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MicroRNA-34a in Colorectal Cancer: Linking Epigenetic Regulation to Tumor Suppression

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ABSTRACT: Colorectal cancer (CRC) persists as a lethal malignancy and ranks as the third foremost cause of cancer-related mortality. The study aimed to evaluate the expression levels of the *miRNA-34a* gene were performed and aby the qRT-PCR to estimate the expression of the target gene and determine levels of Hb, PLT, WBC, and RBC in CRC patient and control. In present study blood samples collected from 60 subjects (20 control and 40 patients with CRC), (females and males) with different aged. The collection of samples and the practical work for this study from January 2023 to November 2023, Concerning The mean value of CBC parameters, the analysis showed that there was statistical difference in Hb (11.84 ± 2.21) and PLT (297.26 ± 127.06) biomarkers in the patient group with P value ≤ 0.05. However, the WBC and RBC showed no significant difference in patient with CRC compared to control group with mean value (7.52 ± 2.69, 4.54 ± 0.71) respectively. Endogenous control gene *U6* miRNA levels were amplified and utilized to normalize the *miRNA-34a* gene levels. U6 showed no-significant variation between patient and control samples accordingly used as housekeeping gene for *miRNA-34a* Which did not show any noticeable change between patient and control group in blood sample. Can conclude from that miRNA-34a could not use as marker for CRC in blood and might be used as biomarker in tissue because it more specific to the regulation role for miRNAs.

Keywords: Colorectal cancer, gene expression, *miRNA-34a*

1. INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer-related mortality globally and the third most prevalent cancer (28). It is a highly heterogeneous illness characterized by tumor formation influenced by both environmental and genetic risk factors, encompassing epigenetic and genetic alterations (1, 2). Fundamental genetic variations among cancers can determine the disease's behavior and prognosis. Due to the constraints of cytotoxic chemotherapy, research has concentrated on creating targeted therapies informed by molecular subtyping (26, 27, 29). In the past twenty years, an increasing array of targeted medicines has proven helpful in inducing tumor regression and enhancing survival rates (3, 4). MicroRNAs, also known as miRNAs, are short noncoding RNA molecules that contain roughly 21-24 nucleotides. They are acknowledged as one of the more important regulatory gene families in eukaryotic organisms. Research has made significant strides in the direction of understanding the intricate roles that microRNAs

play in the control of epigenetics. Because they are epigenetic modulators, microRNAs have the ability to influence the protein levels of target mRNAs without altering the sequences of the genes. The regulation of microRNAs can also be accomplished through epigenetic alterations, such as DNA methylation, RNA modification, and histone modification (5, 25).

Through the selective targeting of mRNAs for degradation or translational suppression, microRNAs are able to influence gene expression. This is accomplished by targeting the 3' untranslated region of mRNAs. As a result, microRNAs can operate as a controller of protein synthesis by effectively diagnosing sequence elements in mRNAs (5). Nevertheless, growing processes has shown that nuclear miRNAs associated with promoters or enhancers gene and can directly intermediate the transcriptional gene. which employ a very important effect on cancer progression by influencing to growth, migration and invasion of tumors (6). The microRNA-34 (miRNA-34) family, a subset of microRNA types, is dysregulated in numerous human malignancies and constitutes a significant group of tumor suppressor genes among miRNAs (7). This type of miRNAs family consists of three members, known as (miRNA-34a, miRNA-34b and miRNA-34c.) All miRNA-34 types are encoded by 2 dissimilar transcriptional units. miRNA-34a is situated at (chromosome 1p36.22) and has an exceptional transcript, whereas miRNA-34 type b and c situated at chromosome 11q23.1 and possess 1 transcript (8).

