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RESEARCH ARTICLE

Immunohistochemistry Study of Expression of TNF-α, TRAF-1, and TRAF-2 in Patients Suffer from Ulcerative Colitis

Ali Jabbar Eidan[1], Dr. Mayada Noori Iqbal[2], Dr. Liwaa Hussein Mahdi[3]

1. MSc Immunology / Assistant lecturer in Department of Basic Medical Science / Faculty of Nursing / University of Kufa. PhD student in Department of Medical Laboratory Technology / College of Health and Medical Technology / Middle Technical University.
2. PhD Immunology / Department of Medical Laboratory Technology / College of Health and Medical Technology / Middle Technical University.
3. M.B.Ch.B, F.I.B.M.S/ Pathology /Department of pathology / Faculty of Medicine / University of Kufa

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Corresponding Author

Ali Jabbar Eidan

Abstract

Background: Ulcerative colitis (UC) is a chronic inflammatory disease of unknown causes. It has involved the inflammatory process of the mucosa and sometimes the submucosa of the large intestinal tract. There are a several theories involve role of the immune mechanisms and cell signals that lead to activation of many intracellular adapter proteins and other markers, which in turn trigger an inflammatory process and death of many colonocytes.

Objective: investigation of the expression of TNF-α, TRAF1and TRAF2 in Iraqi UC patients and also indirectly, explain the direction of intestinal cell signaling toward cell survival or cell death.

Material and Method: A case-control study was conducted at the City of Al-Najaf Al-Ashraf using 56 and 30 individuals of both genders for Patients and Control groups with an overall age range of 18-75 years old. All sera (with negative Epstein - Barr virus (EBV) agglutination test) of all subjects were monitored for Tumor Necrosis Factor-alpha (TNF), TNF Receptor Associated Factor-1 (TRAF-1), and TNF Receptor Associated Factor-2 (TRAF-2) expression by Immunostaining Method. The Data was analyzed by using spss (chi-squire test) program (version 22).

Results: TNF-α, TRAF-1 and TRAF-2 expression were significantly differences in inflamed tissues of UC patients (all at score 4 were 78.6%, 44.6%, 62.5% respectively) than those in control (at Score 3 was 73.3%, at score 2 was 60%, and at score 1 was 56.7% respectively) (P value <0.05).

Conclusion: TNF-α, TRAF-1,and TRAF-2 shown to have an adequate colonoscopic biopsies biomarker for UC and it possibly use as a sensitive assays for monitoring severity of mucosa inflammation.

Recommendation: More work is needed to determine the precise molecular mechanism of TNF-α, TRAF-1and TRAF-2 signaling as well as identify the role of TNF-Rs in such mechanisms.

INTRODUCTION

Ulcerative colitis (UC) is one of the clinical presentations of inflammatory bowel disease (IBD) that affects large intestine lining, causing congestion, edema and ulceration of the mucosa. IBD is a multi-factorial disease with different causes including genetic, innate immune status of the individual and environmental factors [1]. The

etiology of UC is not fully understood, immune factors are reported to be associated with this disease. Higher expression of pro-inflammatory cytokines and intracellular adapter proteins trigger intestinal alterations, setting up a vicious cycle of chronic inflammation [2].

Tumor necrosis factor - alpha (TNF-α) is one of the important pro-inflammatory cytokines. It had receptors, TNF receptor 1 (TNF-R1) and TNF-R2, intermediate the biological functions of TNF-α, while TNF Receptor-associated factors (TRAFs), an intracellular adapter proteins, are involved in TNF-R signaling pathways that are associated with induction of other cytokines, cell survival, proliferation, and differentiation or cell death [3]. Tumor necrosis factor-receptor 1 (TNF-R1) excitation is supposed due to in the formation of two signaling complexes. When TNF binds TNF-R1, TNF-R-associated death domain protein (TRADD), receptor-interacting protein 1 (RIP1), TRAF-1/2, and probably other, as -yet-unidentified molecules, are employed to form complex I which it is suggested to activate downstream cell survival signaling. The second complex (complex II) forms when TRADD and RIP1 associate with FADD and caspase-8 to drive downstream cell death signaling [4].

Death induced by TNF-α is most tightly organized by nuclear factor kappa light chain-enhancer of activated B cells (NF-κB). Adjustment of the response in support of NF-κB keeps cells from apoptosis by TNF-mediated, washout to do so lead to increased cell death. Interestingly, TNF-α, itself is an activator of NF-κB, point out the presence of a complex controlling loop. After binding to TNF-α, TNF-R1 connects the adaptor protein TRADD which enlists caspase-8 via FADD causing the apoptosis. Otherwise, TRADD connected to the kinase RIP, TRAF1 and TRAF2 leading to the inhibitor degradation of NF-κB [5]. TRAFs are a family of proteins firstly implicated in the organization of inflammation, antiviral responses and apoptosis. Currently, seven TRAF proteins (TRAF1–7) have been characterized in mammals. These proteins have no intrinsic enzymatic activity but interact with a series of other proteins [6].

TRAF-1 and TRAF-2 can comprise a heterodimeric complex that implicated in the TNF-α mediated energizing of NF-κB and mitogen-activated protein kinase 8 (MAPK8)/c-Jun N-terminal kinase (JNK) signaling pathways. The complex interacts with inhibitors of apoptosis (IAP) and thus mediates anti-apoptotic signals from TNF-R1 [3]. In exhausted cells, TRAF-2 interacting with eukaryotic translation initiation factor 4GI, a scaffold protein, stops TNF-α signaling [7]. NF-κB signaling pathway, which is activated by TRAF-2, protects cells from endoplasmic reticulum (ER) stress-induced apoptosis [8]. In fact, ER stress plays a crucial role in the development of inflammation [9].

TRAF-1 has been established to be extremely expressed in the epithelial cells of the colon from both the control group and UC patients, although it mediates especially the anti-apoptosis effects [10]. Significant upregulation of TRAF-2 was found in mucosal CD4+ T cells from patients with IBD [11]. TRAF-2 can monitoring, the homeostasis of the colon to prevent the spontaneous development of murine IBD [12].

As TNF-α, TRAF-1, TRAF-2 are very related to inflammation and cell apoptosis, they possibly function as significant roles in the development of UC. In present research**,** we seeking about the expression of TNF-α, TRAF1, and TRAF2 in Iraqi UC patients and also indirectly, explain the direction of intestinal cell signaling toward cell survival or cell death.

PATIENTS AND METHOD

During the period from June/2014 to February/2015, eighty-six individuals from gastroenterology Centre in AL-Sader Medical city of Al-Najaf-Iraq had been recruited for this case-control research. Individuals were divided into two categories:

1. Patients Group: consist of 35 males and 21 females had been clinically diagnosed as UC with an age range of 19-70 years (mean of 41±15.475 years).

2. Control group: Thirty individuals of both genders; 17 males and 13 females, were included in this group who underwent examination by endoscopy due to gastrointestinal symptoms but were investigated to have no inflammation in the bowel. Their age ranges from 18 to 75 with a mean age of 42.67±15.816. Biopsies were collected from both groups and the diagnostic confirmed was depending on clinical, endoscopy, histopathological examinations.

