

## **Role of MicroRNA-663b as an Oncogene in Colorectal Carcinoma on top of familial adenomatous polyposis cells.**

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### **Abstract**

**Background:** Familial polyposis coli (FPC) is almost always benign lesions presenting by bleeding or discovered accidentally during routine colonoscopy. However, malignant transformation could not be excluded and this necessitates early detection in the metaplasia stage.

**Aim:** Evaluation of the ability of estimated tissue expression levels of microRNA-663b (MiR-663b) to distinguish benign from malignant rectal polyps in patients with FPC.

**Subjects & Methods:** Forty-one patients with FPC were assessed clinically, had colonoscopic biopsies, and provided blood samples for estimation of serum carcinoembryonic antigen (CEA) and cancer antigen 19-9 estimate (CA19-9). Tissue samples were separated into two portions, for pathological analysis and PCR analysis to determine the degree of MiR-663b expression in the tissue. Subjects undergoing colonoscopy for other reasons were subjected to a rectal biopsy.

**Results:** The serum levels of CA 19-9 were considerably greater in MRP patients compared to healthy controls. The estimated tissue level of MiR-663b in three benign specimens was greater than the 4th quartile level of control specimens and in the range between the 1st and 2nd quartile levels of malignant specimens; these specimens were thus classified pre-malignant. A multivariate Regression analysis identified elevated miRNA366b gene expression as an independent risk factor for BRP malignant transformation.

**Conclusion:** MiR-663b tissue expression estimation might be utilized to distinguish benign from malignant colorectal polyps. Whenever MiR-663b levels were greater than control levels but below than the levels diagnostic of malignancy, are able to predict the early malignant transformation of benign polyps prior to their being identified by pathology.

**Keywords:** Familial polyposis coli, Malignant transformation, MicroRNA 663b, CA19-9, CEA.

## **Introduction**

Colorectal cancer (CRC) is the most prevalent gastrointestinal malignancy that primarily affects elderly men <sup>(1)</sup>. Ten to twenty percent of CRC patients have a positive family history <sup>(1)</sup>, and around five to seven percent of CRC patients have a well-defined hereditary CRC condition, either non-polyposis or polyposis syndrome <sup>(3)</sup>.

Familial polyposis coli (FPC) is an autosomal dominant polyposis disease caused by a mutation in the adenomatous polyposis gene, a tumor suppressor gene on the long arm of chromosome 5 in band q21 (5q21) <sup>(4)</sup>. FPC often develops in early adolescence, with a 100% lifetime risk of CRC by age 40 if left untreated <sup>(5)</sup>. Twenty five to thirty percent of FPC patients developed "de novo" CRC without clinical or genetic evidence of FAP in family members <sup>(5)</sup>.

Colonoscopy is the preferred diagnostic procedure for FPC to gather specimens for histological examination <sup>(7)</sup>. Endoscopic en bloc resection of malignant polyps is possible <sup>(8)</sup>, but surgery is the cornerstone of curative therapy <sup>(9)</sup>. Despite advancements in diagnostic and treatment techniques for FPC, the problem remains and is reflected by two questions: which polyp will become malignant?<sup>(10)</sup>.

MicroRNAs (miR) are non-coding RNAs consisting of around 22 nucleotides that regulate gene expression by interacting with target mRNAs to inhibit expression <sup>(11)</sup>. Many human illnesses are associated with aberrant expression of microRNAs; prior research identified miR-103a-3p, 143-5p, and 215 as prognostic indicators for colon cancer patients <sup>(12)</sup>. Another study identified five miRNAs that may have prognostic significance for colon cancer <sup>(13)</sup>. Following this, third research advised the use of microRNAs from the 122 and 200 families as predictive biomarkers for colon cancer that has been treated <sup>(14)</sup>.

## **Objectives:**

This study's objectives were to examine the usefulness of tissue expression levels of miRNA-663b as a technique for distinguishing biopsied polyps with malignant alterations from benign polyps.

