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The Relationship between microRNA-125b and Serum Amyloid a Protein (SAA) in the Diagnosis and Prognosis of Colorectal Cancer

H. Jaber Waheed (PhD)[*1], M. Essam Abdalah (PhD)[2], W. Khalid Ahmed (PhD)[3], S. Abdulsattar Oudah Al-Qaysi (PhD)[2]

1.Department of Pharmacology and Toxicology, College of Pharmacy, Mustansiriyah University, Baghdad, Iraq.

2.Department of Clinical Laboratory Science, College of Pharmacy, Mustansiriyah University, Baghdad, Iraq.

3.Department of Chemistry and biochemistry, College of Medicine, University of Fallujah, Fallujah, Iraq.

•**Corresponding Author: H. Jaber Waheed (PhD)**

Address: Department of Pharmacology and Toxicology, College of Pharmacy, Mustansiriyah University, Baghdad, Iraq.

Tel: +964 (790) 2853287. **E-mail:** dr.huda.jw@uomustansiriyah.edu.iq

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ABSTRACT

Background and Objective: Serum amyloid A (SAA) protein increases in case of infection or inflammation, and especially in the serum of cancer patients. Regulation of the genes encoding this protein by microRNAs in cancers has been reported in several studies. The aim of this study is to explore the cumulative effect and interaction between SA1 and miR-125b in serum as a potential diagnostic and prognostic marker for colorectal cancer.

Methods: In this case-control study, 55 patients with colorectal cancer were compared with 50 healthy individuals. SAA levels were measured using the ELISA technique and miR-125b expression was assessed by rtPCR. Alkaline phosphatase was measured using a spectrophotometer and serum ferritin levels were measured and compared by CLIA analyzer.

Findings: There was a significant difference in the mean value of SAA in patients when compared to control (27.43 12.9 µg/ml vs. 4.84±0.8 µg/ml, respectively) ($p<0.0001$). There was a significant change in the gene expression in microRNA-125b expression in patient group, which was 2.76-fold higher than that of control ($p<0.05$). There was a significant difference in serum ferritin levels in patients (53.2±11.4 ng/ml) when compared to control (13.64±5.5ng/ml). There was a significant difference in ALP level when compared between colon cancer patients (94.4±17 IU/L) and control (273.8±83 IU/L). SAA levels significantly increased in colorectal cancer patients in comparison to healthy subjects.

Conclusion: According to the present study, SAA and microRNA-125b are promising markers to diagnose the colorectal cancer.

Keywords: *Colorectal Cancer, Serum Amyloid A (SAA), microRNA-125b.*

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Introduction

Colorectal cancer is one of the most important causes of death in most European countries during the past three decades (1). Early detection of the disease is very important, but the need for a reliable marker still exists. The discovery of novel and non-invasive tumor markers is needed in the prognosis, diagnosis, post-treatment, and the prediction of the therapeutic response of colorectal cancer patients. Several studies suggest that microRNAs play promoter or suppressor roles in different types of diseases, particularly cancers, and show potential as a diagnostic biomarker. The exploration of serum diagnostic markers also provides new insights (2, 3).

Some studies have examined the association between colorectal cancer and constipation, epidemiological and clinical characteristics, and omega-3 intake (4-6).

Serum Amyloid A (SAA) is an acute-phase protein that is overexpressed in cancer (7). MicroRNA-125b (miR-125b) is downregulated in colorectal cancer and associated with poor prognosis. The interplay between these two molecules has been in focus due to the association of low miR-125b levels and elevated SAA expression in colorectal and hepatocellular carcinoma. Several studies investigated the possible mechanisms of interconnection and the impact of this dynamic process on diagnosis, prognosis, and therapeutic strategies in colorectal cancer (8, 9). SAA is known for its pro-tumorigenic but also for its antitumor activity and miR-125b can have oncogenic or tumor-suppressive roles in tumors. The potential impact of this dynamic process on colorectal cancer patients' stratification and the usefulness of this interplay as a diagnostic and prognostic marker, as well as a therapeutic target, is worth investigating. SAA and miR-125b can circulate in blood and their interplay in exosomal cargo or in circulating tumor-related blood cells in colorectal cancer patients may result in systemic activation of cellular signaling. Collectively, these findings highlight the potential targets for therapeutic intervention in colorectal cancer and the complex networks engaged in the regulation of tumor biology. Iron is considered to be an essential microelement in human body. It plays a crucial role in the metabolic functions specially lipid metabolism. Iron is a transition metal and is characterized by having external electrons that can participate in lipid peroxide reactions. The sequence of these events leads to mutations in DNA. This leads to the formation of abnormal (cancerous) cells (10). The genetic expression of ALPs in different types of cancer is complex and depends on many variables. Its abnormal expression is linked to existing molecular pathways. Its association with increased ALP gene expression leads to increased Wnt/β-catenin pathway activity, which is one of the pathways affected by the formation of cancers, including colon, liver, and bone cancers. Beta-catenin moves to the nucleus and increases the expression of ALP gene transcripts, leading to elevated ALP releasing (11).