Exclusion criteria were: (a) formerly diagnosed IBD., (b) Pregnancy., (c) Age < 18 years., (d) Antibiotic use when patients were admitted to the hospital and previous history of medical treatment., (e) Previous history of colectomy., and (vi) Other synchronous infection, chronic diseases or cancer.

Laboratory Analyses: ***1-Antecubital venous blood (3 ml):*** was drawn from each subject of the two groups and was put in tubes. Centrifuge apparatus at 3000 rpm separated sera for 10 minutes and the separated sera was used immediately for Epstein-Barr Virus (EBV)-agglutination Test to exclude cases with EBV infection. ***2-Biopsies:***. Two different mucosal areas were chosen in each patient with UC, including one inflamed colonic segment and one apparently normal mucosal segment, which were distant from the lesion. No congestion, edema or

ulcers were involved. Biopsy mucosal areas were chosen by under endoscopy from the control group. Biopsied specimens were regarded as adequate if they met all of the three following criteria [13]: (a) Diameter _ 3 mm, (b) Penetration into the muscularis mucosa, and (c) < 20% crush artifact.

Biopsy specimen was fixed in 10% neutral buffer formalin for at least 72 hrs. The fixed specimens were then submitted to grade of concentration alcohol 70%, 80%, 90% and 100%, followed by immersed in twice in xylene and embedded in paraffin. Sections of 4µm-thickness were obtained and, used in immunohistochemistry analysis for detection of TNF-α, TRAF-1and TRAF-2. Thymus, tonsil, liver and spleen sections recognized as a positive for TNF-α, TRAF-1 and TRAF-2 respectively using as positive control for every one run of immunostaining. Thymus, tonsil, liver and spleen sections were untreated with primary antibody of TNF-α, TRAF-1and TRAF-2 respectively were considered as negative controls for each set of each run. IHC was performed as described elsewhere using ImmunoCruz™ mouse LSAB Staining System (Code: sc-2050). The primary antibodies used were Anti- TNF-α monoclonal antibody (Monoclonal mouse Anti-human TNF-α Protein, 1ml Santa Cruz Biotechnology, Inc, USA (Code: sc-52746)), Anti- TRAF-1 monoclonal antibody (Monoclonal mouse Anti-human TRAF-1 Protein, 1ml Santa Cruz Biotechnology, Inc, USA (Code: sc-6253)) and Anti- TRAF-2 monoclonal antibody (Monoclonal mouse Anti-human TRAF-2 Protein, 1ml Santa Cruz Biotechnology, Inc, USA (Code: sc-136999)) All above primary antibodies were used a dilution of 1:50.

Counting or scoring of Immunoreactive staining was achieved by calculated the percentage of immunoreactive cells per total number of inflammatory cells and epithelial cells. The proportion of positively stained cells was evaluated as 1, 2, 3 or 4 score by estimation on screening wide areas within each tissue section: 1, <10% stained cells; 2, <30% stained cells; 3, <70% stained cells and 4, ≥70% stained cells, compared with the total cells [14,15].

Statistical Analyses: were performed using SPSS (version 22 Software, IBM, USA). Comparing the differences in the expression studied parameters between UC patients and control using chi-squire test. Missing values were not included in the statistical analysis. P<0.05 was considered to be statistically significant.

RESULTS:

First table and figures (1 and 2) show expression of TNF-α, in the normal colonic mucosa, the majority of TNF-α expression was 22 cases had score 3 (73.3%) in the interstitial space among colonocytes all along the crypt axis (Figure 2c).

In non-inflammatory mucosa, immunohistochemistry of patients revealed the majority of cases (22 out of 56) (39.3%) show a moderate TNF-α expression in inflammatory cells and epithelial cells in colon (score 3), (Figure 2b). While in the inflammatory mucosa, expression of TNF-α was much increased compared to its expression in normal epithelium and no found quite absent. The 44 (78.6%) from 56 biopsy of colon samples, appeared strong expression levels on the epithelial cells surface (score 4) and only 12 cases (21.4%) with (score 3) (Figure 2a). These results were highly significant (P value=0.000).

Table (1): IHC Score of TNF-α express in studied groups.

Studied groups		TNF-α IHC scores				Total	C.C. P-value
		1	2	3	4		
Patients (Inflammatory area)	Count	0	0	12	44	56	$\chi 2$ =56.169 C.C=0.532 P value = 0.000 (HS)
	%	0.0%	0.0%	21.4%	78.6%	100.0%	
Patients (Non-Inflammatory area)	Count	0	18	22	16	56	
	%	0.0%	32.1%	39.3%	28.6%	100.0%	
Control	Count	0	2	22	6	30	
	%	0.0%	6.7%	73.3%	20.0%	100.0%	
Total	Count	0	20	56	66	142	
	% of Total	0	14.1%	39.4%	46.5%	100.0%	

Chi-squire Test (In comparison between the three groups).

$\chi 2$: chi-squire, C.C: Correlation Coefficient, HS: High Sig. at P value < 0.05.

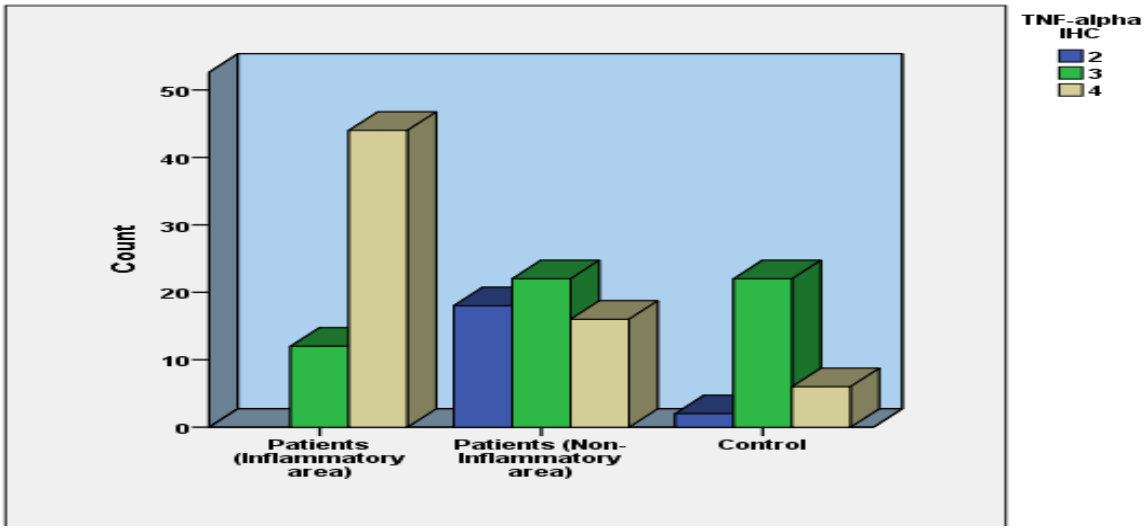
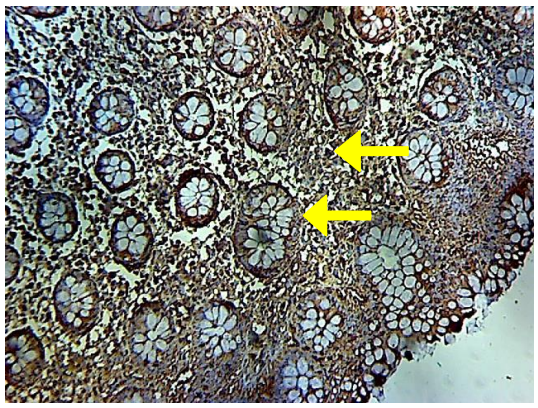
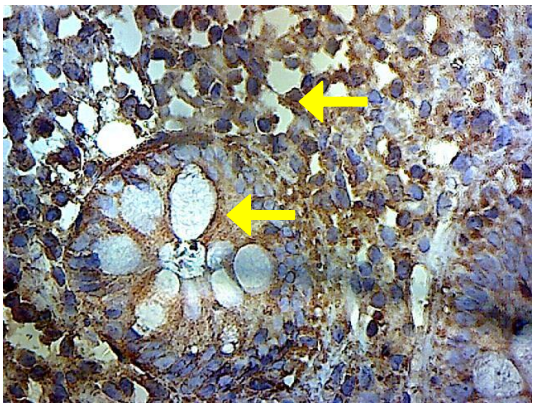


