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# Association of TLR9 Gene Expression with Prior COVID-19 Infection in the Colorectal Cancer Tissues of Iraqi Patients

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## Abstract

Background: It has recently been reported that colorectal cancer is among the most common types of cancer in Iraq. This cancer arises from genetic and hereditary changes in the epithelial cells of the colon or rectum and often progresses from benign adenomatous polyps to invasive cancer.
Methods: A total of 30 Iraqi patients of both genders (11 males and 19 females) with colorectal cancer (CRC) and 15 healthy people (controls) were included in the study. Patients attended the Gastroenterology and Liver Diseases Teaching Hospital, Medical City, Baghdad, between January 2025 and November 2025. Participants underwent diagnosis.
Results: Toll-like receptor (TLR9) gene expression levels were studied in all patient groups. Compared with that in the control group, TLR9 gene expression was significantly greater in colon cancer patients than in rectal cancer patients and control subjects (6.88 ± 0.77, 4.79 ± 0.90, and 1.04 ± 0.19, respectively).
With respect to COVID-19, TLR9 gene expression was significantly higher in the colon cancer group than in the rectum and control groups, which were not infected with COVID-19. The values were 10.213.24±6.15 ,0.88±, and 1.080.23±, respectively, with $P < 0.001$. There was also a significant difference between the two groups of COVID-19-infected and noninfected patients with colon cancer ($P < 0.001$).
In tumor tissue, the size was less than 1 mm, and high TLR gene expression occurred in the patient group compared with that in the control group ($P < 0.001$). Compared with rectal cancer patients and controls, colon cancer patients were significantly more prevalent (7.750.36±2.42 ,1.11±, and 1.210.32±, respectively), according to the group whose tumor tissue size was above 1 mm ($P < 0.001$).
Conclusion: These findings indicate that TLR9 expression may represent a potential tissue biomarker in colorectal cancer. However, larger studies are needed to validate these findings.

**Keywords**
Colorectal cancer, TLR9, Gene expression, COVID-19, Tissue biomarker



**How to Cite:**
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## Introduction

Colorectal cancer is a malignant tumor that poses a global health threat, ranking 3rd among the most common cancers and the second leading cause of cancer-related deaths worldwide [1]. It is the most prevalent gastrointestinal tract cancer, with similar incidence rates among men and women in both developed and developing countries [2].

The development of colorectal cancer results from an interplay of genetic, environmental, and lifestyle factors. The disease typically originates from precancerous polyps and progresses to adenocarcinoma via specific pathways, most notably chromosomal instability (CIN) and microsatellite instability (MSI) [3]. An increase in the number of cases was observed with advancing age, particularly after age 50, and was due to other controllable factors, such as obesity, lack of physical activity, a diet high in red and processed meats, smoking, and excessive alcohol consumption [4].

Another study revealed that COVID-19 infection decreased the number of diagnosed colorectal cancer cases and the proportion of stage 1 cases [5]. Compared with mild cases, severe cases of COVID-19 are associated with higher mean inflammatory marker levels, such as C-reactive protein levels, erythrocyte sedimentation rates (ESRs), ferritin levels, D-dimer levels, and neutrophil-to-lymphocyte ratios [6]. Another study demonstrated that changes in trace element levels can negatively affect biological processes and contribute to carcinogenesis. Both trace element deficiencies and excesses are associated with the development or exacerbation of certain cancers, such as colorectal cancer [7]. Colorectal polyps are characterized by significantly increased levels of monocyte chemoattractant protein (MCP-1, MCP-2, and MCP-3) proteins [8]. These chemokines are also elevated in patients with chronic periodontitis [9]. The regulatory function of the ID family in numerous processes and the upregulation or downregulation of ID genes in tumors underscore their importance in tumor development. Therefore, these proteins could be used as markers for colorectal cancer [10]. Colorectal cancer often develops silently in its early stages, but certain warning signs may warrant further investigation. Common symptoms include rectal bleeding or blood in the stool; changes in bowel habits such as diarrhea, constipation, or hardening of the stool; and unexplained weight loss, abdominal pain, cramps, or bloating [11].

Therefore, this study was designed to examine the role of TLR9 receptor gene expression in the development of colorectal cancer in a sample of Iraqi patients.