The aim of this study is to explore the cumulative effect and interaction between SA1 and miR-125b in serum as a potential diagnostic and prognostic marker for colorectal cancer. Previous studies reported elevated ALP levels in patients with colon cancer. Another study proved its association with other cancerous tumors, such as liver cancer, and explained the association between high ALP levels and metastasis (12). Therefore, the aim of this study is to explore the cumulative effect and interaction between SA1 and miR-125b in serum as a potential diagnostic and prognostic marker for colorectal cancer.

Methods

In this cross-sectional research, 55 patients with colorectal cancer were enrolled in the study. The present study was conducted from March to June 2024 in Oncology Teaching Hospital, Baghdad. This study was ethically approved by the Research Ethics Committee in College of pharmacy/ Mustansiriyah University

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(No. of ethical approval 52 with project no.65 in 9/12/2024). Serum amyloid A levels were measured by ELISA technique supplied by Ray Biotech Co., USA. Serum ferritin levels were measured by fully automated CLIA analyzers. ALP was measured by Reflotron®, supplied by Roche, Germany. miR-125b gene expression was evaluated using RT-PCR and primers were supplied by Alpha DNA Ltd. (Canada). All patients were diagnosed by biopsy during colonoscopy and the diagnosis was confirmed by a physician based on different stages of disease. For comparison, fifty healthy subjects were enrolled in the study.

All patients included in the study were admitted to the hospital for the purpose of undergoing tumor removal operations (Sigmoidectomy), and blood was collected from the patients 12 hours before their operation. About 5-7 ml of blood was obtained from patients. The serum was promptly collected by separating it from the blood and then kept at a temperature of -20 °C for further evaluations of serum amyloid, ferritin and ALP.

mRNA was included in this study. Part of serum sample was placed in Eppendorf tubes containing 200 µl of Triazole reagent and kept at -40 °C for RT-PCR experiments.

Measurement of serum amyloid A: For the determination of serum amyloid A, a sandwich enzyme immunoassay technique was used for the in vivo quantitative measurement of serum amyloid A and the stander curve as shown in figure 1.

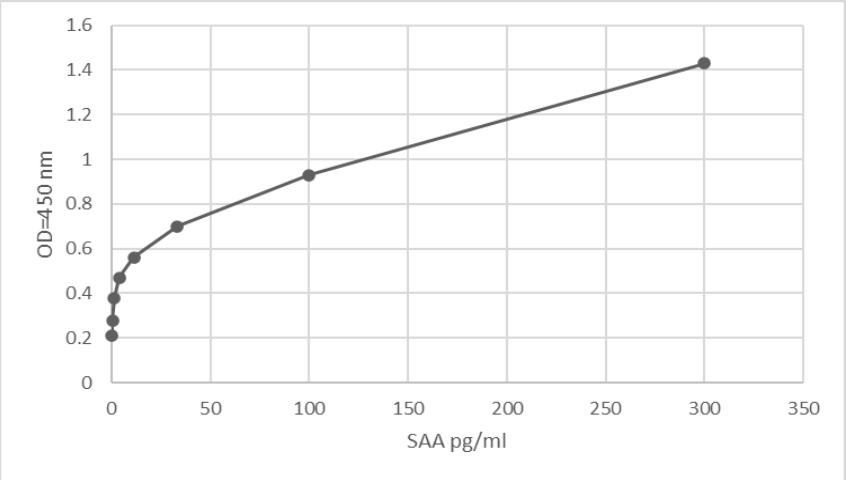

Figure 1. Serum amyloid A standard curve

Measured of other biomarkers: Serum ferritin levels were measured in serum after collection using fully automated CLIA analyzers. ALP was measured in serum using specific kit based on Reflotron® instrument which was supplied by Roche, Germany.

Measurement of MicroRNA-125b expression by RT-qPCR: After serum separation, microRNA was isolated by using (Qiagen miRNeasy Serum/Plasma Kit). The resulting miRNA was used for complementary DNA (cDNA) synthesis using (EasyScript® two-Step gDNA Removal and cDNA Synthesis SuperMix Kit). qPCR with F R primers for miR-125b primers were supplied by (Alpha DNA Ltd. Canada). cDNA was added to the qPCR reaction mixture containing primers to assess the miR-125b gene. qPCR was run on a real-time thermal cycler.

The statistical analysis was done using SPSS (Statistical Package for the Social Sciences) version 26, descriptive and inferential statistics were assessed via independent paired T-test to compare the two groups and $p<0.05$ was considered significant.