## **Design:**

Prospective case-control comparative double-blinded study

## **Setting**

Department of General Surgery in conjunction with the Molecular Biology & Biotechnology Unit, Faculty of Medicine, Benha University

## **Subjects & Methods**

After obtaining approval of the study protocol by the Local Ethical Committee (No: MS 33-6-2021), the study started at on august 2021, all patients presented to the outpatient clinic of General Surgery received a comprehensive clinical examination and radiologic workup after the collection of demographic information and a thorough history about the current disease, family history of comparable disorders, and a

comprehensive medical history. Then, all patients underwent exhaustive laboratory tests (at the hospital lab).

### **Exclusion criteria**

Exclusion criteria include the existence of coagulopathies, liver, renal, or heart illnesses, severe inflammation or tight narrowing of the anal margin.

### **Inclusion criteria**

Patients who were previously diagnosed to have FAP, met the exclusion criteria, and agreed to participate in the study- were enrolled. As a control group, the study comprised ten volunteers who met all inclusion and exclusion criteria, were scheduled for colonoscopy for reasons other than bleeding per rectum and consented to snip-biopsy of the colorectal area.

## **Methods**

### **Colonoscopy and biopsy taking:**

Under a mild intravenous anesthesia, colonoscopy and biopsies were performed on all patients. Biopsy includes most respectable polyps, particularly questionable polyps. Each obtained biopsy was divided into two parts, the first of which was preserved in formalin solution (10%) and sent to the Pathology Department of the Benha Faculty of Medicine for tissue examination using paraffin sections; the results of tissue examination served as the gold standard for comparisons according to the feedback report. The second portion of the collected tissue sample was directly transferred to the Molecular Biology and Biotechnology Unit at Medical Biochemistry department to be stored at -80oC for determination of the tissue miRNA-663b expression levels.

### **Estimation of tissue expression levels of miR-663b**

Using the MiRNeasy Mini Kit (Qiagen, Germany), miR-663b was extracted from homogenized tissue samples according to the manufacturer's instructions. The RNA extracts was stored at -20 for further processing. Two-step real-time PCR with Maxima SYBR Green was used to determine the relative abundance of miR-663b.

1st step: complementary deoxyribonucleic acid (cDNA) was generated using the miScript II RT Kit (Qiagen, Hilden, Germany). Each tube containing the reverse-transcription master mix was transferred to a G-storm thermocycler (Gene Technologies Ltd, United Kingdom) for 60 minutes at 37°C, followed by 5 minutes at 95°C to inactivate the miScript reverse Transcriptase Mix. 2nd step: cDNA was used as a template for real-time PCR using SuperReal PreMix Plus (SYBR Green) (Tiangen, Shanghai). The second phase included the ABI StepOne real-time PCR equipment (Applied Biosystem, Waltham, MA). The employed primers were detailed in Table 1. This is how the real-time cycler was programmed: 40 cycles of initial activation at 95°C for 15 minutes, denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 70°C for 30 seconds were performed<sup>(15)</sup>.

### **Data analysis**

Mir-663b expression was normalized against the housekeeping gene GAPDH. The fold changes were calculated according to the equation of  $2^{-\Delta\Delta ct}$  (Table 1).<sup>(15)</sup>

**Table (1): List of the used primers in the current study:**

Primers' name	Sequence (5'-3')
miR-663bF	CATAATAAATAGGCGGGGCG
miR-663bR	CAGAGCAGGGTCCGAGGTA
GAPDH F	CCACCCATGGCAAATTCATGGCA
GAPDH R	TCTAGACGGCAGGTCAGGTCCAC