## Subjects, Materials, and Methods

Subjects: A total of 30 Iraqi patients of both genders (11 males and 19 females) with CRC and 15 healthy people (controls) were included in the study. Patients will visit the Gastroenterology and Liver Diseases Teaching Hospital/Medical City/Baghdad during the period from January 2025 to November 2025. Participants underwent diagnosis and treatment. They were clinically examined and evaluated by the hospital's consulting medical staff, and accordingly, they were divided into two clinical groups on the basis of the stage of cancer (24 with stage II, 6 with stage III), according to the TNM classification. When patients with colorectal cancer arrived, their tests were reviewed to determine if they had previously been infected with COVID-19. Previous COVID-19 infection in cancer patients was confirmed through medical records; therefore, most patients were infected one year before undergoing endoscopic tissue sampling during the aforementioned study period. A biopsy was taken from the colon and rectum several months after the previous viral infection, and the patients had not received any chemotherapy, radiation, or immunotherapy. The diagnosis of cancer during that period occurs when the biopsy is taken and examined histologically by specialists.

Tissue samples: Endoscopic biopsy fragments were collected by biopsy forceps and were 1 to 3 mm in size. Additionally, a mixture containing 750 µl of triazole was added to the Eppendorf tube and then frozen at -80°C until assessment [12].

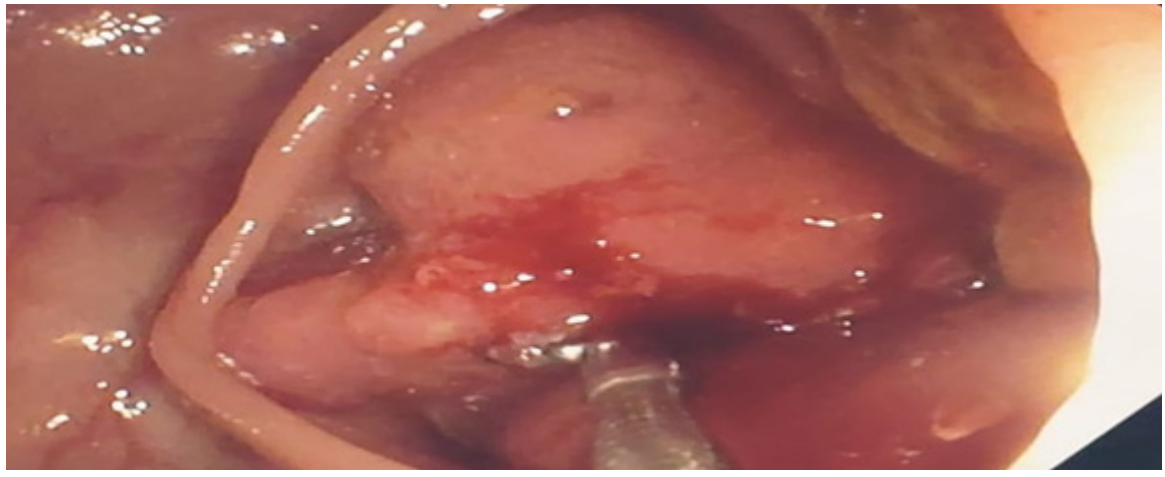

**Figure (1):** Endoscopic biopsy sample from the colorectal lesion during colonoscopy.

During endoscopic examination, prominent mucous protrusion was observed, accompanied by intestinal bleeding and redness. The site appeared smooth; however, inflammation and ulceration were detected in the sample because of the patient's advanced age and history of multiple ulcerations, raising suspicion of a tumor. Therefore, the endoscopic appearance raised suspicion of an early rectal tumor, which was subsequently confirmed by histopathological examination.