MicroRNA-34a has tumor suppressor characters, encourage in cell arrest and apoptosis process with aging function. miRNA-34a has frequently drained abundant consideration owing to its effect of suppressor the tumors. Evidence suggests that miRNA-34a directly targets the 3' UTRs of many oncogenic mRNAs, such as Bcl-2, SIRT1, Fra-1, c-Met, Notch1, Notch2, and PLK1, potentially elucidating its tumor-suppressive characteristics (9). Recently, demonstrate that the miRNA-34a expression not at all unique for estimation as a prognostic and diagnostic biomarker, nonetheless, can impede the growth and metastasis in several tumors (10). With expanding awareness of the probable role of miRNA-34a in the therapy pf many types of cancer (11, 12).

2. MATERIALS AND METHODS

2.1 Subject

The present study was conducted on a total of 60 subjects 20 controls and 40 patients with CRC (females and males) of different ages. Samples were collected from January 2023 to November 2023 from Gastroenterology and liver diseases teaching Hospital /Medical City/ Baghdad and Baghdad teaching hospital/medical city/Baghdad.

2.2 Blood Sample Collection

Five ml of venous blood was collected using a sterile syringe. Divide this blood into 3 ml place it in a gel tube and leave it for 20 minutes to clot at room temperature (25-30°C). The tubes were then centrifuged at 3000 RPM over 15 minutes to separate the serum, and the serum was then stored in Eppendorf tubes at −20°C until used for another chemical assay. The remaining 2 ml used for CBC counts and 250µl placed in Eppendorf tube that contained 750µl of Trizol and kept frozen at −20°C for molecular analysis.

2.3 Ethical approval

The study was performed following the acquisition of both verbal and written consent from the patients before collecting the samples, this case-control study was approved by the Biology Department, College of Science, Mustansiriyah University.

2.4 Measurement of WBC, RBC, Hb, and PLH

The levels of Hb, PLT, WBC, and RBC in blood were determine using a Sysmex XT 2000i analyzer (Kobe, Japan) (13).

2.5 RNA extraction

The RNA was extracted from blood of CRC patients as well as from apparently healthy groups (control) by using TransZol Up Plus RNA Kit (blood). company of kit (Transgen) Then Total RNA was extracted successfully from all samples for patients and controls. The 2000c Nanodrop spectrophotometer (Thermo Fisher Scientific, USA) was used to evaluate the concentration and purity of extracted RNA in order to determine the quality of samples for subsequent analysis in qRT-PCR. The samples ranged in RNA concentration from samples was (62-122) ng/µl, while the absorbance of the samples was measured at two distinct wavelengths to determine RNA purity (260 and 280nm). The presence of an A260/A280 ratio of around 2.0 suggested that the RNA sample was pure.

2.6 Quantitative Real Time PCR (qRT–PCR)

The Complementary DNA reverse transcription was conducted on the same day of RNA extraction. A common primer reaction was applied since it was needed to have cDNA for both the gene in the study and housekeeping gene.

the primers were synthesized and lyophilized by Alpha DNA Ltd. (Canada). **Table 1** provides all of the primer sequences that were utilized in the testing for the *U6* and *miRNA-34a* genes that were conducted as part of this inquiry.

Using the quantitative real-time polymerase chain reaction (qRT-PCR) SYBR Green test, the levels of expression of the *miRNA-34a* gene were assessed in order to quantify the expression of the target gene. This was done in order to understand the relationship between the two. Amplification of the levels of the endogenous control gene U6 miRNA was carried out, and the results were utilized in order to normalize the levels of the *miRNA-34a* gene.

Table (1): Primer used in present study

Primers	**Sequence [5′→3′ direction]**	**primer size bp**	**Product size bp**	**Ta** °C
	MiRNA- 34a			
Forward	TGGCAGTGTCTTAGCTGGTTGT	22	70	62
	MiRNA- U6			
Forward	CTCGCTTCGGCAGCACATATA	21	69	56
miRNA-universal P.	CAGGTCCAGTTTTTTTTTTTTTTVN			
miRNA-universe R.P.	GCGAGCACAGAATTAATACGAC			

2.7 Expression levels fold changes of fold for *miRNA-34a* and *U6* genes were calculated based on the threshold cycle (Ct) readings through constituent of "TransStart® Top Green qPCR Super Mix kit". Each reaction was performed in duplicate. Volume of all required parts according to table (2) was calculated.