Figure (1): Distribution IHC score of TNF-α, in studied groups.

Biopsy	TNF-α (100X)	TNF-α (400X)
a) UC(inflammatory mucosa)		

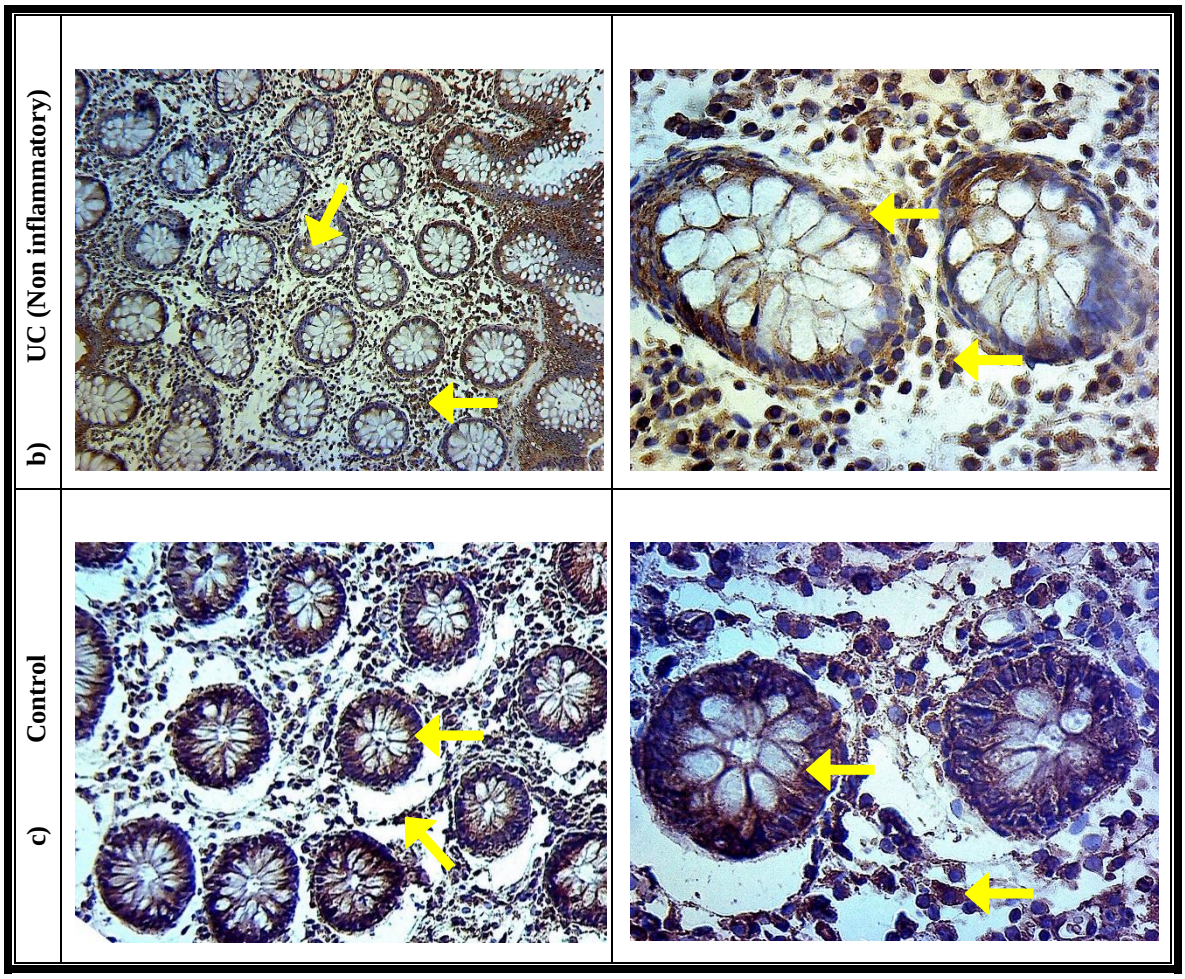


Figure (2): TNF-α Immunostain positivity in both epithelial cells and inflammatory cells in patients with ulcerative colitis.

Table (2) and figures (3 and 4) related to the normal colonic mucosa showed express of TRAF-1, the majority from TRAF-1 expression was 18 cases had (score 2) (60%) of colonocytes cytoplasm along the crypt axis and12 cases (40%) with (score 1) as (Figure 4c).

In non-inflammatory mucosa, immunohistochemistry revealed the majority of cases (43 out of 56) (76.8%) display a weak expression of cytoplasmic TRAF-1 in colonic epithelial cells (score 1), with moderate levels of expression (score 2) clarified by (23.2%) at the surface epithelium and the upper parts of the crypts of patients (Figure 4b).

In inflammatory mucosa, much increasing express for TRAF-1 compared to the normal epithelium and we not found completely lost. From our 56 colon biopsy samples, 31 (55.4%) appeared preserved of TRAF-1 expression in all epithelial cells and inflammatory cells (score 3) but 25 patients (44.6%) showed (score 4) (Figure 4a). These results were highly significant (p value=0.000)

Table (2): IHC Score of TRAF-1 express in studied groups.

Studied groups		TRAF-1 IHC scores				Total	C.C. P-value
		1	2	3	4		
Patients (Inflammatory area)	Count	0	0	31	25	56	$\chi 2$ =160.934 C.C=0.729 P value = 0.000 (HS)
	%	0.0%	0.0%	55.4%	44.6%	100.0%	
Patients (Non-Inflammatory area)	Count	43	13	0	0	56	
	%	76.8%	23.2%	0.0%	0.0%	100.0%	
Control	Count	12	18	0	0	30	
	%	40.0%	60.0%	0.0%	0.0%	100.0%	
Total	Count	55	31	31	25	142	
	% of Total	38.7%	21.8%	21.8%	17.7%	100.0%	

Chi-squire Test (In comparison between the three groups).

$\chi 2$: chi-squire, C.C: Correlation Coefficient, HS: High Sig. at P value < 0.05.