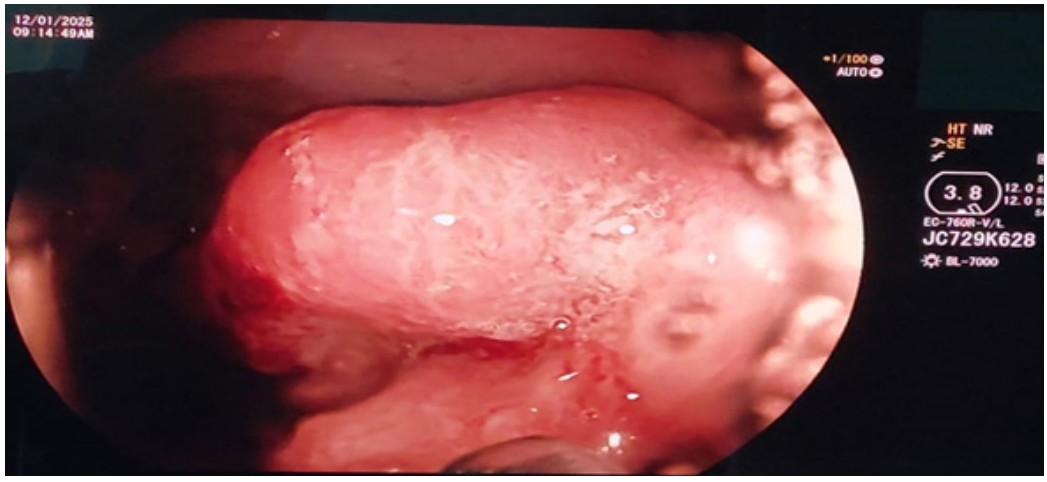


**Figure (2):** Colonoscopic appearance of the colorectal tumor demonstrating mucosal irregularity and luminal protrusion.

A colonoscopy image shows a large polyp in the colon with an irregular mucous surface and changes in the colonic lumen, causing a prominent protrusion within the colonic cavity. The endoscopic appearance suggests the presence of a tumor.

**Methods:**

**RNA extraction**

RNA was extracted using the following steps: 100 mg of tissue was resuspended in 750 µl of TransZol Up and then homogenized using a homogenizer. All the samples were kept at -23°C. Add 200 µl of chloroform to each 750 µl of TransZol Up Reagent. The mixture was vortexed gently for 30 sec and then incubated at room temperature for 3 min. The mixture was centrifuged at 2-8°C and 10,000 rpm for 15 min. The mixture was separated into an upper colorless aqueous phase (containing RNA) and a lower pink organic phase, with an interphase in between. The upper aqueous phase is approximately 50-60% of the total volume of TransZol Up. The colorless upper phase (containing RNA) was transferred to a new RNase-free tube, avoiding contamination from the interphase (second phase) and leaving a portion of the first aqueous phase. An equal volume of 96 -100% ethanol was added. The precipitate and the resulting solution were transferred to a spin column. Centrifuge at 12,000 rpm for 30 sec at room temperature. The spin column, containing 500 µl of CB9, was centrifuged at 12,000 rpm for 30 sec at room temperature. Discard the flow-through. Step 6 was repeated. After confirming that ethanol was added to the spin column, 500 µl of WB9 was added. Centrifuge at 12,000 rpm for 30 sec at room temperature. Discard the flow-through. Step 8 was repeated once, and the column matrix was centrifuged at 12,000 rpm for 2 min at room temperature to remove any residual ethanol and then air-dried for a few minutes. The spin column was placed in a clean 1.5 mL RNase-free tube. After 50-200 µl of RNase-free water was added, the tube was incubated at room temperature for 1 min and then centrifuged at 12,000 rpm for 1 min to elute the RNA. Finally, the RNA extract was stored at -80°C.

**Assessment of RNA concentration and purity**

The concentration and purity of the extracted RNA were assessed to determine sample quality using a OneC NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). The concentrations of RNA ranged from 40 to 111 ng/µl, and the absorbances of the samples, which were measured at two wavelengths (260 and 280 nm), were also used to determine RNA purity. The A260/A280 ratio, approximately 1.97–2.16, indicated the purification of the RNA samples.