Table (2): The qRT-PCR elements were handled in the experiments for *U6* and *miRNA-34a* gene expression.

The Components	**(20 µl rxn)**
"2xTransStart® Top Green qPCR Super Mix"	(10)
Nuclease free water	(4)
Forward Primer (10 µM)	(1)
Reverse Primer (10 µM)	(1)
cDNA	(4)

Conferring to the thermal profile, the cycling protocol was programmed for the subsequent optimized cycles, as given in Table (3) and figure (1) for *miRNA-34a* as well table (4) and figure (2) showed the optimized cycles and the thermal profile of *U6* gene consequently.

Table (3): The thermal profile of *miRNA-34a* gene expressions.

The Steps	**Temperatures (°C)**	**Times (sec.)**		**Cycles**
Enzyme activation	(94)	(30)		1
Denaturation	(94)	(5)		35
Annealing	(62)	(15)		
Extension	(72)	(20)		
Dissociation	55 °C-95 °C			1

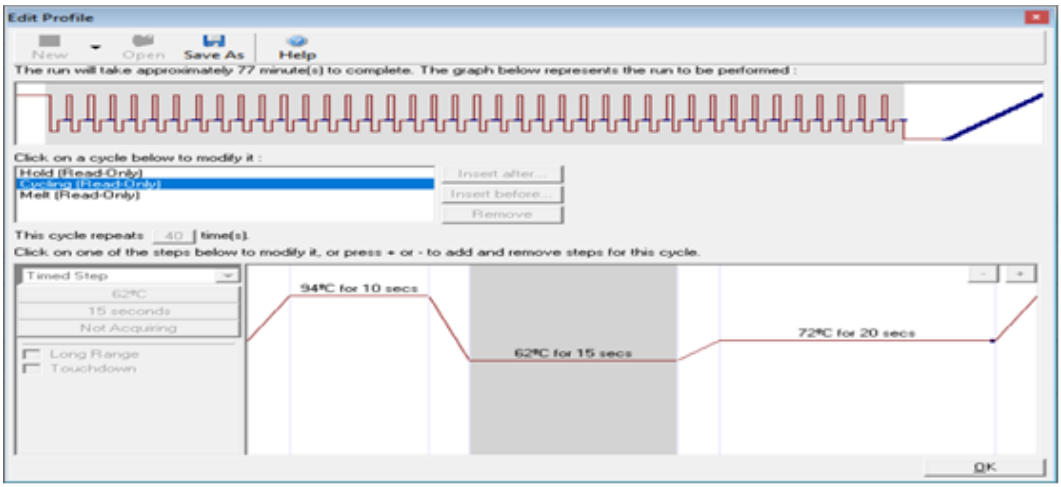

Figure (1): The thermal profile of *miRNA-34a* and gene expression. The image was taken directly from the qRT-PCR machine.

Table (4): The thermal profile of *U6* gene expressions.

The Steps	Temperatures (°C)	Times (sec.)	The Cycles
Enzyme activation	(94)	(30)	1
Denaturation	(94)	(5)	35
Annealing	(56)	(15)	
Extension	(72)	(20)	
Dissociation	55 °C-95 °C		1