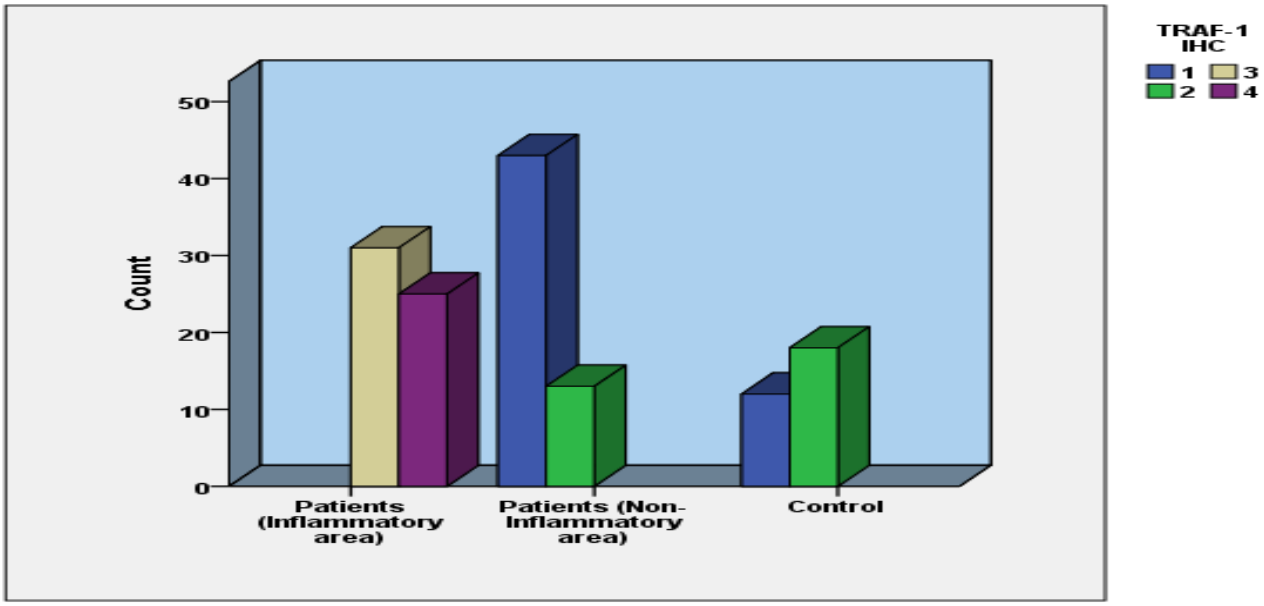


Figure (3): Distribution of IHC score of TRAF-1, in studied groups.

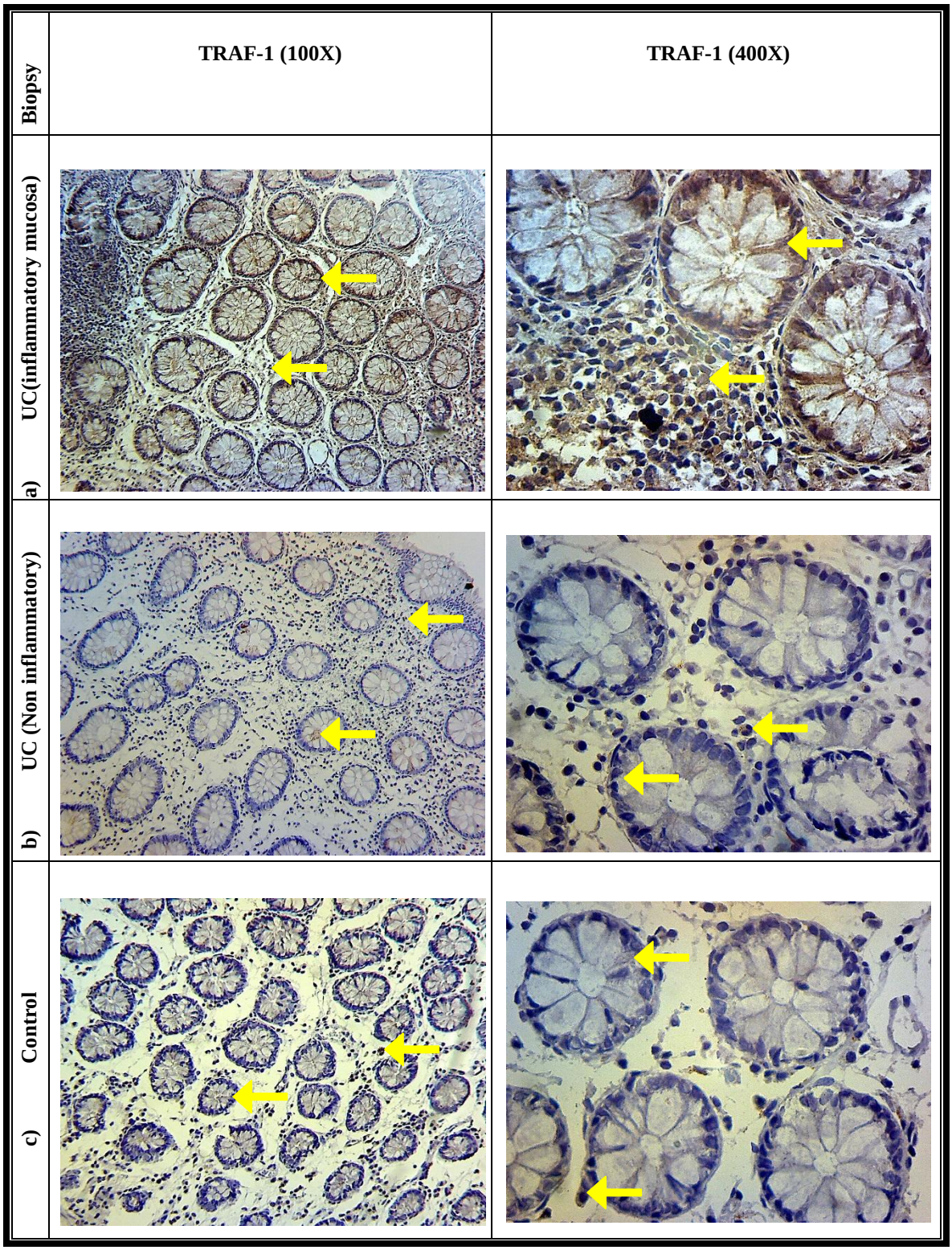


Figure (4): TRAF-1 Immunostain positivity in both epithelial cells and inflammatory cells in patients with ulcerative colitis.

Table (3), figure (5), and figure (6) show expression of TRAF-2 as in the same pattern of TRAF-1 expression, In the normal colonic mucosa, (score 1) in 17 cases appeared weakly expressed for TRAF-2 in colonocytes cytoplasm along the crypt axis and (score 2) in 13 cases (56.7% and 43.3% respectively) (Figure 6c).

Immunohistochemistry of non-inflammatory mucosa, appeared the common of cases (40 out of 56) (71.4%) show a weak cytoplasmic TRAF-2 appearance in colon epithelial cells and inflammatory cells (score 1), accompanied by average levels expression in the upper parts of the crypts and at the surface epithelium (score 2) within 16 cases (28.6%) (Figure 6b).