**TLR9 PCR conditions**

The following primer pair was used: forward primer, AAGCTGGACCTCTACCACGA; reverse primer, TTGGCTGTGGATGTTGTTGT. Sequence size: 3352; product size: 177. For cDNA synthesis from total RNA for mRNA expression, first-strand cDNA synthesis was performed using the EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen Biotech Co., Ltd., Beijing, China) to reverse transcribe total RNA into complementary DNA (cDNA). The procedure was performed in a 20 µl reaction volume, as per the manufacturer's guidelines, with 4 µl of total RNA reverse-transcribed. The random primer was incubated for 10 min at 25°C. The anchored oligo(dT) 18 primer and gene-specific primer (GSP) were incubated for reverse transcription-quantitative polymerase chain reaction (qPCR) at 42°C for 15 min. The reaction was then incubated for 5 sec at 85°C to deactivate the enzyme. The annealing temperature for the primers was calculated according to the manufactur-

er's instructions, and the melting temperature (Tm) of each primer was used to calculate the optimal primer annealing temperature (Ta). The equation requires the primer sequence because specific nucleotides are needed. The equation is as follows: "Melting temperature (Tm) = 2 (A+T) + 4 (G+C)". "Annealing temperature (Ta) = Tm - (2-5) °C". Using the equations above, the melting temperatures for the forward and reverse primers were considered. By comparing the annealing temperatures for the forward and reverse primers, the lowest temperature (°C) was chosen.

**Quantitative expression of real-time PCR:**

**The expression levels of the TLR 9 gene**

TLR9 gene expression levels were measured using reverse transcription–quantitative polymerase chain reaction (qPCR), a sensitive and widely used technique for quantifying steady-state mRNA levels. A quantitative real-time qRT-PCR SYBR Green assay was performed to confirm the expression of the target gene TLR9, after which the RNA levels of the "endogenous control (GAPDH)" gene were amplified and then processed as a normalization control for the RNA levels of the TLR9 gene. qPCR was performed using the "QIAGEN Rotor gene Q Real-time PCR System (Germany)". To evaluate the variation in the fold change and the level of TLR9 gene expression, a "TransStart® Top Green qPCR Super Mix kit" was used, and the threshold cycle (Ct) was measured. Every reaction was performed twice. Summary of the reactions undertaken and assays performed for each range, given that an assay of a concerted process with volume was needed.

**Table (1):** Components of the qPCR mixture used for TLR9 amplification.

| 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) |

The cycling protocol was programmed for the following optimized cycles according to the thermal profile shown in Table 2.

**Table (2):** Thermal profile of TLR 9 gene expression.

| The Steps | Temperatures (°C) | Times (sec.) | The Cycles |
|---|---|---|---|
| Enzyme activation | (94) | (30) | 1 |
| Denaturation | (94) | (5) | 40 |
| Annealing | (58-60) | (15) | |
| Extension | (72) | (20) | |
| Dissociation | 55 °C-95 °C | | 1 |

**Annealing temperature for TLR 9 58 and GAPDH 60

**Calculation of the expression levels of TLR9**

The (2–ΔCt) relative cycle threshold is used to determine the variation in the fold change in the quantified expression of the mature RNAs. It is the relative gene expression ratio of the patient group over the control group. Values higher than 1 indicate upregulated or upregulated gene expression, values between zero and one indicate low and downregulated expression, respectively, and the regulation of the gene, and a value of 1 indicates that no variation occurred. The target gene expression levels were normalized by setting appropriate thresholds to obtain accurate Ct values provided by the qPCR instrument. By using real-time cycler software, the threshold cycle (CT) was calculated for each sample. Duplicate running was applied for all the samples, in addition to the calculation of the mean values. The Ct values for the housekeeping genes (GAPDH) and the target genes (TLR9) tested in patients and controls were recorded. The calculations were as follows: CT The variance among the CT values (ΔCt), which is also called the "normalized raw data" for every target gene and the housekeeping gene, was calculated by subtracting the selected normalization factor from the "Ct value of each gene of interest". "ΔCt (control)=CT (gene)-CT(HKG) " ΔCt (patient)=CT (gene)-CT(HKG)" Last, the following formula is used to calculate the expression level ratio: 2-ΔCt = normalized expression ratio 2. ΔΔCT The double delta Ct value (ΔΔCt) of the genes in the current study was calculated by subtracting the ΔCt value of each assessment patient group from that of the control group as follows: ΔΔCt=ΔCt(patient)-ΔCt(control). The 2-ΔΔCt method was used to obtain the expression fold change for every gene, which represents the relative fold change. Therefore, the results are expressed as the fold change in the expression level of the target gene normalized to that of the endogenous control (housekeeping gene) and relative to that of the calibrator, which is the target gene in

control subjects. Finally, the fold-change values for assessing gene expression were calculated as follows: "fold change =2 -ΔΔCt normalized expression ratio".[13]