## Grouping

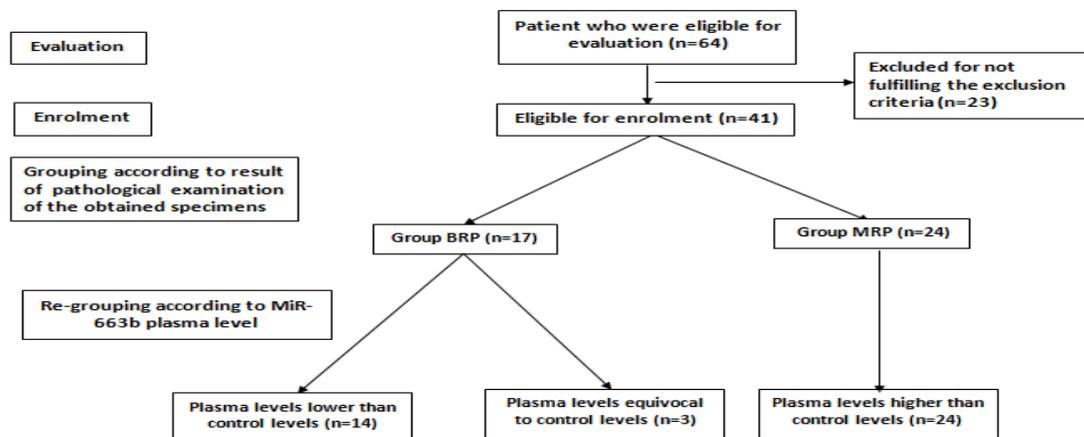
Patients were grouped according to the result of histopathological examination of the excised polyps into a benign rectal polyp (BRP) and malignant rectal polyps (MRP).

## Statistical analysis

One-way analysis of variance (ANOVA) was applied to the obtained data to determine the statistical significance of the difference between the means of more than two groups. Using the Chi-Square test, the connection between two qualitative variables was analyzed. Spearman's correlation analysis was utilized to evaluate the strength of link between two quantitative variables. Using generalized linear models, regression analysis was utilized to predict risk factors when the dependent variable was categorical. A p-value is deemed significant if it is less than 0.05 at a 95% confidence range. Statistics analyses were conducted using the social science statistical package (IBM SPSS Statistics for Windows, Version 25.0, 2017, Armonk, NY: IBM Corp.). A p-value is deemed significant if it is less than 0.05 at a 95% confidence range.

## Results:

During the study period of August 2021 through January 2022, 64 patients presenting with per-rectal bleeding were evaluated; 41 patients were included in the study, while 23 patients were eliminated for not meeting the inclusion criteria (Fig. 1). Histopathological investigation identified 17 BRP samples and 24 MRP samples. At the time of enrollment, demographic, clinical, and laboratory data revealed non-significant differences across groups and in contrast to the control group (Table 2).



**Figure 1: Consort Flow sheet**

**Table 2: Enrolment data of studied patients and controls**

Variables	Group	Control (n=9)	BRP (n=17)	MRP (n=24)	Significance of difference between		
					Control & BRP	Control & MRP	BRP & MRP
Age (year)		40.3±18.9	45.2±17.5	41.3±10.4	0.766	0.976	0.593
Gender; M:F		4:5	9:8	14:10	0.292	0.140	0.121
Smokers (%)		1 (11.1%)	2 (15.4%)	5 (17.9%)	0.774	0.216	0.269
BMI (kg/m <sup>2</sup> )		31.2±2.4	29.8±3.1	28.7±4.5	0.482	0.059	0.647
Hemoglobin (g/dl)		13.1±1.16	11.3±1.3	10.2±2.1	0.003	0.004	0.299
WBC (X10 <sup>9</sup> /L)		5.7±1	8.1±2.5	7.6±1.7	0.569	0.356	0.109
Platelets (×10 <sup>9</sup> /L)		247±51.5	246.5±59	241±61.6	0.687	0.199	0.187
Serum creatinine (mg/dl)		0.77±0.18	0.88±0.25	0.88±0.27	0.139	0.145	0.809
Serum albumin (g/dl)		4.3±0.3	4±0.55	4.2±0.6	0.059	0.765	0.068
Serum bilirubin (mg/dl)		0.67±0.15	0.62±0.14	0.69±0.18	0.236	0.519	0.051
Serum AST (U/L)		21.8±5.8	25.6±6.1	25±4.9	0.055	0.062	0.819
Serum ALT (U/L)		19±5.2	23.1±7.8	22.8±4.5	0.102	0.098	0.124
Serum ALKP (U/L)		9.3±1.4	12.7±8.6	14.9±8.5	0.084	0.072	0.069

Data are presented mean, standard deviation, ratios, numbers, and percentages; BRP: Benign rectal polypi; MRP: Malignant rectal polypi; BMI: Body mass index; WBC: white blood cell count; AST: Aspartate transaminase; ALT: Alanine transaminase; ALKP: Alkaline phosphatase; P<0.05 indicates significant difference