In inflamed tissues of mucosa, expression of TRAF-2 was prevailing increased comparing to the normal epithelium and present study did not appeared completely lost. From our 56 colon biopsy samples, 35 (62.5%) revealed the keeping for TRAF-2 expression in epithelial cells and inflammatory cells (score 4) while 21(37.5%) patients had (score 3) (Figure 6a). These results were highly significant (P value=0.000).

Table (3): IHC Score for TRAF-2 in studied groups.

Studied groups		TRAF-2 IHC scores				Total	C.C. P-value
		1	2	3	4		
Patients (Inflammatory area)	Count	0	0	21	35	56	$\chi2$ =145.145 C.C=0.711 P value = 0.000
	%	0.0%	0.0%	37.5%	62.5%	100.0%	
Patients (Non-Inflammatory area)	Count	40	16	0	0	56	
	%	71.4%	28.6%	0.0%	0.0%	100.0%	
Control	Count	17	13	0	0	30	
	%	56.7%	43.3%	0.0%	0.0%	100.0%	
Total	Count	57	29	21	35	142	
	% of Total	40.1%	20.4%	14.8%	24.6%	100.0%	

Chi-squire Test (In comparison between the three groups).

$\chi2$: chi-squire, C.C: Correlation Coefficient, HS: High Sig. at P< 0.05.

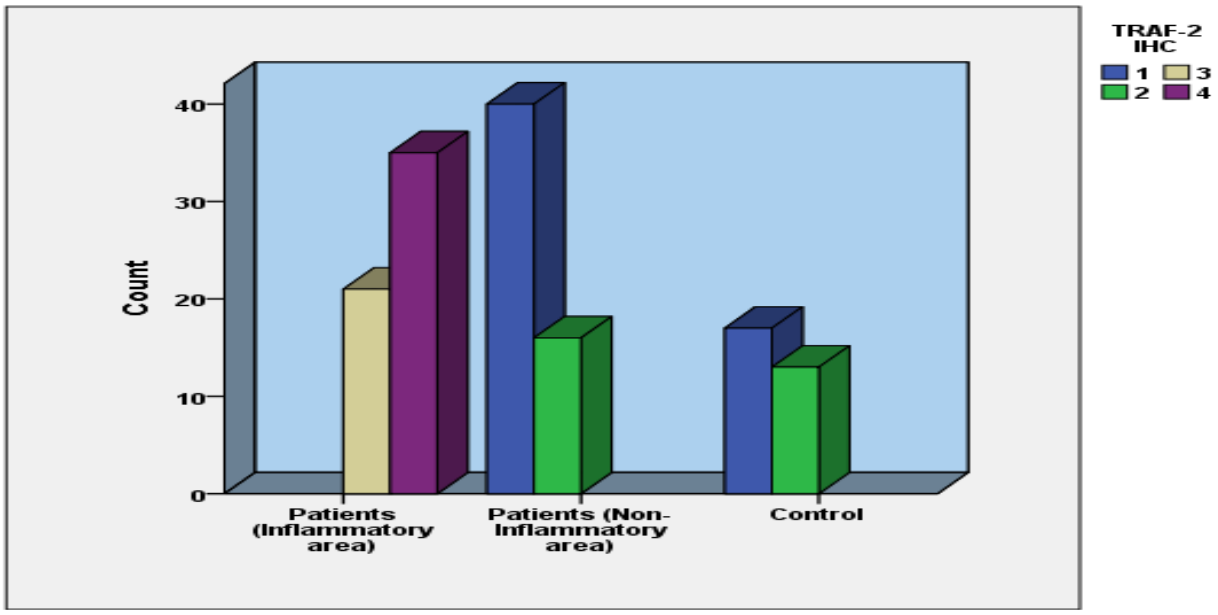


Figure (5): Distribution IHC score of TRAF-2 in studied groups.

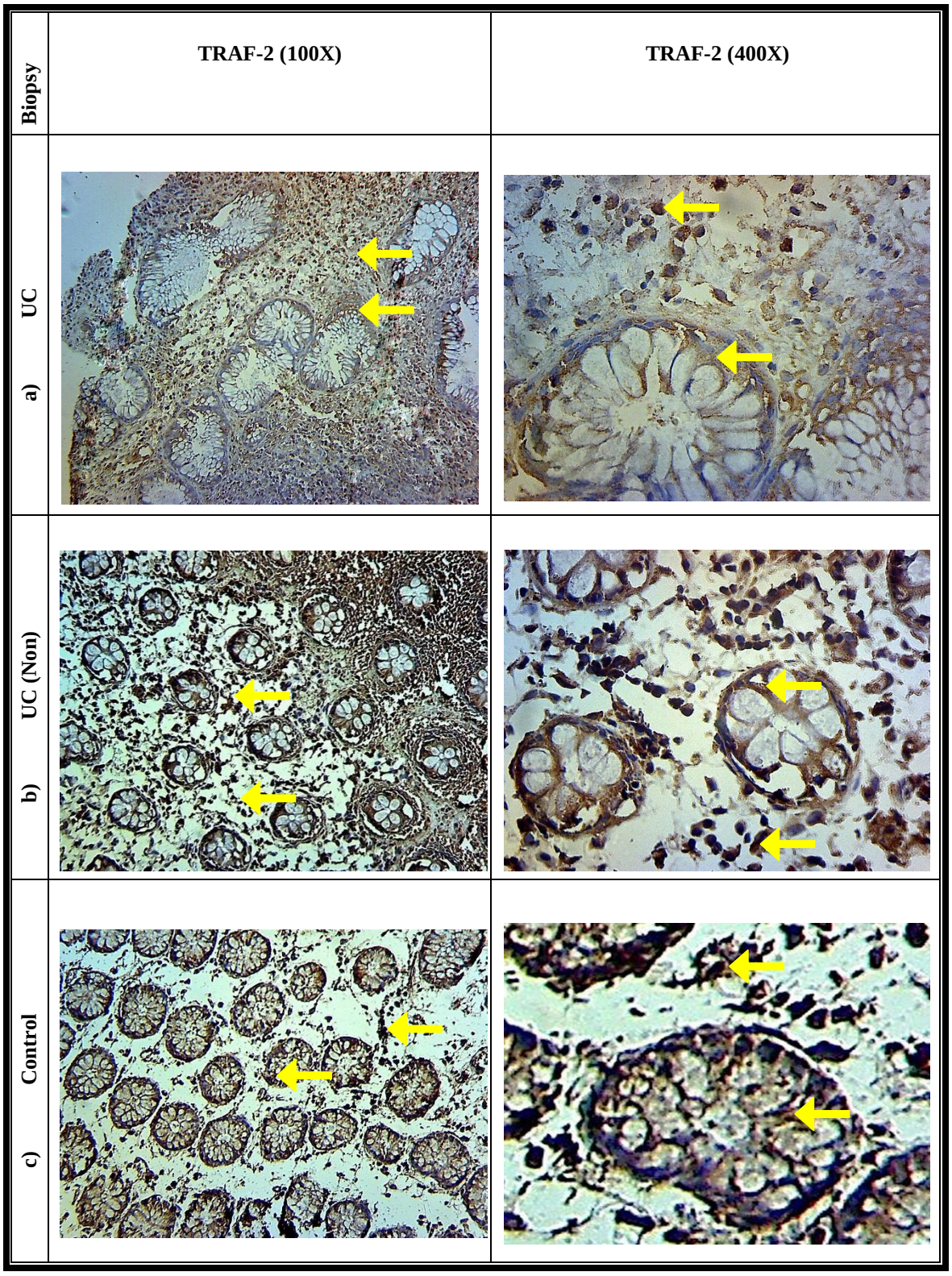


Figure (6): TRAF-2 Immunostain positivity in both epithelial cells and inflammatory cells in patients with ulcerative colitis.

