RESEARCH ARTICLE

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TLR4, IgA and *EpCAM* Expression in Colorectal Cancer and Their Possible Association with Microbiota as a Pathogenic Factor; An Immunohistochemical and Genetic Study

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Abstract

Background: The pathogenesis of inflammatory bowel disease (IBD) and colorectal cancer (CRC) is thought to be related to immune response against gut microbiota. *TLR4*, *IgA*, and EpCAM have a role in intestinal local immune response and their altered expression related to both IBD and CRC. Lipopolysaccharide (LPS) is the main activator of *TLR4*. The objective of this study is to evaluate the possible role of intestinal microbiota in the pathogenesis of IBD and CRC through expression of *TLR4*, *IgA* and *EpCAM*. **Methods:** One hundred five cases were divided into (Group 1/ Control: 10 sections of normal colonic mucosa, Group 2/CRC: 51 cases, Group 3/IBD: 44 cases). Immunohistochemistry for *TLR4*, *IgA*, and *EpCAM* was done. LPS was assessed in all groups. *TLR4* gene and protein expression were assessed in colorectal cancer cell line by RT-PCR and immunocytochemistry. **Results:** There was a significant correlation between *TLR4* and tumor grade (P value 0.003 and 0.01 respectively). A significant correlation was found between *IgA* expression and T stage (P value 0.02) and between *EpCAM* expression and histologic type (P value 0.02). In comparison of CRC patients to controls; there was a statistically significant different expression of *TLR4* positivity, *IgA* positivity and *EpCAM* (P value <0.001, 0.004, <0.001 respectively). Patients with CRC were compared to colitis patients and there was a statistically significant different expression of *IgA* positivity and *EpCAM* expression (P value <0.001). There was significant higher expression of *TLR4* in CRC cell line than the fibroblast by both PCR and immunocytochemistry (P-value: 0.003 and 0.024 respectively). LPS level in CRC patients was significantly higher than the control and IBD groups (P values <0.001 and <0.001 respectively). **Conclusion:** *TLR4*, *IgA*, *EpCAM* expression in both CRC and IBD might be related to the pathogenic role of microbiota and could represent potential prevention modalities and therapeutic targets.

Keywords: Microbiota- IBD- Colon Cancer- *TLR4- IgA- EpCAM*

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Introduction

Incidence rate of IBD increased in the past few decades in Western countries and it varied considerably depending on the region. In 2017, UC incidence rates ranged from 0.97 to 57.9 per 100,000 in Europe, 8.8 to 23.14 per 100,000 in North America, and 0.15 to 6.5 per 100,000 in Asia and the middle east [1]. In Egypt, the incidence has increased in past years, a recent Egyptian registry reported incidence of Crohn's disease to be 0.8% and ulcerative colitis to be 10.4% in 250 samples of colonoscopic biopsies [2]. The age-standardized prevalence rate of IBD in Egypt increased from 17.9 to 26.7/100 000, between 1990 and 2017 with a 48.9% change [3].

Colorectal cancer (CRC) represents the third most common malignancy globally (10%) and the second leading cause of cancer deaths (9.4%). In 2020, more than 1.1 million new cases were diagnosed, and 576,858 CRC deaths occurred [4]. The incidence of CRC is believed to increase due to aging of population and popularity of Western lifestyle [5]. In Egypt, the incidence of colorectal cancer in young population; below the age of 40 is high

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representing around 35% and the young population has lower 5-year survival rate 25% compared to 75% in older population [6].

The intestinal environment includes trillions of commensal microorganisms that maintain homeostasis with the host immune system. However, the defect of innate immune system as mucosal destruction or leakage of epithelial barrier can harm the beneficial host-microbiome balance [7, 8]. The pathogenesis of IBD and CRC is thought to be related to aberrant immune response against gut microbiota [9].

Immunotolerance is a specific condition in which the immune system shows an unresponsiveness or hyporesponsiveness to foreign harmless antigens or self-antigens [10]. Many mechanisms have been proposed, by which immunotolerance is maintained through regulation of activated T cells. These include T-cell anergy, regulatory T cells (Tregs) producing immunosuppressive cytokines and activation-induced T-cell apoptosis from undefined sites [11]. Because of their specific physiological feature, immunotolerance needs to be well established in some of human organs including the gut [12]. Many factors are involved in maintaining an immunotolerant environment in the gut during ingestion of dietary antigens including *IgA*, commensal bacteria Tregs, dendritic cells (DCs) CD8 T cells, γδ T cells, regulatory B cells, and massive cytokines, such as transforming growth factor (TGF)-b1 and interleukin 10 [13-16].