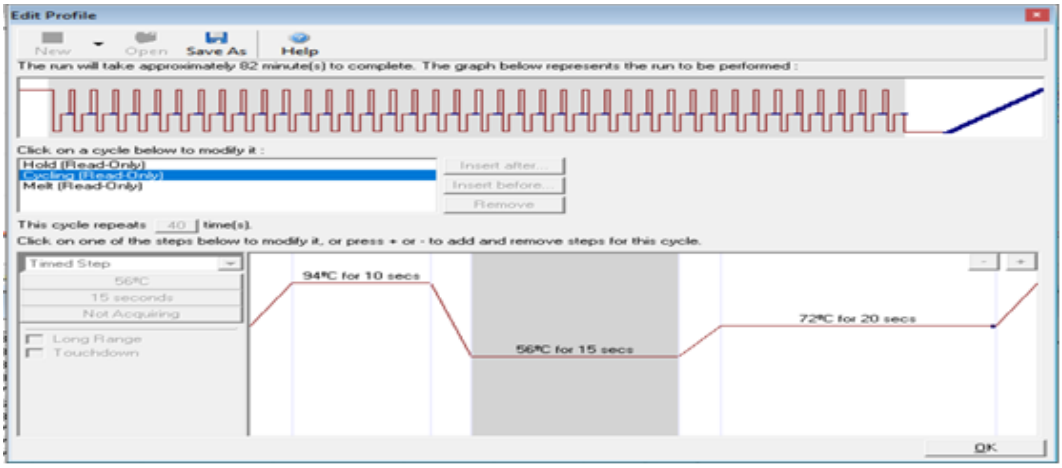

Figure (2): The thermal profile of *U6 and* gene expression. The image was taken directly from the qPCR machine.

2.8 Statistical analysis

Results were analyzed related to the objectives and presented according to the general description of the sample. Microsoft Excel 2010 and SPSS (version 25) software was used for statistics analysis. Microsoft package (Excel and Word). The data are expressed as mean ± SD, Differences were considered significant when p values was $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Complete blood count distribution that related with CRC

Concerning The mean value of CBC parameters, the analysis showed that there was statistical difference in Hb **(**11.84 ± 2.21**)** and PLT **(**297.26 ± 127.06**)** biomarkers in the patient group with P value ≤ 0.05. However, the WBC and RBC showed no significant difference in patients with CRC compared to control group with mean value (7.52 ± 2.69, 4.54 ± 0.71) respectively. As illustrated in Table (5).

Table (5): Comparison between patients and control groups in WBC, RBC, Hb and PLT.

Groups		WBC	RBC	Hb	PLT
Patients	**Mean**	7.52	4.54	11.84	297.26
	Std. Deviation	2.69	0.71	2.21	127.06
	Std. Error of Mean	0.43	0.11	0.35	20.34
Control	**Mean**	7.97	4.53	13.21	206.18
	Std. Deviation	1.74	0.53	1.04	62.04
	Std. Error of Mean	0.52	0.16	0.31	18.7
p-value		0.6	0.9	**0.05***	**0.02***

Numerous studies have highlighted the significant involvement of platelets in cellular carcinogenesis, identifying them as crucial regulators in thrombosis and inflammation. Among the current findings, Heightened platelet counts is agreement with Dudiki *et al.*, 2023(14) and ; Zhu *et al*., 2023(15). Demonstrated that cancer cells activate thrombopoietin, which stimulates several interleukins to produce platelets. Platelet-derived thrombopoietin in the circulation promotes bone marrow macrophage proliferation, which boosts platelet production (16).

The reduced level of Hemoglobin (Hb) in the current study agrees with many recent study (17-19). Complete blood counts (CBCs) were obtained from patients diagnosed with colorectal cancer (CRC) from primary health clinics. The results of the study showed that there was a gradual change in the parameters of the CBCs in both groups at least fifteen months prior to the diagnosis of CRC. The CBC was shown to have decreased in a statistically meaningful manner in patients who had advanced-stage colorectal cancer (20). This is because the differences in CBC are primarily intended to be incorporated into prediction models in order to identify individuals who are at an increased risk of getting metastatic colorectal cancer, which will facilitate early diagnosis.

