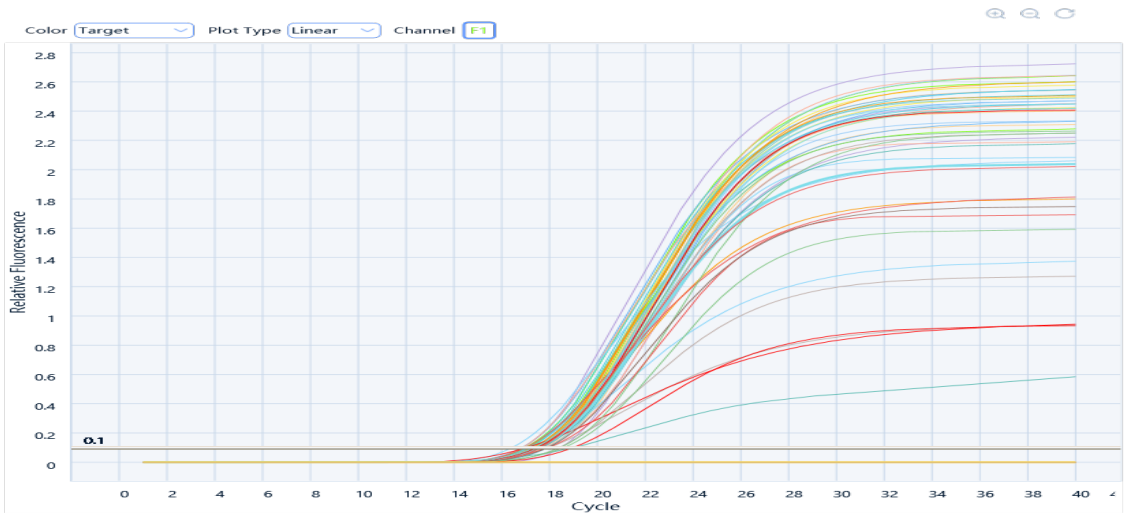
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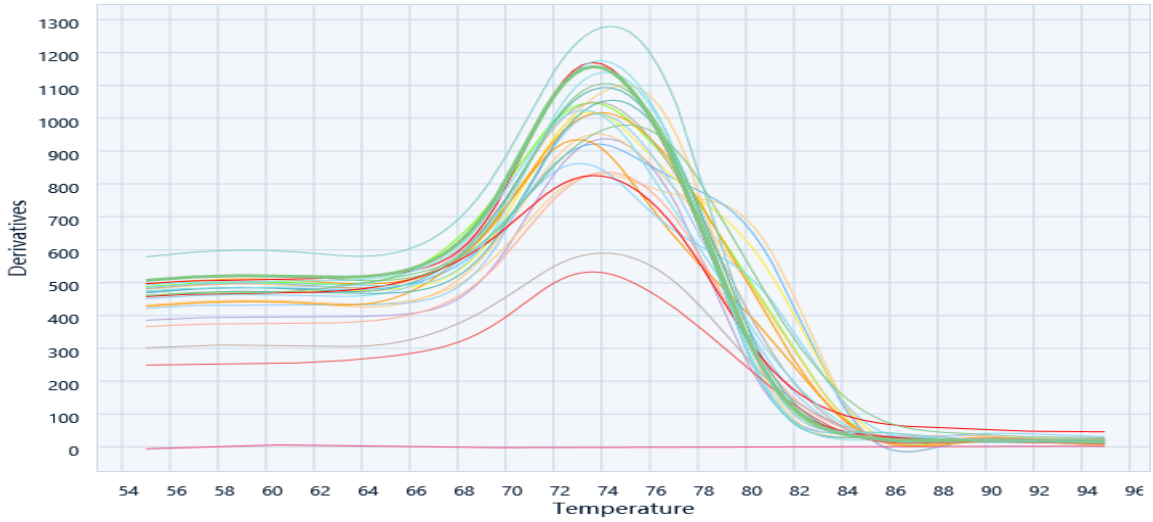
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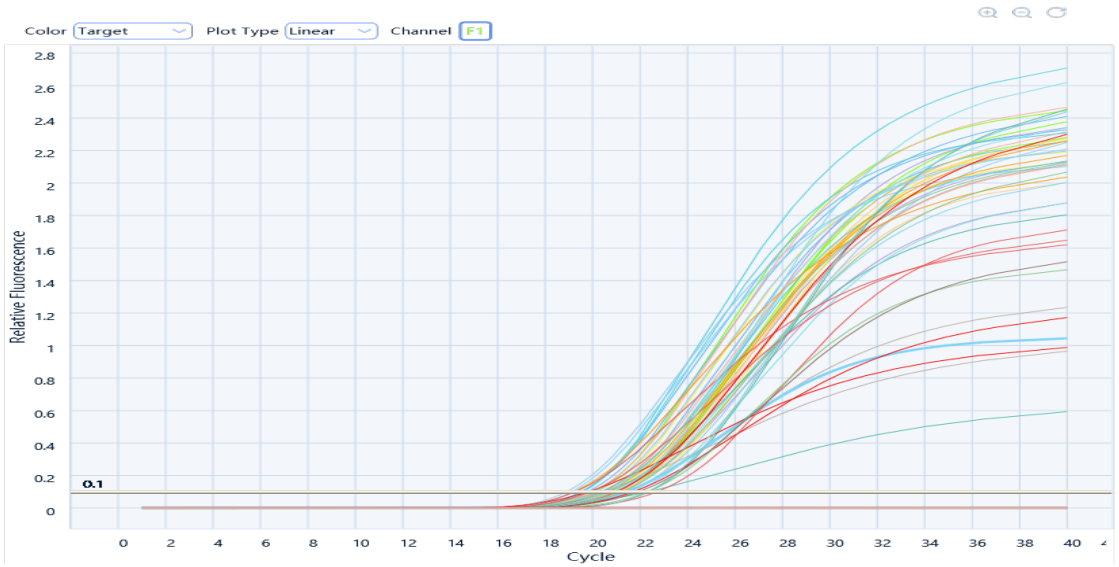
c