Toll-like receptors (TLRs) which are present on immune cells have crucial role in immune response to different pathogens. At least 11 mammalian TLRs have been identified and are involved in recognition by immune and nonimmune cells of pathogen-associated molecular patterns, such as lipopolysaccharides (LPSs), viral double-stranded RNA, and unmethylated CpG islands [17]. The role of toll-like receptors is to induce the maturation of DCs leading to activation and differentiation of T-cells into effector T-cells which is responsible for adaptive immune response. Thus, TLR signaling is the link between the host innate and adaptive immune response [18]. Extracellular vesicles (EVs) are lipid bilayer-enclosed, cell-derived particles that are released by all cellular organisms including bacteria. Extracellular vesicles are heterogenous structures, and this is due to variety of types and functional states of the releasing cells as well as of the different biogenetic routes. EVs are involved in several homeostatic processes, including the essential, ubiquitous involvement of EVs in fundamental immune mechanisms and immune-mediated disease processes [19-21].

EVs play important roles in cancer development. Cancer cells secrete extracellular vesicles (EVs), which are a unique form of communication between cells that can facilitate cell proliferation and survival, help the tumour microenvironment develop, and enhance invasive and metastatic activity [22]. EVs tend to localize in the intestinal tract associated with epithelial cell adhesion molecule (*EpCAM*). Inhibition of *EpCAM* expression in colon aggravates murine IBD and the protective effect of EVs from intestinal epithelial cells with decreased *EpCAM* on murine IBD is impaired [23].

In the present work we aimed to investigate the suggestive role of microbiota via assessing LPS levels in sera of IBD and CRC cases in comparison to control cases. Also, to interpret role of immunohistochemical expression in pathogenesis of IBD and CRC through *TLR4*, *IgA*, and *EpCAM* expression and their clinicopathologic correlations and possible implications in clinical practice.

Materials and Methods

Tissue collection

This is a retrospective study included total 105 cases divided into three groups; group 1 (control representative of normal colonic mucosa): 10 sections prepared from free margins of specimens received as Hemicolectomy specimens for cases diagnosed as Hirschsprung disease or CRC, group 2: 51 sections prepared from the tumor in cases diagnosed as CRC, and group 3: 44 cases diagnosed as IBD. All cases were retrieved from the archival files of the pathology labs of Ain Shams University Hospitals. The histopathology reports were reviewed to record clinicopathologic data of the patients.

Haematoxylin and Eosin-stained sections of 4-5 μm thickness were examined. The tumor tissues were examined by the authors and confirmed to be colonic carcinoma. The pathological features were obtained as tumor size, grade, pathological stage, lympho-vascular invasion and presence of tumor budding. The tumor grade was determined based on the WHO criteria [24]. The tumor stage was evaluated using the current AJCC/UICC TNM staging (8th edition, 2017) of colorectal carcinoma [25]. Paraffin blocks with adequate tissue were selected and used for preparation of three positively charged slides for performance of *TLR4*, *IgA* and *EpCAM* immunohistochemistry and for *TLR4* gene expression by real time PCR.

Immunostaining Procedure

For staining, we used an automated stainer (Ventana Benchmark) with primary antibodies used as presented in Table 1.

Cell Culture

The human colorectal cancer cell line; CRC group (Caco3, CCL-247™) and normal Adult Human Primary Dermal Fibroblasts; HDFs group (PCS-201-012™) were purchased from American Type Culture Collection (ATCC). Both cell lines were cultured and grown in 6 well plates/ each cell line using RPMI-160 culture media with 10% Fetal Bovine Serum (FBS) supplemented with 1% penicillin/streptomycin and 2mM glutamine. Cells were incubated in a humidified atmosphere at 37°C CO2 incubator and media changed every 2 to 3 days. We assessed *TLR4* gene expression in both cell lines using quantitative RT-PCR. Additionally, we measured the immunostaining ability of both cell lines against the *TLR4* antibody (Santa Cruz Biotechnology; sc-10741) using immunocytochemistry technique.