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Results

The characteristics of patients and control, including age, gender, smoking status, drinking alcohol, disease duration and stage, are shown in Table 1.

The present shows that there was a significant difference in the mean of SAA in patients when compared to control (27.43±12.9 vs. 4.84±0.8, respectively) with ($p<0.0001$), as shown in Table 2.

There was a highly significant difference in serum ferritin levels in patients (53.2±11.4 ng/ml) when compared to control (13.64±5.5ng/ml).

Moreover, there was a significant difference in ALP level when comparing colon cancer patients (273.8±3 IU/L) with control (94.4±17 IU/L).

Table 1. Patients' characteristics

Characteristic	Patients (n=55) Number(%)	Healthy (n=50) Number(%)	p-value
Age (Mean±SD)	62.32±4.73	59.3±8.22	0.076
Gander			
Female	25(45.45)	34(68)	0.04*
Male	30(54.54)	16(32)	0.06
Smoking			
Yes	30(54.55)	20(40)	0.001**
No	25(45.45)	30(60)	0.03*
Alcohol drinker			
Yes	4(7.27)	0(0)	0.76
No	51(92.73)	50(100)	0.34
Stage			
Stage 0	0(0)		
Stage I	3(5.45)		
Stage II (A, B,C)	18(32.27)	-	-
Stage III (A,B,C)	17 (30.9)		
Stage IV(A,B,C)	7(12.7)		
Recurrent	10(18.18)		
Duration of disease (year)	1.4±0.8	-	-

*p-value<0.05, **p-value<0.001

Table 2. Clinical parameters

Parameter	Patients	Control	p-value
SAA (µg/ml)	16.54±9.7 (7.62-29.17)	4.84±0.8 (3.53-5.26)	0.001**
TGF-β (ng/mL)	14.4±7.14 (13.22-25.83)	6.63±2.67 (1.73-9.16)	<0.05*
S.Ferritin (ng/ml)	31.32±14.83 (16.37-77.38)	16.96±6.1 (9.37-24.6)	<0.05*
ALP (IU/L)	202.6±92.16 (116.6-387.14)	88.24±17.3 (63.14-109.65)	0.001**

*p-value<0.05, **p-value<0.001

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The internal control gene is used in genetic studies to compare the tested gene with it. In the current experiment, MiRU6 was used as a housekeeping gene, which is commonly used in miRNA RT-qPCR (Figure 2). In the current study, RT-qPCR assay analyzed microRNA-125b expression while comparing its expression in both study groups. miRNA gene fold expression was assessed. ΔCt value was measured by subtracting Ct of microRNA-125b from the Ct of miRNAU6. As shown in Table 3, the changes in the gene expression fold in microRNA-125b expression for patients' group was 2.76 times higher than that of control. Amplification plots of microRNA-125b in study groups were used based on RT-qPCR (Figure 3).

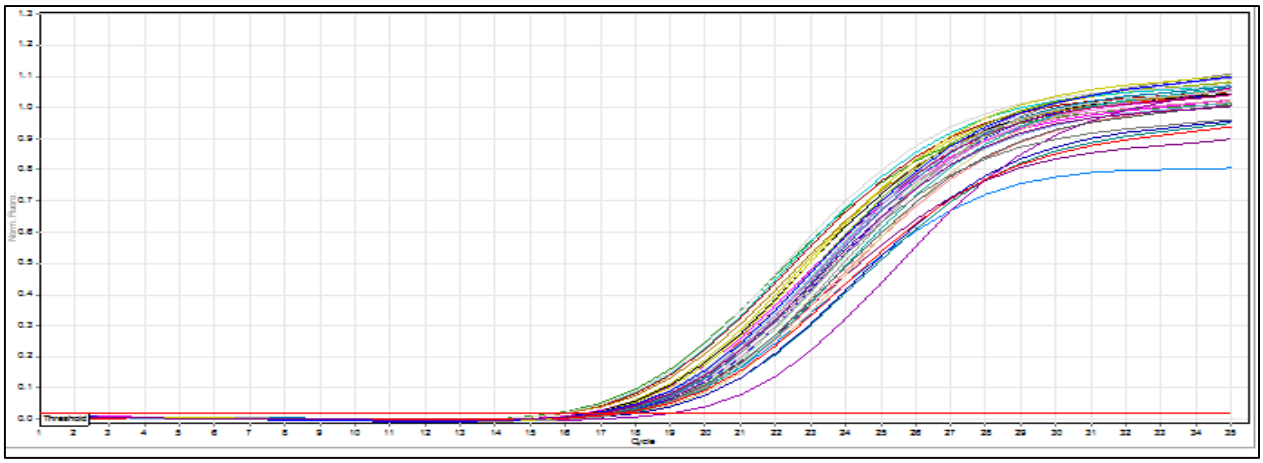

Figure 2. MiRU6 amplification plots by qPCR (The photograph was taken directly from Qiagen Rotor gene qPCR apparatus).