**Statistical Analysis:**

The results were statistically analyzed using SPSS (Statistical Package for Social Sciences) version 23. Their data are presented as the mean ± standard error (S.E.), and differences between means were assessed by ANOVA (analysis of variance), followed by the Duncan test, which was used to compare the three study groups. and ROC tests, assumptions required for ANOVA, such as data normality and variance homogeneity, were evaluated before statistical analysis.

## Results

TLR9 gene expression was significantly higher in colon cancer patients than in rectal cancer patients and control subjects (6.88 ± 0.77, 4.79 ± 0.90, and 1.04 ± 0.19, respectively), as shown in Table 3, with P < 0.001.

**Table (3):** Comparison between the two groups of patients and the control group.

| Gene | Control | Colon CA patients | Rectum CA patients | P value |
|---|---|---|---|---|
| | Folding (Mean±S.E.) | | | |
| TLR9 | 1.04±0.19 a | 6.88±0.77 c | 4.79±0.90 b | <0.001** |

Different letters indicate significant differences between the groups. Similar letters indicate nonsignificant differences

The data in Table 4 reveal no significant difference between the sexes of the patients, but there was a significant difference between the two patient groups and the control group for both sexes (P < 0.001).

**Table (4):** Comparison between sex and TLR9 expression in the study groups.

| Gene | Sex | Control | Colon CA patients | Rectum CA patients | P value |
|---|---|---|---|---|---|
| | | Folding (Mean±S.E.) | | | |
| TLR9 | Male | 1.21±0.31 a | 7.42±1.40 b | 4.74±1.85 b | <0.001** |
| | Female | 0.84±0.18 a | 6.53±0.93 b | 4.82±1.06 b | |
| **P value** | | **0.8 NS** | **0.5 NS** | **0.9 NS** | **-** |

Different letters indicate significant differences between groups, whereas identical letters indicate nonsignificant differences. Control male (8) female (7), colon male (6) female (9), rectum male (5) female (10)

In the age group under 60 years, TLR9 gene expression was significantly greater in patients with colon cancer than in patients with rectal cancer and in the control groups (5.91±1.0, 1.59±0.46, and 0.66±0.14, respectively), with P < 0.001. Compared with the other groups, the group over 60 years of age had significantly greater TLR9 gene expression in patients with colon cancer, with P < 0.001. There was also a difference in age between the groups of patients with rectal cancer (p <0.01), as shown in Table 5.

**Table (5):** Comparison between the age of patients with colon and rectal cancer and TLR9 gene expression.

| Gene | Age (Yr.) | Control | Colon CA patients | Rectum CA patients | P value |
|---|---|---|---|---|---|
| | | Folding (Mean±S.E.) | | | |
| TLR9 | ≤60 | 0.66±0.14 a | 5.91±1.0 b | 1.59±0.46 a | <0.001** |
| | >60 | 1.79±0.24 a | 7.74±1.11 c | 5.29±0.96 b | |
| **P value** | | **0.4 NS** | **0.2 NS** | **0.01*** | **-** |

Different letters indicate significant differences between groups, whereas identical letters indicate nonsignificant differences. Control <60 (10), control >60 (5), colon <60 (7) >60 (8), rectum <60 (2) >60 (13)

As shown in Table 6, compared with the control group, the group with an average body mass index between 21 and 25 had significantly greater TLR9 gene expression in the tissue of patients with colon and rectal cancers (P < 0.001).