RNA Isolation and RT-qPCR

Extraction of total RNA from tissues of all groups was achieved using Direct-zol RNA Miniprep Plus

(Cat#R2072, ZYMO RESEARCH CORP. USA). Afterwards, quantity and quality were evaluated by Beckman dual spectrophotometer (USA). Reverse transcription was performed on extracted RNA using SuperScript IV One-Step RT-PCR kit (Cat#12594100, Thermo Fisher Scientific, Waltham, MA USA) followed by PCR. 48-well plate StepOne instrument (Applied Biosystem, USA) was used in a thermal profile as follows: 10 minutes at 45 ºC for reverse transcription, 2 minutes at 98 ºC for RT inactivation and initial denaturation by 40 cycles of 10 seconds at 98ºC, 10 seconds at 55 ºC and 30 second at 72 ºC for the amplification step. After the RT-PCR run the data were expressed in Cycle threshold (Ct) for the target gene and housekeeping gene. The ΔΔ Ct method was used for normalization of variation in the expression of target gene; *TLR4* referring to the mean critical threshold (CT) expression values of β-actin housekeeping gene. The relative quantitation (RQ) of target gene is quantified according to the calculation of 2 -ΔΔCt method. Primers sequences for *TLR4* gene and β-actin housekeeping gene were; forward 5′- GCCTTTTCTGGACTATCAAG -3′ and reverse 5′-AATTTGAAAGATTGGATAAG -3′; forward 5′- CCTGTTCCTCCCTGGAGAAG -3′ and reverse 5′-CACTGTGTTGGCATACAGGT -3′ respectively.

Immunocytochemistry

Immunocytochemistry (ICC) for the analysis of *TLR4* protein expression. ICC was used to detect the immune-positive cells for *TLR4* in the CRC versus HDFs cells. ICC was performed in four sequential steps. Firstly, the cells were seeded on a 24 well plate for 24 hours for adherence. Secondly, fixation of the cells in 100% methanol/10min at room temperature in addition to permeabilization with 0.25–0.5% Triton X-100 in PBS/10 min. Thirdly, incubation for 24 h with *TLR4* primary antibody (1:500, Santa Cruz, Europe) was performed. Last step, washing after the immunostaining step was applied and secondary antibody anti-rabbit IgG (1:1000) was added for 1 h. Finally, the percentage number of *TLR4* immuno-expression cells was quantified in five images/ each group using the Image-Pro Plus program.

Estimation of LPS levels in serum

The serum levels of Lipopolysaccharides (LPS) were measured in serum samples from healthy control subjects, patients with IBD, and CRC cases using the ELISA kits (from Abbexa LTD, Cambridge, UK) according to the manufacturer's procedure. LPS is contained in the cell wall of Gram-negative bacteria.

Ethical considerations

The study protocol has been approved by Ain Shams University, Faculty of Medicine Research Ethics Committee (REC) FWA 000017585 with IRB approval number: FMASU R 130/2021 and followed the declaration of Helsinki regarding ethical considerations.

Statistical methods

For the statistical data analyses, SPSS program version 25, Excel and Project R software were used to calculate descriptive statistics of the variables and construct tables with expression scores of *TLR4*, *IgA* and EPCAM.

Quantitative data were presented as minimum, maximum, mean and SD. Qualitative data were presented as count and percentage. Chi square test (or Fisher Exact) was used to compare qualitative data between different groups. P value < 0.05 was considered statistically significant.

Results

TLR4 staining pattern was membranous expression in the neoplastic cells. 45 (88.2%) colorectal cancer cases showed positive staining, and 6 (11.8%) cases showed negative staining. Staining intensity was evaluated as, 0 represented negative staining (Figure 1a) and no immunoactivity, 1 represented a weak positive immunoactivity (Figure 1b), 2 represented a moderate intensity of staining, and 3 represented a strong intensity of staining (Figure 1c, Figure 1d). The percentage of *TLR4* positive tumor cells was scored on a scale of 0–4 (0: no staining; 1+: ≤ 10%; 2+: 11–30%; 3+: 31–50%; 4+: >50%). Furthermore, the *TLR4* percentage expression was divided as low or high based on the score (low for score 0-2 and high for score 3 and 4) [26]. *IgA* staining pattern was membranous ± cytoplasmic. Twenty-six (51.0%) cases were positive to *IgA* expression and 25 (49.0%) cases were negative. Expression pattern was either membranous apical (Figure 1e), membranous apical and lateral (Figure 1h), membranous and cytoplasmic (Figure 1f). Degree of expression in mucosal plasma cells was divided into low expression (when density of mucosal plasma cells was less than 50% of stromal inflammatory cells) (Figure 1e) and high expression (density of mucosal plasma cells more than 50%) (Figure 1f, Figure 1h). *EpCAM* staining pattern was membranous± cytoplasmic. (Figure 1i, Figure 1j, Figure 1l) and scored as 0: negative (Figure 1k), Weak, Moderate (Figure 1i), Strong [27]. Sixteen cases (31.4%) were positive for *EpCAM* expression, and 35 (68.6%) cases were negative.