Moreover, Virdee *et al*., indicates that hemoglobin levels may fluctuate due to blood loss, such as in rectal bleeding associated with CRC, resulting in an increase in platelet levels to assist the body in building clots to halt the bleeding (18). Furthermore, report a statistically significant difference in mean white blood count (WBC) levels between individuals diagnosed with CRC and those without a diagnosis within a six-month period for both males and females (t-test, all $p < 0.05$), particularly in patients with inflammatory diseases associated with CRC. The findings from these studies on CBC count were inconsistent with those of **Virdee et al. (2019**), indicating that no significant adjustments have been implemented in general practice to utilize such data for the early detection of the disease(21).

3.2 Gene Expression of U6 by using Real time qPCR

The mean value of *U6***,** the housekeeping gene used in the present study for *miRNA34a* gene is shown in Table (6) and show plots amplification in figure (3). The range of mean value for U6 in blood samples of patient group was 15.95-18.96 with a mean ± SD **(17.74± 0.70)**, for the control group it ranged was from 16.61-18.64with a mean ± SD **(17.58±0.59)**. Non-significant difference was shown between patients and control groups samples.

Table (6): Mean and Std Deviation of *U6* expression blood samples

Groups		Mean	Std. Deviation	Range
Patients	***U6* in Blood**	17.74	0.70	15.95-18.96
Control		17.58	0.59	16.61-18.64
P-value		**0.3 NS**		

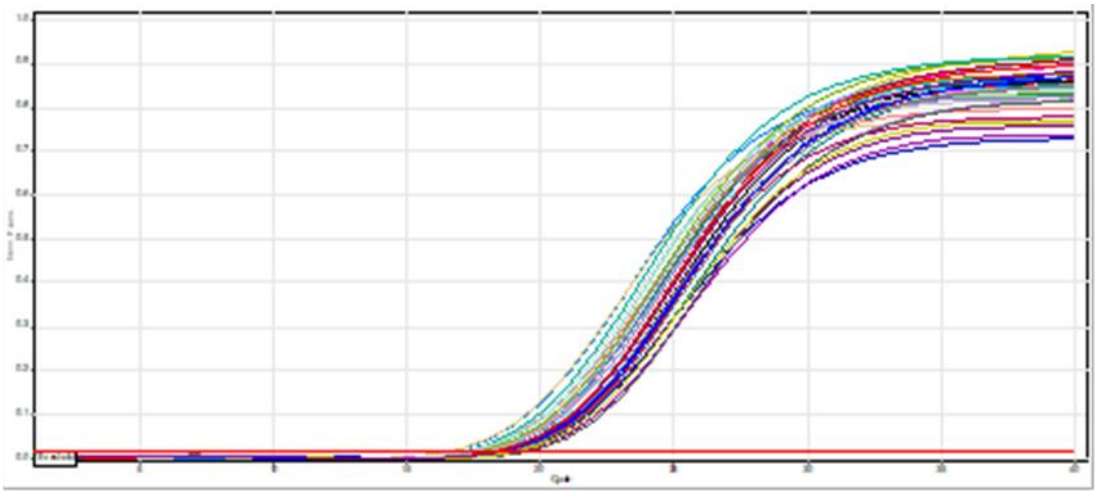

Figure (3): U6 gene amplification was represented by qRT-PCR samples encompassing all research groups with CT ranges (16-19). The pictures were obtained via the Qiagen Rotor Gene Q qPCR device.

3.3 Quantitative Expression of *miRNA-34a* genes by Q-PCR technique

Through compared the ΔCt of *miRNA*-34a (25.54) and the *U6* house keeping gene (17.74), The fold express of miRNA-34a in Blood sample is (1.01) in patient with CRC as compared with healthy which was (1) in tissue. So, the expression of *miRNA-34a* was not noticeable with fold (1.01) and the control folding number is (1). All aspects are showed in Table (7) and the plots of amplification showed in figure (4).