Color Target
Plot Type Linear
Channel F1
Relative Fluorescence
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d


Derivatives
Temperature

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Color Target
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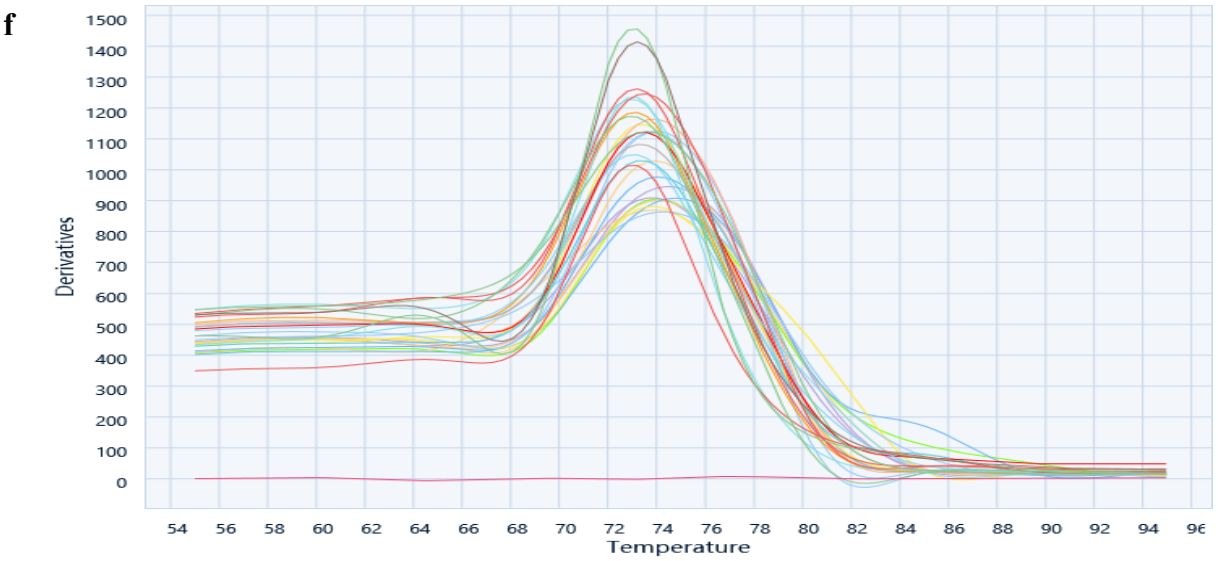
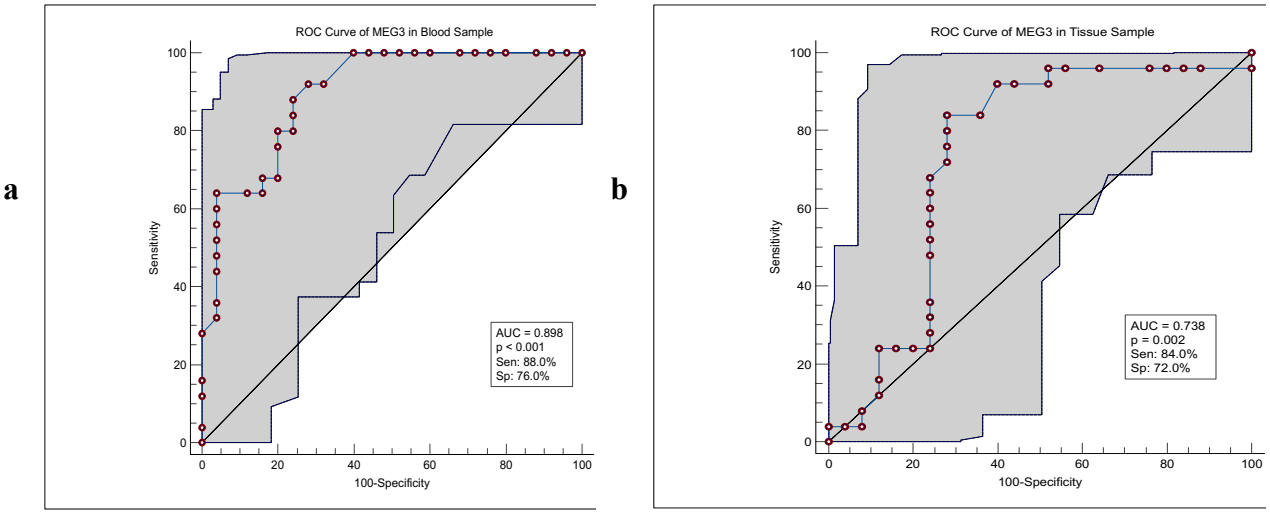