Immunohistochemical expression of *TLR4* (Figure

Table 1. Primary Antibodies Used in the Present Study

Marker	*TLR4*	*IgA*	*EpCAM*
Clone	pAb clone (A5258) ABclonal, Woburn, MA 01801, United States	Mouse mAB (47C12): sc-69785 Santa Cruz Biotechnology, Inc. Bergheimer Str. 89-269115 Heidelberg, Germany	Mouse mAB (C-10): sc-25308, Santa Cruz Biotechnology, Inc.Bergheimer Str. 89-269115 Heidelberg, Germany
Dilution	1:100	1:100	1: 100
Expression	Membranous	Membranous ±cytoplasmic	Membranous

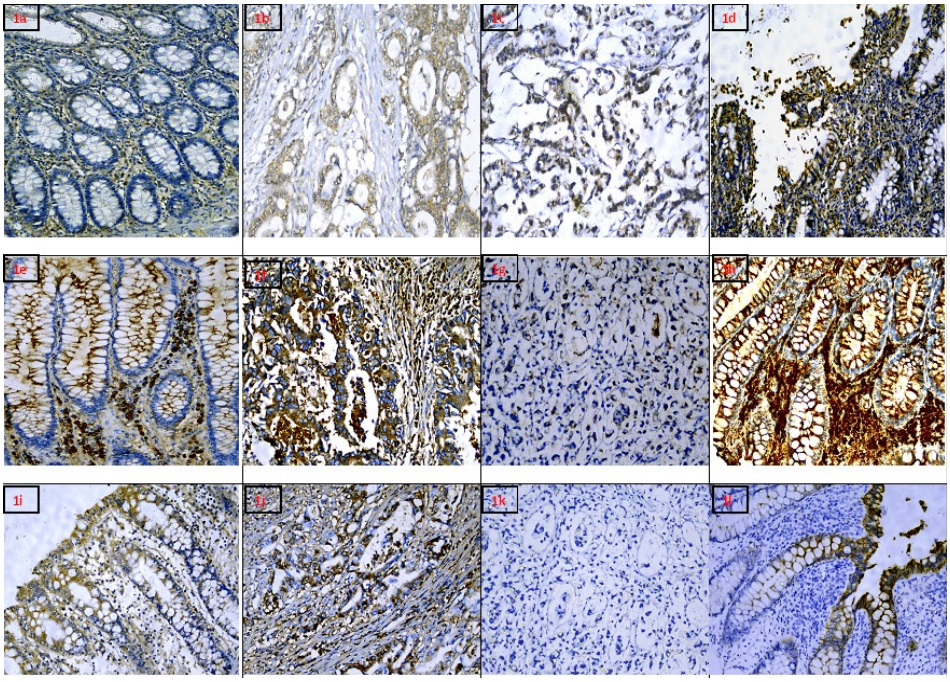

Figure 1. a, Control group showing negative TLR4 expression in colonic epithelium; b, A case of moderately differentiated adenocarcinoma showing Mild TLR4 expression; c, A case of mucoid adenocarcinoma showing strong TLR4 expression; d, A case of ulcerative colitis showing strong TLR4 expression in (Fig.1a-1d: TLR4x200); e, Control group showing Positive membranous apical expression of IgA in colonic epithelium with low mucosal plasma cell expression; f, A case of moderately differentiated adenocarcinoma showing strong membranous and cytoplasmic IgA expression in neoplastic epithelium with high mucosal plasma cell expression; g, A case of mucoid adenocarcinoma with weak IgA expression; h, A case of ulcerative colitis showing strong membranous apical and lateral IgA expression in colonic epithelium with high mucosal plasma cell expression (Figure 1e-1h: IgAx200); i, Control group showing positive membranous and focal cytoplasmic EpCAM expression in colonic epithelium; j, Moderately differentiated adenocarcinoma with strong EpCAM expression; k, A case of mucoid adenocarcinoma with negative EpCAM immunostaining; l, A case of ulcerative colitis showing positive moderate membranous expression in colonic epithelium. (Figure 1i-1l: EpCAMx200)

1d), *IgA* (Figure 1h) and *EpCAM* (Figure 1l) in cases of IBD was just scored as positive and negative as samples were obtained by endoscopic biopsy and tissues were limited for assessment. There was a significant correlation between *TLR4* intensity and both histologic type and tumor grade (P value 0.003 and 0.01 respectively). No significant correlation between *TLR4* intensity and other clinicopathological parameters. No significant correlation was detected between *TLR4* percentage expression and clinicopathological parameters.