Estimated blood levels of CEA were significantly (P<0.001) higher in samples of MRP patients compared to control samples and to samples of BRP patients (P=0.046), with significantly (P=0.004) higher levels in samples of BRP patients than control samples. However, the estimated serum CA 19-9 levels were significantly (P=0.028) higher in samples of MRP patients than controls, while serum CA19-9 levels estimated in samples of BRP patients were non-significantly higher than in control samples and non-significantly lower than in samples of MRP patients. Plasma levels of MiR-663b gene expression were significantly (P<0.001) higher in specimens of MRP patients compared to specimens of BRP patients and control specimens, with significantly (P=0.035) higher expression levels in specimens of BRP patients than control specimens (Table 3).

Intriguingly, the estimated tissue level of MiR-663b in three BRP-diagnosed specimens (3.258, 3.451, and 3.356) was higher than the 4th quartile level of control specimens (1.698), and was in the range between the 1<sup>st</sup> (3.222) and 2<sup>nd</sup> (5.097) quartile levels of malignant specimens, indicating that these specimens were pre-malignant.

**Table 3: Estimated levels of the studied biomarkers in samples of the studied patients and controls**

Variables	Group	Control (n=9)	BRP (n=17)	MRP (n=24)	Significance of difference between		
					Control & BRP	Control & MRP	BRP & MRP
Serum CEA (ng/ml)		0.71±0.29	5.53±3.6	8.1±4.6	<0.001	0.004	0.046
Serum CA 19-9 (U/ml)		13.56±4.3	16.9±5.2	19.6±7.6	0.480	0.028	0.084
Tissue MiR-663b expression level		1.281±0.2	1.076±1.1	4.73±0.53	0.035	<0.001	<0.001

Data are presented as mean, standard deviation, ratios, numbers, and percentages; BRP: Benign rectal polypi; MRP: Malignant rectal polyps; CEA: Carcinoembryonic antigen; CA: Cancer antigen; P value <0.05 indicates a significant difference

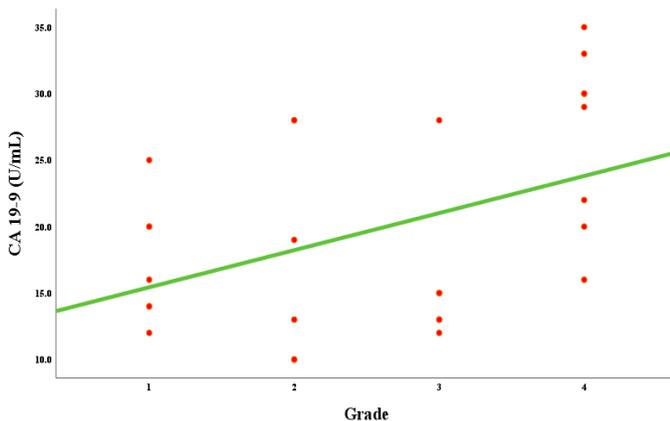
All patients who had MRP underwent surgical resection and macroscopic evaluation showed a median tumor size of 5; range: 1-12 cm. Pathological grading of excised tumors defined 7 specimens of grade I, 6 specimens of grade II, 7 specimens of grade III, and 8 specimens of grade IV. Estimated serum CEA levels showed non-significant differences between samples of patients who had MRP that were categorized according to malignancy grade, while estimated levels of serum CA 19-9 and plasma levels of MiR-663b showed significant differences (Table 4).