DISCUSSION:

1) Expression of TNF-α:

A present immunohistochemical analysis showed score for TNF-α higher than the score of another study conducted by Dionne S. *et al*. in which the middling to strongly inflamed tissues (scores 2 and 3) appeared more TNF-α than inflamed tissues with (score 1) [16]. Low TNF-α release in non-inflamed specimens perhaps reflection for the small number of CD14+ cells existing in nearly normal mucosa. Moreover, implicated IBD mucosa is the location of dense accumulation of recently immigrated CD14+ macrophages, secret large amounts of pro-inflammatory cytokines [17, 18]. High levels of mucosal TNF-α secreted in UC might be results to infiltration of macrophages, which showed the migration of large numbers into the inflamed mucosa and sub mucosa of the intestinal lumen during active UC [19].

A recent study used a pokeweed mitogen (PWM) and Lipopolysaccharide (LPS) to stimulate TNF-α release from lamina propria mononuclear cells (LPMC) isolated biopsy from UC patients and controls, the results showed greater releasing from inflamed tissues. Also Rugtveit *et al*. [19], who found that PWM-stimulated LPMC from implicated IBD tissue released greater TNF-α than cells which isolation from controls. The significant role of newly enlist macrophages is corroborative by the observation that PWM and LPS induced TNF-α released threefold higher from severe inflamed UC tissues. Previous studied appeared the secretion patterns of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) from LPMC which isolated from colonic biopsies from UC patients, they showed that the spontaneous secretion of TNF-α by isolated LPMC was very low from normal and non-involved UC mucosa [20]. Conversely, the spontaneous secretion of TNF-α by LPMC which obtaining from involved UC mucosa was high in comparison with the control group. We suggested that determination of pro-inflammatory cytokines secretion might a sensitively test for oversight the severity of mucosal inflammation of disease.

Monocyte and macrophage activation by normal bacteria and their products could be altering physiological epithelial ion transport and transepithelial electrical resistance [21]. These changes may elucidatethe chronic diarrhoea in such patients. Dionne *et al*. demonstrated that the immune response elicited by staphylococcal enterotoxins B perhaps allow stimulation of abnormal immunological responses and potently increased TNF-α release from histologically normal colonic mucosa suggests that it could be an important mediator in the initiation of the inflammatory cascade [22] Superantigens induced greater TNF-α release in inflamed tissues. This might due to increased total T -cell numbers, increased subpopulations of T cells related to gut homing or modifications dependent onto the mucosal environment, express of MHC on antigen presenting cells or co-stimulatory molecules, such as ICAM, LFA-3 [23]. It spotted that the TNF-α released from un stimulated biopsies was higher in colonic inflammation patients [24].

Nevertheless, no a clear object to presume that only macrophages secrete TNF-α in inflamed bowel then it is now well proven that T cells can also secrete this cytokine [25]. Also, many mediators might produce in bowel with inflammation; it has lately exhibited that IL-1 secreted by macrophages of lamina propria is raised in UC patients [26]. Other explication is that TNF-α may released by cellular sources others than T cells and macrophages, including fibroblasts, eosinophils, mast cells, epithelial and paneth cells [27]. Mast cells showed releasing of TNF-α after contact with bacteria causing neutrophil influx into the lung and peritoneum [28], whereas invasion epithelial cells with bacteria induced the release of TNF-α and chemokine [29]. Regarding, T cells were not essential to induce this inflammatory response [22].

A greater TNF-α release in inflammatory area than in non-inflammatory area of UC (Figure 4.3a and 4.3b) was agreement with recent data that confirmed the releasing of TNF-α greatly influenced by severity of inflammation. Furthermore, when the biopsies were classified according to their inflammation severity score, there was a significantly difference between inflammatory area of UC and normal control. Therefore, colonic TNF-α can be used as a marker to distinguish UC tissue.

It is also improbable that production of TNF-α is particular for UC disease and that as more enteropathies are studied, TNF-α production could be found to be common features. The studies of the cytokine levels in IBD at the actual sites of inflammation in the intestine led to a paradigm in which UC is a Th2-associated disease, whereas in CD the Th1/Th17 cytokines play a major role driving the inflammatory response [27].

TNF-α, a type 1 cytokine, played a major role in UC pathogenesis in present study. It was over-expressed in patients with an active disease. Such evidence tightly mirrors the high ability of infliximab in prompting and keeping absolution in moderate-to-severe UC patients [30].

By a number of different mechanisms, TNF-α may regulate, amplify, and perpetuate inflammation of mucosa. TNF-α caused increasing the adhesion molecules endothelial-leukocyte adhesion molecule- (ELAM-1), intercellular adhesion molecule-I (ICAM-1), and vascular cell adhesion molecule-I (VCAM-1) expression on endothelial cells, which participate to the increased adhesion of leukocytes to the mucosal vascular system [21]. TNF-α also enhances the IFN-γ induced expression of MHC class II on intestinal crypt epithelium [31]. TNF-α

produced by sub epithelial cells may reduce epithelial integrity [32] particularly in the presence of IFN-γ [33]. IFN-γ may also cooperation with TNF-α in several pro-inflammatory ways, increased the expression of adhesion molecules and MHC class I and II, [34] and up regulating macrophage production of free radical [35]. There is clearly of possibility within the intestine for direct activation of sub epithelial macrophages by LPS from the luminal flora, particularly after any breach of epithelial integrity. In UC epithelial permeability is increased, and it is noteworthy that faecal diversion perhaps protective [36].

The perivascular production of TNF-α, also concurrent with known patterns of complement deposition, is likely to be particularly damaging. It binds to high affinity endothelial cell surface receptors, and acts to increase inflammatory cell recruitment by upregulating expression of both adhesion molecules and the chemotactic cytokine IL-8. TNF-α can be inducer to activation and degranulation of granulocytes and macrophages, with the resultant in secretion of oxygen radicals, nitric oxide, prostaglandins, leukotrienes and proteases [37]. TNF-α could exacerbate inflammation by increasing permeability the endothelial cell and epithelial cell, and also can stimulate and activate cytotoxic T cells. TNF-α had capability of stimulating different cell types to increase the secretion of potent chemotactic cytokines such as IL-8 [38].

Furthermore, epithelial cells are constantly shed from the tips of villi at an estimated rate of 1400 cells/villus/24 h [39]. The epithelium forms a barrier between the body and the GI lumen, so shedding poses a threat to the integrity of the barrier. In sanitary persons, the barrier is maintained by a rearrangement of tight connection proteins around the shedding cell, which plugs the gap created by the extrusion process [40, 41].