There was a significant correlation between *IgA* expression and T stage (P value 0.02). There was a significant correlation between *EpCAM* expression and histologic type (P value 0.02). (Tables for immunohistochemical results are listed in supplementary files). In comparison of colorectal cancer patients to controls; there was a statistically significant different expression of *TLR4* positivity, *IgA* positivity and *EpCAM* (P value <0.001, 0.004, <0.001 respectively) (Table 2). Patients with colorectal cancer were compared to colitis patients and there was a statistically significant different expression of *IgA* positivity and *EpCAM* expression (P value <0.001) (Table 3).

Furthermore, Patients with colitis were compared to controls. There was a significant expression of *TLR4* positivity and *IgA* expression in mucosal plasma cells (P value <0.001) (Table 4). There was a statistically significant higher expression of *TLR4* in CRC cell lines than the fibroblast control group by both PCR (as fold change of gene expression) (Figure 2) and

Table 2. Comparison between Colorectal Cancer Patients and Controls

		Colorectal Cancer		Control		P value
		N	%	N	%	
TLR4	Negative	6	11.80	8	80.00	<0.001 HS
	Positive	45	88.20	2	20.00	
IgA	Negative	25	49.00	0	0.00	0.004 HS
	Positive	26	51.00	10	100.00	
EPCAM	Negative	35	68.60	0	0.00	<0.001 HS
	Positive	16	31.40	10	100.00	

*Chi square test (FE, Fisher Exact)

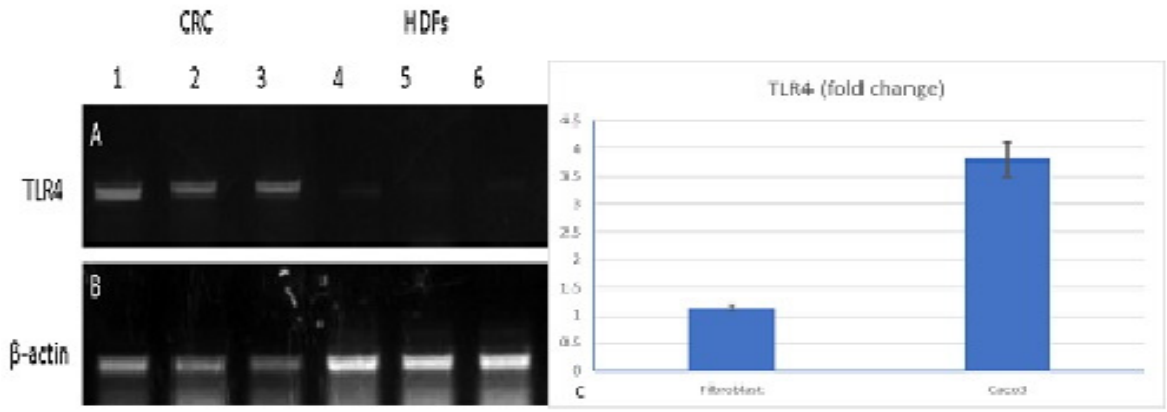

Figure 2. Agarose Gel Electrophoresis Showed PCR Products of TLR4 Gene with the Quantitative Aanalytical Data in All the Studied Groups: A: lanes 1-3: showed high density TLR4 gene in CRC group and 4-6 lanes: showed low density TLR4 gene in HDFs group; B: lanes 1-6: showed β-actin housekeeping gene PCR products in both CRC and HDFs groups; C: The quantitative fold change data for TLR4 gene expression in all studied groups. There was a significant increase expression of TLR4 gene in CRC patients (3.81±0.32, p value <0.001) compared to IBD patients (1.13±0.06, p value <0.001).

immunocytochemistry (protein expression as percentage of immune positive cells) (Figure 3). The mean serum level of LPS in colorectal cancer patients (300.59 ±164.65) was significantly higher compared to both the control group (111.67 ± 22.04) and the IBD group (152 ± 29.82) at P values <0.001 and <0.001 respectively. (Table 5). Meanwhile, no statistically significant difference was found when control group is compared to IBD group. (Table 6) (Figure 4). The clinicopathologic data were listed in supplementary files.

Discussion

One of the first studies that emphasized the role of *TLR4* in CRC is that by Tang and colleagues to investigate the expression of *TLR4* in human colon carcinoma cells and its function. They concluded strong correlation of *TLR4* and human colorectal carcinoma cells, and this expression led to immunosuppression and apoptosis resistance in cancer cells, which can augment the immune escape phenomenon in colon cancer cases. Although it was a preclinical study on Colon cancer cell line and the method of *TLR4* detection was PCR not like our study by IHC, still it highlighted the important role of *TLR4* in CRC carcinogenesis [28].