**Table 4: Estimated levels of the studied biomarkers in samples of patients who had MRP categorized according to tumor grade**

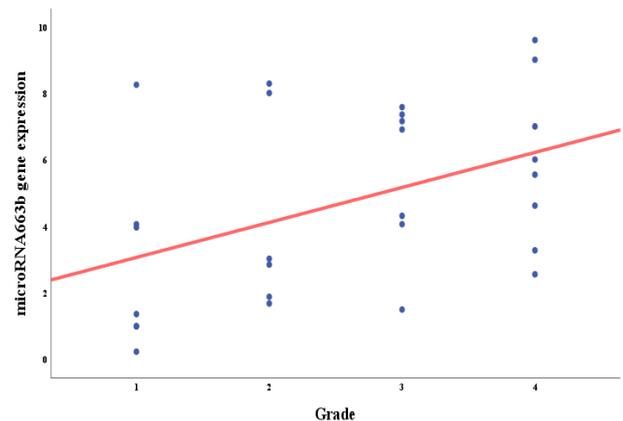
	Grade I	Grade II	Grade III	Grade IV	P value
Serum CEA (ng/ml)	3.6 (2.5-18.2)	5 (1.5-10)	9 (3.2-27.2)	9 (3-18.2)	0.549
Serum CA 19-9 (U/ml)	16 (12-25)	16 (10-28)	15 (12-28)	29 (16-35)	0.013
Plasma MiR-663b expression level	1.4 (0.23-8.3)	2.9 (1.7-8.3)	6.9 (1.5-7.6)	5.8 (2.6-9.6)	0.023

Data are presented in median and interquartile range; CEA: Carcinoembryonic antigen; CA: Cancer antigen; MiR: MicroRNA; P value <0.05 indicates a significant difference

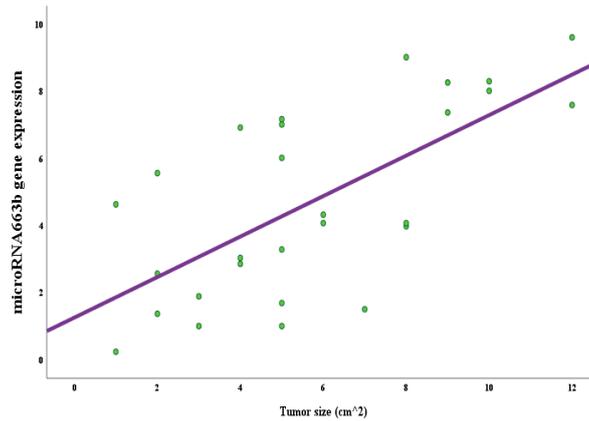
Spearman's correlation analysis showed a positive significant correlation between tumor grade and serum CA19-9 ( $r_s=0.430$ ,  $p=0.022$ ) as shown in figure 2a and plasma level of MiR-663b ( $r_s=0.437$ ,  $p=0.020$ ) as shown in figure 2b, while the correlation with serum CEA was non-significant ( $r_s=0.289$ ,  $p=0.136$ ). Concerning tumor size, the correlation was highly significant with plasma level of MiR-663b ( $r_s=0.675$ ,  $p<0.001$ ) as shown in figure 2c, while was non-significant with CEA ( $r_s=0.039$ ,  $p=0.842$ ) and CA19-9 ( $r_s=0.159$ ,  $p=0.429$ ).



**Fig. (2a): correlation between serum CA 19-9 and tumor grade**



**Fig. (2b): correlation between plasma level of MiR-663b and tumor grade**



**Fig. (2c): correlation between the tissue expression level of MiR-663b and tumor grade**

Regression analysis was conducted for the prediction of malignancy on top of BRP- defined higher serum CEA and CA19-9 levels, and miRNA366b tissue gene expression level as the predictors for the risk of malignancy on top of BRP. However, in multivariable analysis, a higher miRNA366b gene expression level was considered an independent risk predictor of malignant transformation of BRP (Table 5).

**Table (5): Regression analysis for prediction of malignancy on top of BRP**

Covariates	Univariate		Multivariate	
	p	OR (95% CI)	p	OR (95% CI)
Age	0.704	0.995(0.972-1.019)		
Gender	0.184	1.538 (0.943-2.509)		
Smoking	0.128	1.963(0.824-4.676)		
CEA	0.008	1.149(1.044-1.264)	0.053	1.324(0.996-1.759)
CA 19-9	0.015	1.074(1.014-1.137)	0.072	1.231(0.982-1.544)
Alkaline phosphatase	0.201	1.069(0.727-1.112)		
MiR663b tissue level	0.004	1.317(1.603-2.585)	0.029	2.577(1.412-6.255)

CEA: Carcinoembryonic antigen; CA: Cancer antigen; MiR: MicroRNA; OR: odds ratio; CI: confidence interval.