In spite of the epithelial barriers are largely well-kept during TNF-induced cell shedding, it does start to be unsuccessful at some locations [42]. Only single epithelial cells of healthy persons are shed from a specific site. Nevertheless, TNF-α exposure increased the rate of shedding, and multiple cells are derived from a single site. In this case, tight junction proteins might not redistribute widely enough to seal the large gap or microerosion left by the loss of multiple cells. This loss of barrier role could be involved in the pathogenesis of IBD. Confocal laser endomicroscopy has identified epithelial cell shedding and sites of local barrier dysfunction [43].

Earlier studies declared that inflammatory cytokines show significant roles in the IBD pathogenesis [21, 44, and 45]. In particular, intestinal mucosal biopsy specimens from patients with UC appear increased TNF-α mRNA and protein expression beyond the amounts observed in healthy control subjects [46]. Different observations suggested that accurate measurement of TNF-α in serum could give pathogenetically and clinically important information in IBD [47].

Murch *et al*. noticed TNF-α protein in mucosal tissues of UC and CD patients by IHC and ELISA, clarify that TNF-α positive cell were more numerous in submucosa and lamina propria of UC patient's specimens[48]. Dionne *et al*. [46] studied the expression TNF-α mRNA in biopsy of mucosal specimens of IBD patients and establish that TNF-α mRNA expression was greater in CD patients than in UC patients.

In view of the high expression of TNF-α producing cells in active UC, and the well characterized effects of this cytokine, it is probable that TNF-α makes an independent giving to inflammatory tissue damage [47].

2) <u>Expression of TRAF-1:</u>

TNF-Receptors Associated Factors (TRAFs) are the molecules that onto correlation of the TNF-R by a TNF ligand reach first in interaction with the triggered TNF-R, at first acting as docking molecules for kinases and additional effector proteins that are recruited to the activated receptor. TRAFs later control the subcellular relocalization of the receptor-ligand complex and they modulate the extent of the response by governing the degradation of key proteins in the pathway [49].

According to our knowledge, there are two studies demonstrated the expression of TRAF-1. One study has been published and analyzed TRAF-1 expression pattern in UC and it depend on mRNA expression rather than IHC and showed that TRAF-1 mRNA was highly expressed in UC patients, but the same phenomenon was also observed in normal control, and biopsy did not showed differences between inflamed and non-inflamed tissues [10]. Another paper by Yu Qi *et al*., which investigated the patterns of TRAF-1 and TRAF-2 expression (mRNA and IHC) in UC patients and showed that TRAF-1 was expressed mainly in inflammatory cells and epithelial cell membrane, while a small amount distributed in the cytoplasm in UC patients and controls. TRAF-1 expression the mucosa of colonic patients with UC was significantly higher compared with controls [3]. Similarly, in the present study, overexpression TRAF-1 showed to be existent in colonic mucosa of UC patients. In inflamed UC tissues, the expression of TRAF-1 was appeared higher than that in non-inflamed tissue; indicate that TRAF-1 was up regulated in the process of inflammation. Inflammation of UC does not always affect the whole colon. Although the non-inflamed segments showed normal under endoscopy, changes of proteins expression are not fully understood.

Many investigators have contributed to knowledge filed of the plethora of TRAF-1-interacting partners. A variety of binding proteins of TRAF-1 inclusive receptors, kinases, adaptors and regulator proteins have been fixed by yeast-two hybrid screening, *in vitro* bound and over-expression experiments. As review showing TRAF-1 directly interacts with the cytoplasmic domains of distinct members of the TNF-R superfamily such as CD30, 4-1BB, OX40, ATAR/HVEM, TRANCE-R/RANK, AITR, EDAR, TAJ/TROY [50, 51]. TRAF-1 can also be recruited to the TNF-R1 and TNF-R2 through its interaction with TRADD [52] and TRAF-2 [53] respectively.

Furthermore, family members of TNF-R- in general operate more than one TRAF family members for signaling, often activating similar pathways and even the identical downstream effectors. For this reason , the levels of expression of the different TRAF-family members and downstream effectors to be expected it play an essential role in the consequence to the response [50].

TRAF-1 can be induced by several featured types' stimuli such as IL-1, TNF-α, CD40 ligand [54] or EBV infection [55]. It demonstrated by *in vitro* and *in vivo* activation of T cells and B cells using a variety of sensitively which results in a strong up-regulation of TRAF-1 [56]. So, the high expression level of TRAF-1 in UC may be due to high expression level of TNF- α.

Schwenzer and coworkers [55] accomplished *in vitro* DNA binding assays, organizer-driven reporter assays, and RNase protection assays with the human *TRAF-1*gene. They showed that *TRAF-1*gene promoter containing various functional κB sites is highly inducible by NF-κB. These results describe why different stimuli which activate NF-κB also can stimulate TRAF-1 expression. A previous study [57] suggested that TRAF-2 can prompt TRAF-1 expression by the NF-κB signal transduction pathways. in spite of TRAF-1 itself cannot activate NF-κB, it is believed that TRAF-1 is contribute in the NF-κB regulation, may be by formation of heterodimer with TRAF-2 as well as TRAF-1 itself is a target gene of the NF-κB signaling pathway, it might do as a feedback regulator of NF-κB activation [58].

The important function of TRAF-1 is supposed to be suppression of TNF-α or T cell receptor which mediated apoptosis. After binding of TNF-α to TNF-R1, TRADD recruits ancillary proteins such as TRAF-1, TRAF-2, cIAP1 and cIAP2 [59] which can suppress TNF-α dependent activation of caspase-8 resulting prohibition of apoptosis [60]. The accurate manner by which TRAF-1 overexpression inhibits the stimulation of apoptosis is not clear. As TRAF-1 does not include any identified catalytic domain and TCR-induced apoptosis of CD8+ T cells is intermediated by signaling complex of TNF-R2, it is most likely that overexpression of TRAF-1 inhibition the apoptosis that mediated with TNF-R2- by changing the components of signaling complex of TNF-R2. It is also potential that TRAF-1 may mediate an as-yet-to-be determined anti-apoptotic signal during antigen-prompted cell death of mature T cells [59, 61]. Only a recently study was shown that TRAF-1 shifts the quality of integrated TNF-R1-TNF-R2 signaling from induction of apoptosis to pro-inflammatory NF-κB signaling [62].

Interestingly, TRAF-1 has ability altered into a pro-apoptotic version after cleavage by an upstream initiator caspase-8 during TNF-α-induced apoptosis. Caspase-8 cleaves TRAF-1 into two fragments. High expression of the C-terminal TRAF-1 fragments but not N-terminal fragments reinforce the TNF-R1- and Fas-mediated apoptosis. Henkler and coworkers elucidated that TRAF-1 and its cleavage product selectively interfere with the TRAF-2 recruitment to some TNF-R family members. They also demonstrated that the C-terminal portion of TRAF-1 but not full-length version blocks activation of IKK through a physical interaction with IKK. [59].