Dietary habits have changed in the last decades which could have a great impact colorectal carcinogenesis. Diet may disturb the immune response and provoke inflammation. Moreover, diet and early exposure to antibiotics can extremely affect the composition of the intestinal microbiota. Many studies highlighted that the microbiota could certainly reveal a ‘‘missing link’’ in the close interaction between dietary factors and CRC. The intestinal microbiota exhibits a high diversity in cultures that eat less-processed high-fiber diets [29]. Such data are of potential clinical relevance considering recent epidemiological trends in CRC in North America with an increased risk in younger people [30].

In our study, *TLR4* percentage expression was not associated with any pathological parameter (tumor size, site, histological type and grade, lympho-vascular invasion, perineural invasion, T stage and lymph node metastasis), while *TLR4* intensity was significantly associated with mucinous carcinoma (p= 0.003) and

Table 3. Comparison between Colorectal Cancer Patients and Colitis Patients

		Colorectal Cancer		Colitis		P value
		N	%	N	%	
TLR4	Negative	6	11.80	5	11.40	0.95 NS
	Positive	45	88.20	39	88.60	
TLR4 Intensity	Mild	8	17.80	8	20.50	0.94 NS
	Moderate	27	60.00	23	59.00	
	Strong	10	22.20	8	20.50	
IgA	Negative	25	49.00	0	0.00	<0.001 HS
	Positive	26	51.00	44	100.00	
EPCAM	Negative	35	68.60	5	11.40	<0.001 HS
	Positive	16	31.40%	39	88.60%	

*Chi square test (FE, Fisher Exact)

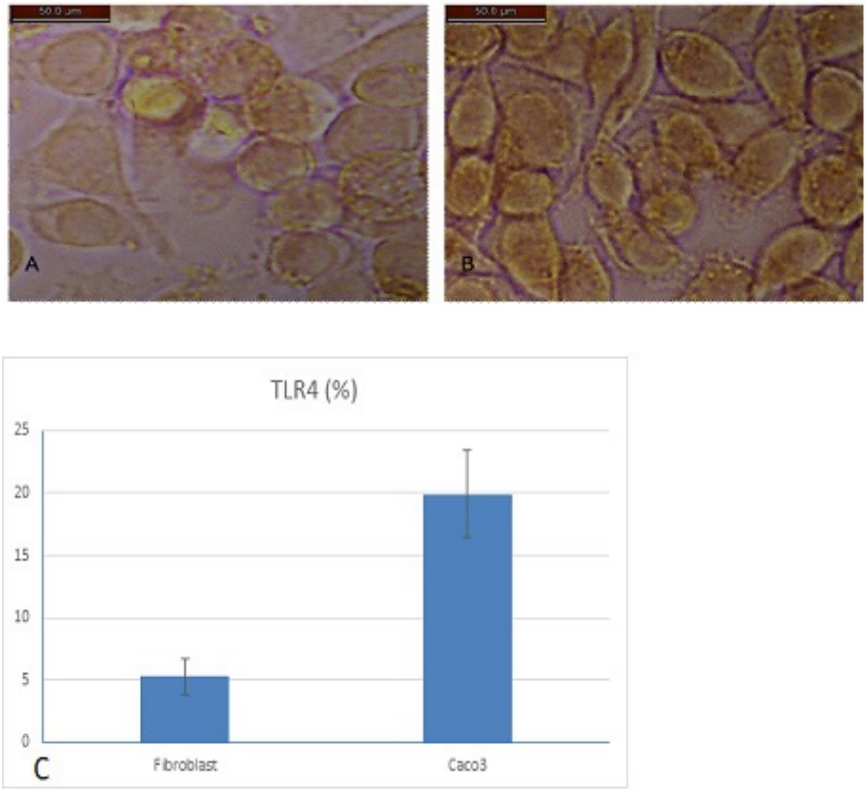

Figure 3. This Figure Showed ICC of TLR4 Protein Expression in Human Fibroblast Cells (A) and human adenocarcinoma cells (B) (in vitro study) with the quantitative analytical data (C). There was a significant increase expression of TLR4 protein in Caco3 cell line (19.9±3.49, p value <0.001) compared to fibroblast cell line (5.13±1.45, p value <0.001).

high grade (p= 0.01). In a recent work by Beilmann-Lehtonen et al. [31], *TLR4* was significantly correlated with gender (p = 0.006) and histological tumor grade (p < 0.001). *TLR4* expression was not correlated with other factors like age, the histological type of tumor, or tumor stage. *TLR4* expression was negative prognostic marker in stage II colon cancer (p = 0.017), a finding that could be implemented in the selection for the group of patients with stage II colon cancer that could get benefit from adjuvant therapy.