**Table (6):** Comparison between the body mass index of patients with colon and rectal cancer and TLR9 gene expression

| Gene | BMI | Control | Colon CA patients | Rectum CA patients | P value |
|---|---|---|---|---|---|
| | | Folding (Mean±S.E.) | | | |
| TLR9 | 15-20 | - | 8.06±1.47 a | 5.81±1.51 a | <0.001** |
| | 21-25 | 1.04±0.19 a | 6.10±0.80 b | 3.90±1.04 b | |
| **P value** | | **-** | **0.16 NS** | **0.2 NS** | **-** |

Different letters indicate significant differences between groups, whereas identical letters indicate nonsignificant differences. Control <25 (15), colon <20 (6) <25 (9), rectum <20 (7) <25 (8)

In smokers, TLR9 gene expression gradually and significantly increased in colon cancer patients compared with that in rectal cancer patients and controls (7.34±1.16, 4.55±1.25, and 1.14±0.22, respectively) at P < 0.001, as shown in Table 7.

**Table (7):** Comparison between smoking status and TLR9 gene expression in the study groups

| Gene | Smoking | Control | Colon CA patients | Rectum CA patients | P value |
|---|---|---|---|---|---|
| | | Folding (Mean±S.E.) | | | |
| TLR9 | NO | 0.64±0.16 a | 6.20±0.86 b | 5.07±1.39 b | <0.001** |
| | Yes | 1.14±0.22 a | 7.34±1.16 c | 4.55±1.25 b | |
| **P value** | | **0.8 NS** | **0.4 NS** | **0.7 NS** | **-** |

Different letters indicate significant differences between groups, whereas identical letters indicate nonsignificant differences. Control NO (3), Control Yes (12), colon NO (6) YES (9), rectum NO (7) YES (8)

There was no significant difference between the two groups of patients with stage II cancer, but there were significant differences between the two groups of patients with stage III cancer at P < 0.05, and there was also a significant difference between stages two and three of rectal cancer at P < 0.05, as shown in Table 8.

**Table (8):** Comparison between stages of cancer with respect to TLR9 gene expression

| Gene | Stage | Colon CA patients | Rectum CA patients | P value |
|---|---|---|---|---|
| | | Folding (Mean±S.E.) | | |
| TLR9 | II | 6.62±0.95 a | 5.36±1.06 a | < 0.05* |
| | III | 7.96±0.31 a | 2.53±0.49 b | |
| **P value** | | **0.5 NS** | **0.05*** | **-** |

Different letters indicate significant differences between groups, whereas identical letters indicate nonsignificant differences. Colon II (12), III (3), rectum II (12), III (3)

TLR9 gene expression was significantly higher in the colon cancer group than in the rectum and control groups, which were not infected with COVID-19. The values were 10.21±0.88, 6.15±3.24, and 1.08±0.23, respectively, with P < 0.001, as shown in Table 9. There was also a significant difference between the two groups of COVID-19-infected patients and noninfected patients with colon cancer (P < 0.001).

**Table (9):** Comparison between COVID-19 and TLR9 gene expression in patients with colorectal cancer

| Gene | COVID infection | Control | Colon CA patients | Rectum CA patients | P value |
|---|---|---|---|---|---|
| | | Folding (Mean±S.E.) | | | |
| TLR9 | NO | 1.08±0.23 a | 10.21±0.88 c | 6.15±3.24 b | <0.001** |
| | Yes | 0.92±0.34 a | 5.22±0.53 b | 4.58±0.96 b | |
| **P value** | | **0.9 NS** | **<0.001**** | **0.4 NS** | - |

Different letters indicate significant differences between groups, whereas identical letters indicate nonsignificant differences.
Control NO (11) YES (4), colon NO (5) YES (10), rectum NO (2) YES (13)

As shown in Table 10, the tumor tissue size was <1 mm, and TLR9 gene expression was higher in the patient group than in the control group (P < 0.001). High significance was observed in colon cancer patients compared with rectal cancer patients and controls (7.75±1.11, 2.42±0.36, and 1.21±0.32, respectively) for tumors whose tissue size was greater than 1 mm (P < 0.001).

**Table (10):** Comparison of tumor size and TLR9 gene expression between the study groups

| Gene | Tumor Size | Control | Colon CA patients | Rectum CA patients | P value |
|---|---|---|---|---|---|
| | | Folding (Mean±S.E.) | | | |
| TLR9 | ≤ 1 mm | 0.88±0.21 a | 5.90±1.01 b | 5.66±1.12 b | <0.001** |
| | > 1 mm | 1.21±0.32 a | 7.75±1.11 b | 2.42±0.36 a | |
| **P value** | | **0.8 NS** | **0.17 NS** | **0.037*** | - |

Different letters indicate significant differences between groups, whereas identical letters indicate nonsignificant differences.
Control < 1 mm (8), > 1 mm (7), < 1 mm (7), > 1 mm (8), < 1 mm (11), > 1 mm (4)

The results of this study revealed that TLR9 was the most significant indicator of CRC sensitivity.