## Discussion

In the current investigation, tissue levels of MiR-663b gene expression were shown to be dysregulated in individuals with colorectal polyposis illness compared to levels calculated for healthy colorectal tissues. This data suggests a potential function for microRNA in the etiology of colorectal polyps and is consistent with earlier research indicating such a connection<sup>(16-19)</sup>.

In comparison to control tissues, the expression levels of MiR-663b were much lower in benign polyp tissue and significantly greater in malignant polyp tissue, as determined by histological inspection. These results suggested a possible role for increased MiR-663b in the development of colorectal polyp-associated cancer. In accordance with these findings, prior research observed a significant increase in the expression levels of miR-663b in CRC cells and showed that the overexpressed miR-663b increased CRC cell proliferation, migration, and invasion, and prevented apoptosis<sup>(20)</sup>. Another study found considerable overexpression of miR-663b in tissue samples and cell lines from colorectal cancers and concluded that miR-663b plays a

crucial role in the development of CRC <sup>(21)</sup>. Subsequently, one investigation demonstrated the connection between several clinicopathological characteristics of CRC and MiR-663b expression levels <sup>(22)</sup>.

Intriguingly, the estimated tissue level of MiR-663b in three cases of benign polyps was greater than the fourth quartile of the control samples, but between the first and second quartiles of the malignant samples. This discovery sheds information on the potential initiation of malignant transformation that histopathology either overlooked or was unable to identify. In addition, our study and proposal revealed that the tissue expression level of MiR-663b may be used to detect cases of early transformation prior to clinical or pathological manifestation. In support of this hypothesis, statistical studies identified the higher performance of estimated MiR-663b as a diagnostic test for differentiating benign and malignant polyps compared to tumor markers hence, MiR-663b might be regarded as a diagnostic and prognostic biomarker.

In accordance with these findings, prior research demonstrated that miR-663b might serve as a diagnostic biomarker for CRC <sup>(20-22)</sup>. Furthermore, another study discovered that the expression levels of miR-663b in tissues of primary or metastatic CRC are extremely equivalent and concluded that the expression levels of MiR-663b had comparable predictive qualities for the prognosis or treatment response in patients with advanced CRC <sup>(23)</sup>.

In a trial to investigate the role of miR-663b in the malignant transformation of benign to malignant polyps, a preliminary study revealed that miR-663b targets the CCND2 gene, which codes for cyclin D2, resulting in overexpression of this cyclin. Cyclin D2 forms a complex with CDK kinase to regulate the cell cycle G1/S transition and promote cell proliferation <sup>(24)</sup>. Recent research indicates that overexpression of miR-663b can promote CRC cell proliferation, migration, and invasion via its regulation of the Wnt/-catenin pathway by suppressing the adenomatous polyposis coli 2 <sup>(20)</sup>- which is a direct target of miR-663b- and its suppression results in uncontrolled proliferation of intestinal epithelial cells and is linked to the earliest stages of colorectal carcinogenesis <sup>(25)</sup>. Another study attributed the tumorigenic activity of upregulated miR-663b expression to activation of the Ras/Raf signaling activity <sup>(21)</sup>, with a subsequent increase in expression of YAP1, which is translocated to the nucleus and interacts with transcription factors to induce gene expression <sup>(26)</sup>. Additionally, upregulated miR-663b activates the CD44, which promotes carcinogenesis through the Wnt/-catenin signaling pathway in tumors <sup>(27)</sup>.

## **Conclusion**

It may be possible to distinguish benign from malignant colorectal polyps based on the tissue expression level of MiR-663b. Expression levels of MiR-663b, if it was greater than control levels but below than levels diagnostic of malignancy, may be able to predict the early malignant transformation of benign polyps prior to histological diagnosis.

## Limitation

To evaluate the feasibility of using plasma levels as a non-invasive diagnostic modality, the plasma expression level of MiR-663b was to be determined and compared to the tissue expression levels

## Recommendations

Larger-scale studies are necessary to define distinguishing cutoff points for cases susceptible to develop malignancy.

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