Additionally, our study showed a significant correlation of intensity of *TLR4* and mucinous histology. In the most of studies addressing the prognosis of mucinous as to non-mucinous colorectal adenocarcinoma, overall survival of mucinous colorectal adenocarcinoma patients tended to be poorer than non-mucinous colorectal adenocarcinoma patients [32]. Also, it was reported that mucinous histology tends to be higher in younger population [32], a finding can open a new field to understand the missing explanation of increasing incidence of CRC in young population which may be correlated to the change in the gut microbiota due to change in dietary habits and the excessive use of

Table 4. Comparison between Colitis Patients and Controls

		Control		Colitis		P value
		N	%	N	%	
TLR4	Negative	8	80.00	5	11.40	<0.001 HS
	Positive	2	20.00	39	88.60	
IgA in mucosal plasma cells	Low	10	100.00	0	0.00	<0.001 HS
	High	0	0.00	44	100.00	
EPCAM	Negative	0	0.00	5	11.40	0.57 NS
	Positive	10	100.00	39	88.60	

*Chi square test (FE, Fisher Exact)

Table 5. Comparison of the Levels of LPS in Control Subjects, IBD and, Colorectal Cancer Patients

	Control Mean + SD	IBD Mean + SD	CRC Mean + SD	P value
LPS (ng/mL)	111.67+22.04	152.00+29.82	300.59+164.65	<0.001

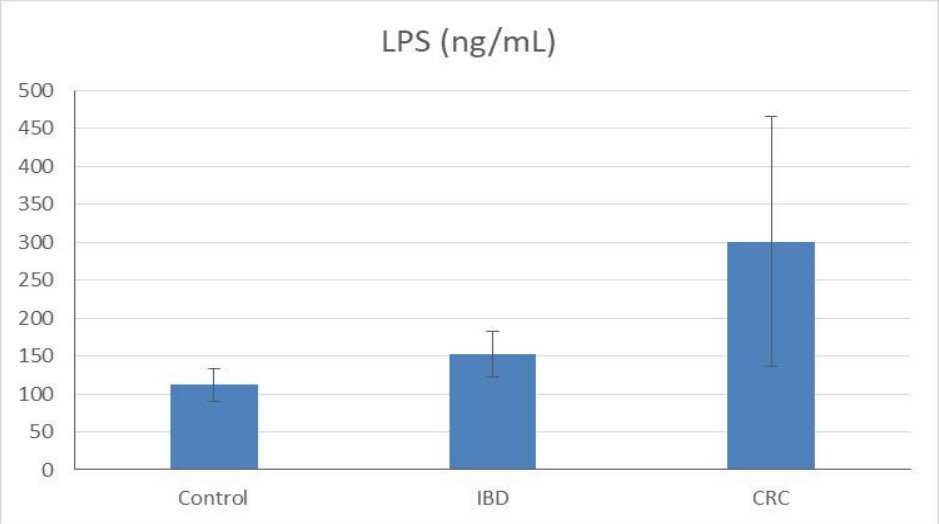

Figure 4. The Lipopolysaccharides Level in the Serum (ng/mL) of the Different Study Groups

Table 6. Pairwise Comparisons between Control Subjects, IBD and, Colorectal Cancer Patients Regarding LPS Levels

	Control	IBD
IBD	0.983	
CRC	<0.001	<0.001

Post-hoc pair wise comparison (P value between each group)

antibiotics. Our study showed a significant correlation between *IgA* and T stage. Our results were consistent with other studies which confirmed the same finding where patients with higher *IgA* have the worse prognosis on follow up [33]. A high intra-tumoral proportion of the *IgA* isotype was also associated with negative prognosis in the KRAS-mutant subtype of lung adenocarcinoma as well as bladder cancer [34].

There was a significant correlation of *EpCAM* expression in our study regarding the histological type, where there was loss of expression of *EpCAM* in 100 % of cases with mucinous carcinoma (p=0.02), while no correlation was found between it and other pathological parameters. In Han et al. [35] study, *EpCAM* overexpression was found to be higher in patients with high grade, with vascular invasion and higher T and N, with metastatic patients and with tumor budding. The loss of *EpCAM* expression was positively associated with tumor differentiation, tumor stage, vascular invasion, depth of tumor invasion, lymph node metastasis, distant metastasis, and tumor budding in CRC patients No correlation was found between the loss of *EpCAM* expression and tumor location or the tumor histology.