Table 3. MicroRNA-125b expression in the current study groups

Groups	Means Ct of microRNA-125b	Means Ct of U6	ΔCt (microRNA-125b)	$2^{-\Delta Ct} * 10^7$	Fold of gene expression
Colorectal cancer patients' group	37.05	17.10	15.95	206	2.76
Control group	32.62	17.06	15.56	157	1.00

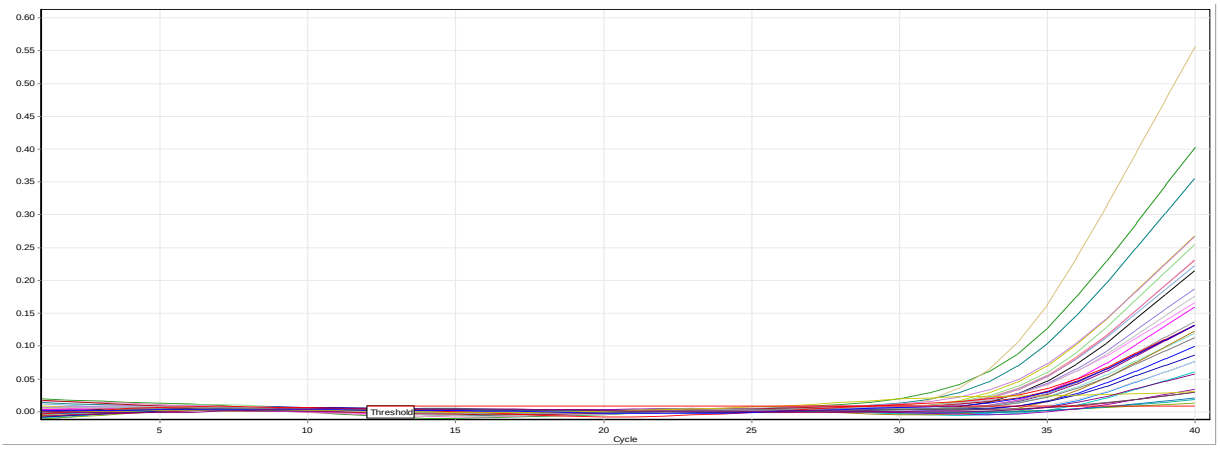

Figure 3. MicroRNA-125b amplification schemes by qPCR (The image was taken from Qiagen Rotor gene qPCR apparatus).

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Discussion

The current study was designed to assess the selective biomarkers (SAA, S. ferritin and ALP) in colon cancer and compare between them to record which one is more sensitive to detect the disease.

The current study showed that there was a significant increase in the arithmetic mean of SAA for patients with cancer of different stages when compared to healthy controls.

Recent studies suggest that SAA is effective in several steps in the inflammatory process, such as adhesion and migration and other processes which activate matrix metalloproteinases enzyme which stimulate the extracellular matrix (ECM) and regulate cell migration (12, 13). SAA may affect carcinogenesis via stimulating the transcription factor and NF-κB (14).

The interaction between SAA and miR-125b is complex and can be regulated by different signaling pathways, which are often dysregulated in colorectal cancer and many other factors (15). We propose the molecular mechanisms by which serum amyloid A and miR-125b can regulate their levels and exert feedback loop mechanisms. Oxidative stress, mitogenic kinase oncogene, oxidative stress signaling, and WNT/β-catenin are major signaling pathways that mediate the dysregulation of SAA and miR-125b biology in CRC. Not only does serum amyloid A targets several genes involved in tumor biology, comprising cancer stem cells, cell proliferation, Wnt signaling, cell migration, and more, but it also represents an additional intertwined mechanism to connect SAA and miR-125b in critical cellular functions and physiological processes (16).

The current study showed that serum ferritin was significantly increasing in colon cancer patients compared with control. It has been proven that the best indicator of iron stores is the ferritin protein. The treatment of elevated ferritin has also been added to many cancer protocols so that it can be adopted as a tumor indicator in these cancers. Despite this, the use of ferritin as a cancerous function is not absolutely approved, especially in cancer of the digestive system, due to the presence of many indicators and molecules that are affected by this type of cancer and because of its increase in colon cancer, it is affected by the bleeding condition that the patient may be exposed to, which causes anemia (17).

The present study shows that the ALP level increases significantly in patients compared with healthy subjects. In many studies, elevated levels of ALP were found in patients with cancer, especially when during metastasis, so its measurement was used to assess cases of cancer metastasis (18, 19).

miR-125b expression significantly increases in colorectal patient group when compare to control. SAA, Ferritin, and ALP levels significantly increase in colon cancer patients in comparison to healthy subjects. According to the present study, SAA and miR-125b are a good indicator for the diagnosis of colorectal cancer.

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