Harmonious with the anti-apoptotic function of TRAF-1, epithelial cells without TRAF-1 were high sensitive to apoptosis induced by TNF-α [63], and TRAF-1-deficient dendritic cells exhibited severely weakened survival in reply to TNF-α and CD40Ligand [64]. Additionally, obligatory expression of TRAF-1 in T cells close reactive T cells apoptosis consequently preventing antigen-induced tolerance [61].

Interfering with TRAF-1 role might subsequently improve TNF-R1 and TNF-R2 replies and hence be exhibition to chronic inflammation and autoimmunity. In this consideration, expand of TNF produced by reactive leukocytes is a common feature of several autoimmune diseases, comprising rheumatoid arthritis (RA), CD, UC and other chronic inflammatory diseases. Production too much of TNF-α can lead synovial inflammation and degradation of articular cartilage and bone, which are public features of RA. In UC, great levels of TNF-α reason colon inflammation [65]. So, though if TNF-α level stay normal, targeting TRAF-1 might raise the responsiveness of T lymphocytes and triggering autoimmunity [50].

Although expression of TRAF-1 is expressively higher in non-inflamed tissue of patients than in control and it possibly be activated early in UC patients, further studies are required to testing whether TRAF-1 may be trigger before the onset of the disease.

3) <u>Expression of TRAF-2:</u>

According to our knowledge, only one research on a murine model has been analyzed TRAF-2 expression patterns in UC and proposed that TRAF-2 may possibly control the colon homeostasis to prevent UC [13]. As well as, another recent paper by Yu Qi *et al*., which analyzed TRAF-1 plus TRAF-2 expression patterns in patients with

UC and showed that TRAF-2 was expressed mostly in inflammatory cells and the epithelial cell membrane, while a small amount was prevalence in the cytoplasm in UC patients and controls. TRAF-2 expression in the colonic mucosa of patients with UC was not considerably higher than, those in control [3].

In the present study, TRAF-2 was revealed to be existent in colonic mucosa of UC patients and control, and high expression of this protein may be common in UC. In inflamed UC tissues, the expression of TRAF-2 was remarkably upper than in non-inflamed tissue, proposing that TRAF-2 was upregulated in the process of inflammation.

Together TRAF-1 and TRAF-2 are implicated in TNF-R signal transduction pathways [66]. TRAF-1 frequently works jointly with TRAF-2 to compose a heterodimeric complex. In the complex, TRAF-2 interacts straight with TNF-R2, which is an activator of JNK like TNF-R1. The complex is not only related with the TNF-α intervened activation of JNK and NF-κB but also cooperates with IAP, which prompts the anti-apoptosis process. Earlier studies have suggested that TRAF-2 could be employed to TNF-R1 indirectly through a specific interface with TRADD, which ensures the employment of IAP and inhibits the activation of caspases [67].

A natural question gets up as to when or which situation does TNF-R1 stimulation and lead to apoptosis or cell survival? One likely answer might be through the mitochondrial liberation of Smac protein through JNK activation [68]. Smac may react with cIAPs and take away them from TRAF-1 and TRAF-2. Two probable answers may untruth on the NF-κB-inducible protein c-FLIP. In the lack of NF-κB activation and c-FLIP, TNF-R1 can encourage cell death through a cytoplasmic complex containing TRADD, RIP1, FADD, and caspase-8 activation [69]. Another possible answer, the greater affinity between TRADD and TRAF-2 propose that TRADD may be a sturdy inducer of TRAF-2 signaling. [70].

Furthermore, researches showed that much expression of the survival receptor TNF-R2 sensitizes cells to TNF-α prompted apoptosis [71,72]. Moreover, TRAF-2 seems to be positive signals inducers for cell growth and proliferation which mediated by kinase cascades and the genes induction of NF-κB. Once these molecules are rich, TNF-R activation acts to promote a stress response. [73]. Therefore, availability and function of TRAF-2 proteins may control a cell survival barrier involving either a stress response or programmed cell death. However, TRAF-2 has controlling role on apoptosis may be cell dependent and/or TNF-R dependent. Yet, there is no proof backing an anti-apoptotic activity for TRAF-2 in colonic epithelial cells of UC patients.

A recent research [74] proposed that TRAF-1 and TRAF-2 superior form the TRAF-1 :(TRAF-2)2heterotrimer, which could interacts with cIAPs further toughly comparing with TRAF-2 alone. Additional study [75] appeared that TRAF-2 work together with TRAF-3 might regulator the development and survival signal transferred to B cells, and played a double role in regulating B cell homeostasis. Another recent study has shown a tumor suppressor character for TRAF-2 in B cells. The B cells lacking functional TRAF-2 in transgenic mice revealed the development of Small B cell lymphoma / Chronic Lymphocytic leukemia with high rate [76].

Similarly, T- cells that play critical roles in the expansion of UC, current researches have revealed that TRAF-1 and TRAF-2 are demanded in the T cell response to 4-1BB signaling, and 4-1BB recruits TRAF-1 and TRAF-2 to mediate survival signaling in T cells. Yet the TRAF-2 is necessary in the glucocorticoid inducible TNF-R-linked protein-signaling pathways [77].

Animals which lack TRAF-2 appeared normally birth, but reduced the body weight was expressively comparing to control littermates and the knockout animals became rusted and died early within 3 weeks. With the exemption of thymus and spleen, all organs seem normal but with reduced sizes. Remarkably, overexpression of the TRAF-2 transgene managed to splenomegaly and lymphadenopathy. So, in spite of TRAF-2 has a clear anti-apoptotic functions, it can moreover link TNF-R family members to pro-apoptotic pathways [78].

Based on these hypotheses, TNF-α ability to prompt apoptosis within FADD and caspase-8 promote survival through TRAF-2 recruitment and NF-κB induction [79, 80].

Consequently, it is reasonable to think that suppression of NF-κB activation in UC could be intermediated, as a minimum partly, by TNF-R/TRAF-2 driven mechanisms. In addition, high expression of TRAF-2 in inflamed tissue of patients might be considered one of the mechanisms share to the expansion of UC, additional studies are needed to explore whether TRAF-2 can be triggered before the disease onset.

CONCLUSION

In this study, we obtained increased expression of TNF-alpha, TRAF-1 with TRAF-2 in active UC patients. The TNF-α, TRAF-1,and TRAF-2 are an adequate colonoscopic biopsies biomarker for UC patients. In addition, it might be sensitively assays for observing mucosal inflammation severity.

The TRAF-1 and TRAF-2 proteins were implicated in the intracellular signaling pathways leading from TNF-Rs. These indicating steps are strongly interrelated in a network of additional signaling molecules and regulating effectors the overall outcome of the cellular TNF-α response. In overall, TRAF proteins, especially the TRAF 2

molecule, are the focus of regulatory network. An particular comprehension of the understood regulatory operation will finally allow us to predict a exact cellular response in progress by TNF-α and may then develop the usage of the pathophysiological aspects of TNF-α.

RECOMMENDATION

Extra work is required to decide the accurate molecular mechanism of TRAF-1 and TRAF-2 signaling. For example, is TRAF-2 in monomeric or in a constitutive trimeric state before recruitment to receptors? Is the activation of downstream effectors dependent on oligomerization or on conformational alterations induced by receptor interface? What is the exact molecular basis for this activation? Finally, the maximum challenge will be in translating the structural and functional studies into potential therapy.

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