Another study conducted by Gaghana et al, [36] reported a significant relationship between *EpCAM* expression and colorectal adenocarcinoma with lymphatic invasion and metastasis. However, they didn't find a relationship between *EpCAM* expression and histopathological grade.

So according to these findings, *EpCAM* mediates the adhesion, proliferation, migration, invasion, stemness, and epithelial-to-mesenchymal transition of tumor cells as the disease progresses [37]. Moreover, Han et al. [35] suggested that *EpCAM* expression may be associated with the process of carcinogenesis, while the loss of *EpCAM* is correlated with tumor progression, metastasis, and poor prognosis. The current study failed to prove the prognostic role of *EpCAM* with the non-significant correlation with other prognostic factors, mostly due to limited sample size.

A significant correlation was detected in *TLR4*, *IgA*, and *EpCAM* expression between control specimens and CRC cases in our study regarding (in vivo specimens) and there was a statistically significant higher expression of *TLR4* in CRC cell lines in comparison to the control fibroblast group by PCR and immunocytochemistry.

Wang et al. [26] reported that *TLR4* expression was absent or very weak in the normal mucosae collected from biopsy samples and cancer margin samples. This finding is consistent with the findings in the present study. The expression of *TLR4* was also absent or weak in adenomas Compared with normal mucosae and adenomas, while the expression of *TLR4* was detected in a high proportion of cancers [38, 39].

In an informative meta-analysis done by Han et al. [35], 5 trials included 331 patients with CRC and 402 normal controls, the frequency of *EpCAM* expression was higher in CRC. In the same meta-analysis, two studies of 154 CRC patients and 56 benign colonic lesions revealed no significant difference regarding the *EpCAM* expression between normal mucosa samples and CRC patients.

This discrepancy is mostly due to the nature of the control. In some studies, the control was normal mucosa [40-43], while in 2 studies of them using comparisons with different controls, the control was benign colonic lesions [40, 43]. In our study, the control was the non- malignant surrounding margins of tumor specimens, a finding that is compatible with that of Mokhtari and Zakerzade, [27] as their study was done on tissues of patients with colon adenocarcinoma, together with normal tissues around them as control. In this study, 100 % of normal tissue showed positive staining to the *EpCAM*.

A highly significant correlation was detected in *IgA*, and *EpCAM* expression between colitis group and CRC cases in our study. Our data indicate that an important

feature of local mucosal immunity associated with colitis involves the *IgA* response. Other data revealed increase in colonic mucosal plasma cells in ulcerative colitis [44] though other reports reported opposite data. These discrepancies can be attributed to methodological problems and the selection problem of samples due to histopathological variability within specimens, especially in cases of active inflammatory bowel disease [45]. Other study reported the detection of *IgA* and IgG in fecal specimens from humans with intestinal infections [46].

In the current study, LPS serum level was significantly elevated compared to both the control group and the IBD group. These data are concordant with de Waal et al. [47] study which reported that LPS is significantly higher in plasma from CRC patients, compared to healthy people. Furthermore, several research have revealed that LPS can promote the release of immunosuppressive cytokines and proangiogenic chemokines, hence promoting tumour growth and metastasis [48, 49].

From the explored data we conclude that altered *TLR4*, *IgA*, *EpCAM* expression in both CRC and IBD might be related to the pathogenic role of microbiota in initiation and progression of both lesions and could represent potential prevention modalities and therapeutic targets. Also, LPS may contribute to the development of CRC and, subsequently, could be used as a screening biomarker.

Author Contribution Statement

DS, MMS, HA, AT: Idea, study plan, design research methodology; MMS, FS, LS: Collection and analysis of data, photographing and scoring of immunohistochemical results of pathology specimens on in vivo samples; DS, AT, NE, SE: preparation of cell lines and PCR, writing methods and results of PCR analysis and immunocytochemistry; GR, AG, HA: Collection and analysis of data, supervision of accuracy of scoring methods and reported clinicopathologic data, writing and revision of manuscript; All authors (MMS, AT, HA, DS, FS, GR, NE, SE, AG, LS) have shared equally and contributed significantly to writing, drafting, revision of manuscript and continuous follow up of the whole work and shared significantly with their ideas in the intellectual content.

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Study Approval

This work was permitted by the research committee of the Faculty of Medicine, Ain Shams University.

Ethical Approval

The study protocol has been approved by Ain Shams University, Faculty of Medicine Research Ethics Committee (REC) FWA 000017585 with IRB approval number: FMASU R 130/2021 and followed the declaration of Helsinki regarding ethical considerations.

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