Figure 1. qRT-PCR output of molecular parameters. a. displays the amplification curves of *MEG3* (y-axis: Relative Fluorescence Units (RFU); x-axis: Cycle number). b. shows the corresponding melt curves (y-axis: -d/dT (Fluorescence); x-axis: Temperature (°C). The figure also illustrates specific qRT-PCR amplification of *miR-424-3p* and *miR-424-5p* (y-axis: Relative Fluorescence Units (RFU); x-axis: Cycle number) and melt curves (y-axis: -d/dT (Fluorescence); x-axis: Temperature (°C)) confirming reliable quantification of upregulated *miR-424-3p* (panels c, d) and *miR-424-5p* (panels e, f).



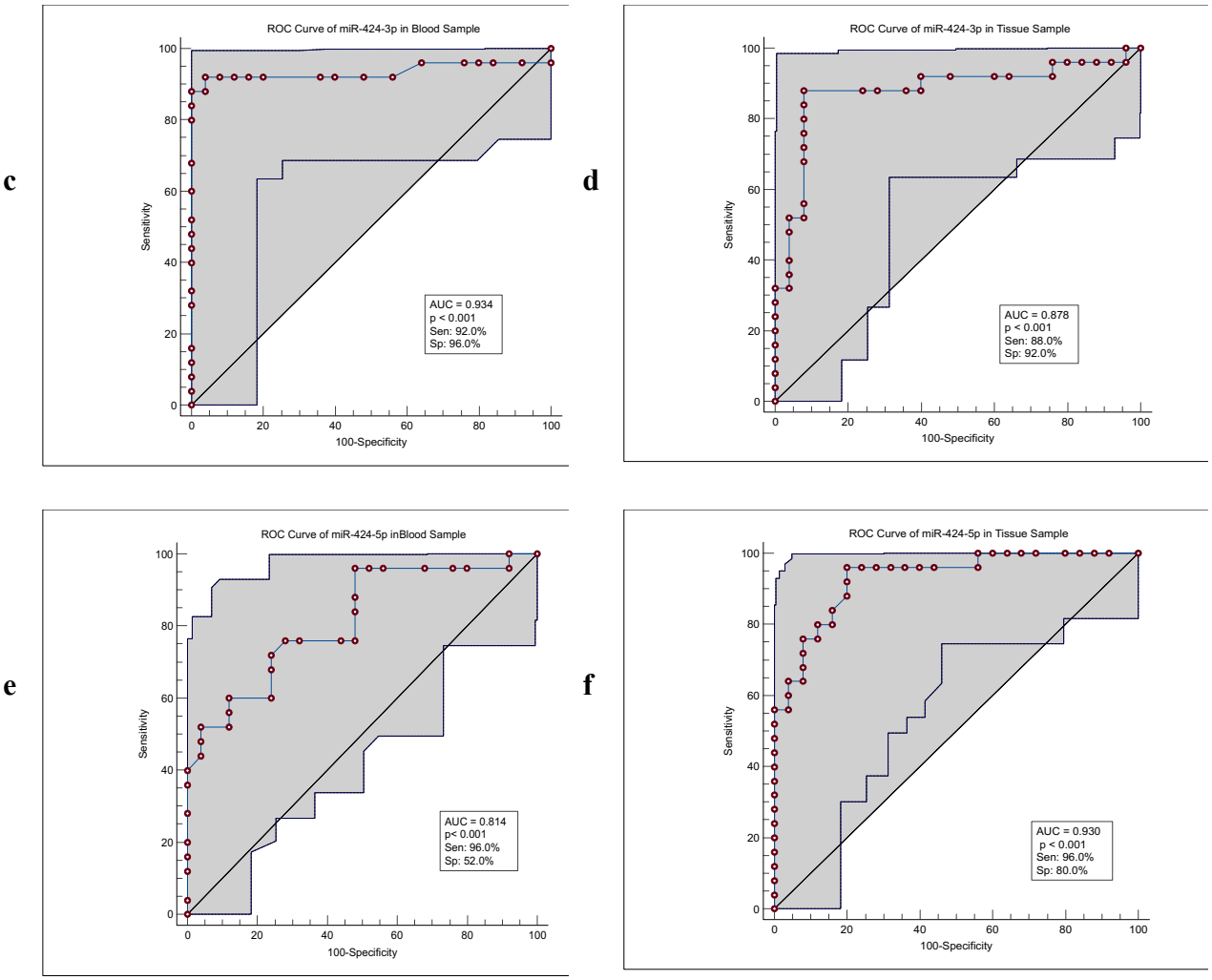


Figure 2. Receiver Operating Characteristic (ROC) curve analysis of *Maternally Expressed Gene 3* (*MEG3)* and *microRNA-424* (*miR-424)* isoforms for Colorectal Cancer (CRC) diagnosis. Individual ROC curves demonstrate diagnostic performance across blood (B) and tissue (T) samples: (A) MEG3-Blood, (B) MEG3-Tissue, (C) miR-424-3p-Blood, (D) miR-424-3p-Tissue, (E) miR-424-5p-Blood, and (F) miR-424-5p-Tissue, with Area Under the Curve (AUC) values ranging from 0.738 to 0.934.

Contributions: Khalid R. Majeed, conceptualization, methodology, writing (review and editing); Esraa Hussein Shamsa, methodology, visualization, funding acquisition, writing (original draft); Nada Nizar Kadhum, methodology, data curation, writing—original draft; Safa Alshattawi, methodology, project administration, resources, writing (review and editing); Maryam Qasim Mohammed, conceptualization, methodology, statistical analysis, writing (review and editing). All authors reviewed and approved the final manuscript.

Conflict of interests: the authors declare no competing interests.

Ethics approval: the study proposal, including its description and consent form, was reviewed and approved by the College of Science Research Ethics Committee at Mustansiriyah University (Approval ID: BCSMU/1222/00036Z in January,1,2023), and all procedures were conducted in accordance with the ethical principles of the Declaration of Helsinki. Written informed consent was obtained from all participants.

Consent for publication: informed consent was obtained from all individual participants included in the study.

Consent to participate: written informed consent was obtained from all participants prior to enrollment.

Availability of data and materials: datasets are available from the corresponding author upon reasonable request.

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Online supplementary materials

Table 1. Relative expression levels of lncRNA MEG3 and miR-424 isoforms in blood and tissue samples across study groups.

Table 2. Diagnostic performance of *MEG3* and *miR-424* isoforms for CRC detection across blood and tissue samples.

Table 3. DeLong test comparisons of ROC AUC differences between MEG3 and miR-424 indicators.

Table 4. Univariate logistic regression analysis of study variables associated with CRC status.

Table 5. Spearman rank correlation coefficient among study parameters.