Table (7): Expression of miRNA-34a in blood samples

groups	*sample*	*Means Ct of miRNA-34a*	*Means Ct of U6*	*ΔCt*	$2^{-\Delta Ct}$	*experimental group/ Control group*	*Foldi ng*
Patients	**Blood**	25.54	17.74	7.80	0.00448	0.00448/0.0042 5	**1.01**
Control		25.42	17.54	7.87	0.00425	0.00425/0.0042 5	**1.00**
p-value		**0.2**					

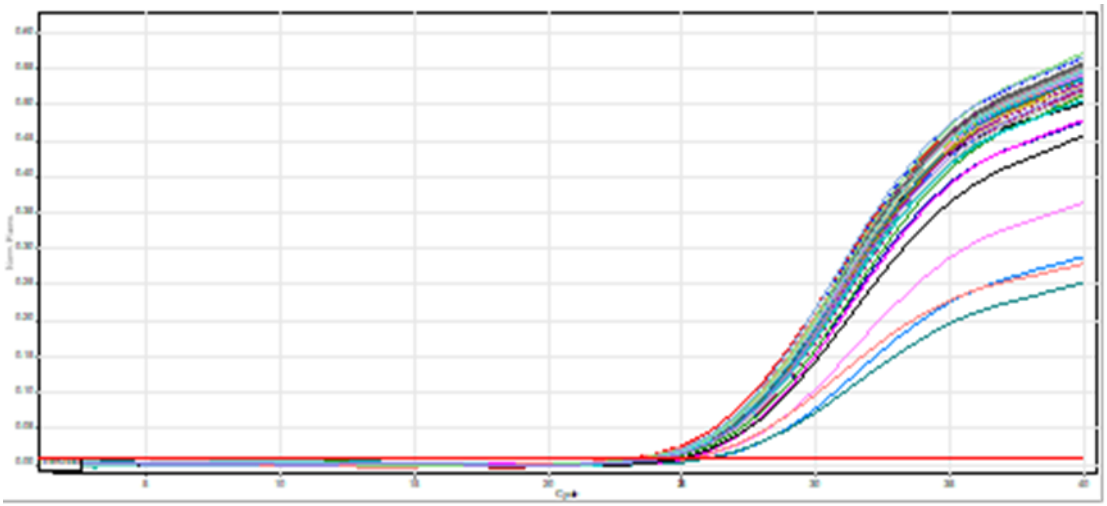

Figure (4): The amplification of the *miRNA-34a* gene was illustrated using qRT-PCR samples encompassing all research groups with CT ranges (24-27). The pictures were obtained via the Qiagen Rotor Gene Q qPCR device.

U6 is a kind of short nuclear RNA (snRNA) that is extremely conserved across all of the species that it is connected with throughout the whole animal kingdom. When it comes to the processing of mRNA precursors, the U6 short non-coding RNA, which is located in the middle of the spliceosome, plays a role (22). U6 is characterized by a high degree of stability. U6 is the internal control gene that is employed the most frequently in miRNA-RT-qPCR assays (23). *MiRNA 34-a* not show any difference between both groups in blood sample while in various previous study this type of miRNA show highly significant variation in tissue sample (12). Moreover, with regard to the expression of the *miRNA-34a* gene among the groups that were investigated, the group that was diagnosed with colorectal cancer demonstrated a significantly lower level of gene expression when compared to patients who had adenomatous polyps and healthy controls. The amount of miRNA-34a can be used to differentiate between colorectal cancer and initial adenomas, since this is the conclusion that can be drawn. Additionally, miRNA-34a in colorectal cancer can be utilized as a predictive marker (24).

4. CONCLUSION

The non-significant difference was showed in U6 sample approved the theory to use as endogenous reference for miRNA-34a. While the non-significant difference of miRNA-34a expression in our blood sample illustrated that miRNA-34a could not use as biomarker to CRC patient which is not agree with our previous study in CRC tissue sample which showed highly variation between patient and control groups. that mean the gene expression of regulatory gene is mor specific in tissue samples.

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