**Table (11):** ROC curve analysis of TLR9 gene expression in tissue cancer

| Gene Expression | AUC | Cutoff | Sensitivity | Specificity | Asymptotic 95% Confidence | | P Value |
|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | |
| TLR9 | 0.96 | 1.9 | 90% | 93% | 0.907 | 1.0 | <0.001** |

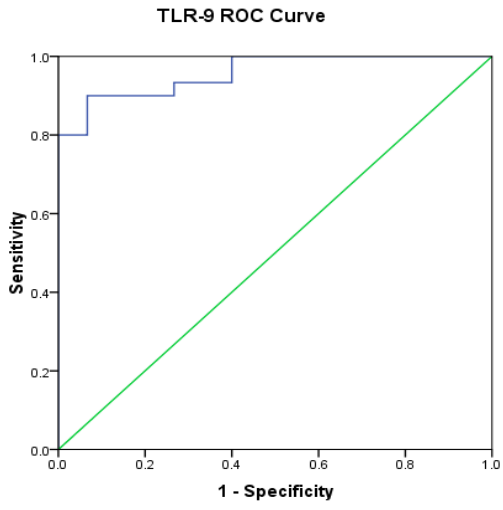


**Figure (3).** Receiver operating characteristic (ROC) curve demonstrating the diagnostic performance of TLR9 gene expression for distinguishing colorectal cancer patients from healthy controls.

As shown in Fig. 3, TLR9 was the most significant indicator of colon cancer sensitivity.

## Discussion

TLR9 gene expression was significantly higher in colon cancer patients than in rectal cancer patients and control subjects. This increase may be related to the development of inflammatory bowel disease. Inflammation results in the release of pro-inflammatory cytokines. These cytokines activate transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which upregulate TLR9 gene expression [14]. In terms of sex, males contained more tumor-associated macrophages, neutrophils, and dendritic cells. Their presence increases total TLR mRNA in cancer tissue [15]. The female hormone estrogen can regulate the expression of toll-like receptors (TLRs). Estrogen receptors interact with the NF-κB and interferon regulatory factor (IRF) pathways, which may promote the gene expression of toll-like receptors in cancerous tissues. In colorectal cancer, hormone levels are disrupted, leading to elevated toll-like receptor levels compared with those in healthy female tissues. [16]

In the age group under 60 years, a significant increase in TLR9 gene expression was observed in colon cancer patients compared with that in rectal cancer patients, and in the age group over 60 years, a significant increase in TLR9 gene expression was observed in colon cancer patients compared with that in the other groups. attributed to changes in the oral gut microbiota. With advancing age, the prevalence of harmful bacteria increases, leading to increased exposure to unmethylated CpG DNA and, consequently, direct activation of TLR9. Continued activation results in increased TLR9 gene expression [17].

In terms of body mass index, there was a significant increase in TLR9 gene expression in the tissue of patients with colon and rectal cancers compared with that in the control group (between 21 and 25). This may be related to the fact that the body of an obese person contains large amounts of fat; as a result of consuming large quantities of food, the body receives more calories than the normal limit does. This excess caloric intake disrupts the function of a specific hormone called guanylin, which is secreted in the intestines and plays a crucial role in preventing colon cancer. Deficiencies in the secretion of this hormone lead to the cessation of the expression of hormone receptors located within the colon wall. The function of these receptors is to prevent abnormal cell growth. Consequently, some cells begin to grow, leading to the development of colon cancer [18].

Most patients with colorectal cancer do not experience weight gain because of loss of appetite, as well as intestinal problems caused by ulcers. All these factors led to weight loss, and therefore, cancer patients with weight gain were not included in the study; thus, overweight and obese patients were not included in the study population. In smokers, there was a gradual, significant increase in TLR9 gene expression in colon cancer patients compared with that in rectal cancer patients, and previous studies suggest that nicotine damages the epithelial cells of smokers, leading to the release of autologous DNA and mitochondrial DNA. It also alters the intestinal barrier, increasing exposure to bacterial CpG DNA, a direct ligand of the TLR9 receptor. Oxidative stress enhances TLR signaling pathways, resulting in increased upregulation and continuous activation of the TLR9 receptor in colon tissue. [19], such that persistent TLR9 stimulation (from gut bacteria or damaged cells releasing DNA resulting from nicotine exposure) leads to the activation of signaling pathways such as the NF-κB pathway. This leads to long-term production of the inflammatory cytokines IL-6 and tumor necrosis factor alpha (TNF-α) and chronic inflammation, which are considered risk factors for colon cancer. According to the stages of cancer, stage II cancer indicates that the higher expression of TLR9 in rectal cancer than in stage III cancer suggests a robust innate immune response during the early stages of disease. As cancer progresses and can metastasize, immune evasion mechanisms may suppress TLR9 signaling, leading to decreased expression of TLR9 in stage III tumors. [20]

Previous COVID-19 studies suggest that SARS-CoV-2 infection may influence interferon signaling pathways that can affect TLR9 expression, blocking interferon production and interfering with interferon signaling when viral proteins inhibit interferon responses, reducing the activation of Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling and decreasing the transcription of interferon-stimulated genes, including TLR9, in colon cancer patients with COVID-19 infection compared with those without viral infection. [21] Patient recruitment was limited to eligible colorectal cancer patients with a documented history of COVID-19 during the study period. emphasizing that our findings should be interpreted as preliminary observations requiring validation in larger, multicenter studies. High TLR9 levels are linked to faster tumor progression and poorer survival, whereas low TLR9 levels are associated with better outcomes [22].

The results of this study also revealed that TLR9 gene expression is a key marker of CRC sensitivity. This was due to the expression levels reflecting tumor aggressiveness, inflammation, metastasis, immune evasion, and the chemotherapy response. Elevated TLR9 expression was associated with faster tumor growth and worse prognosis, whereas reduced TLR9 expression was correlated with improved outcomes, which is consistent with the findings of previous research. High TLR9 levels are linked to faster tumor progression and poorer survival, whereas low TLR9 levels are associated with better outcomes [22]. Although our study did not directly measure the most specific molecular pathways, microbial alterations, or effects of tobacco smoking on the microbial intestine, our results are consistent with the pathways described. These mechanisms may partially explain the observed findings in our patients. This observation in younger rectal cancer patients should be considered exploratory and requires validation in larger studies. However, further direct molecular and metagenomic studies are needed in future research in this area

to definitively validate these proposed pathways within this specific experimental context. We recommend that subsequent multicenter studies use larger patient groups, thereby strengthening the validity of these exploratory findings. These findings require confirmation in larger multicenter studies before clinical application; thus, subgroup findings should be considered exploratory and require validation in larger studies.

## Conclusion

These findings indicate that TLR9 expression may represent a potential tissue biomarker in colorectal cancer. Although the findings related to stage III colon and rectal cancer are promising, they should be considered exploratory and require confirmation in larger multicenter studies.

## Author declarations

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### Ethical clearance

This study was approved by the Institutional Ethics Committee of the Department of Biology, College of Education for Pure Science Ibn Al-Haitham, University of Baghdad, Baghdad, Iraq (Approval No. EC-70; January 2, 2025). All procedures involving human participants were conducted in accordance with the ethical standards of the institutional research committee and the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants prior to their enrollment in the study.

### Data Availability Statement

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

### Author Contributions

H.S.R.: Conceptualization, methodology, investigation, laboratory work, data collection, formal analysis, data interpretation, and writing of the original draft.

H.Y.M.: Methodology, supervision, data interpretation, critical revision of the manuscript, and final review.

A.H.A.: Study design, supervision, interpretation of the results, manuscript review, and final approval of the manuscript.

All the authors have read and approved the final version of the manuscript and agree to be accountable for all the aspects of the work.

### Conflict of Interest

The authors declare that they have no competing interests.

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