Investigation of Serum TNF-alpha, TRAF-1, andTRAF-2 in Patients Suffer from Ulcerative Colitis

التقصي عن عامل نخر الورم (نوع الفا) والعامل المتعلق بمستقبلات عامل نخرالورم الأول والثاني في مصول المرضى المصابين بالتهاب القولون التقرحي

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الخلاصة:

خلفيةالبحث:التهاب القولون التقرحي (UC) هو مرض مزمن مجهول السبب يتصف بالتهاب الغشاء المخاطي وأحيانا التهاب الطبقة تحت المخاطية من الأمعاء الغليظة. هناك عدة نظريات تنطوي على دور آليات المناعة وإشارات الخلية التي تؤدي إلى تفعيل العديد من البروتينات المتحولة داخل الخلايا وعلامات أخرى، وهذا بدوره يقدح عملية الالتهاب وموت الكثير من خلايا القولون.

الهدف: التقصي عن مستوى عامل نخر الورم نوع الفا والعامل المتعلق بمستقبلات نخر الورم الأول والثاني في مصول المرضى المصابين بالتهاب القولون التقرحي وكذلك معرفة دور تلك العوامل في تشخيص المرض.

المنهجية:في دراسة مقارنة أجريت في مدينة النجف الأشرف باستخدام 56 و 30 شخصا من كلا الجنسين للمرضى الصابين بالتهاب القولون التقرحي ومجموعة التحكم مع الفئة العمرية 18-75 سنة. تم جمع الأمصال من كل المجاميع بعد التأكد من الاختبار السلبي لفيروس ابشتاين بار (EBV). وقد تم قياس مستوى نخر الورم عامل ألفا TNF-alpha والعامل المتعلق بمستقبلات نخر الورم الأول والثاني (TRAF-1)، (2- TRAF) باستخدام تقنية الامتزاز الأنزيمي (ELISA). وباستخدام برنامج الـSPSSتم تحليل البيانات واجراء اختبار Tوكذلك، تم استخدام منحنى الميزة (ROC) لتحديد القيمة التشخيصية لتلك العوامل المدروسة.

النتائج:ان مستوى المصل للعامل TNF-ألفا، TRAF-1 وTRAF-2 أعلى بكثير في المرضى الذين يعانون من التهاب القولون التقرحي مما هو عليه في مجموعة السيطرة (مستوى المعنوية لكل العوامل المدروسة يساوي 0,000) وتبين أن العوامل المدروسة تملك قيمة تشخيصية ممتازة للتشخيص المرض حسب المنطقة تحت منحى الميزة من 698،0-000،1 وبمستوى معنوية اقل من 0,001 (P value <0.001).

الاستنتاج: في ضوء النتائج التي تم الحصول عليها في الدراسة الحالية، نخلص إلى أن هناك زيادة في مستويات كل من العاملTNF-ألفا، وTRAF-1 وTRAF-2 في المرضى الذين يعانون من القولون التقرحي. ويبدو أن تفعيل TNF-ألفا، TRAF-1، وTRAF-2 قد تكون الأحداث الأولى في التسبب في المرض .

التوصيات:لغرض تحديد السيتوكينات الموالية للالتهابات ومراقبة تطور المرض ومن اجل تحديد التوقيت المناسب للتدخل في المرض يجب قياس معلمات مناعية ووراثية أخرى، بما في ذلك خلايا-T وأختيارات أخرى.

ABSTACT

Background: Ulcerative colitis (UC) is a chronic inflammatory disease of unknown causes. It has involved the inflammatoryprocess 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 serum TNF-alpha, TRAF-1 and TRAF-2 levels in active Ulcerative Colitis and also, identified the diagnostic possibility of the studied parameters in diagnosis of UC patients.

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 level of Tumor Necrosis Factor-alpha (TNF), TNF Receptor Associated Factor-1 (TRAF-1), and TNF Receptor Associated Factor-2 (TRAF-2), by ELISA Method. All these Data was analyazed by using spss (T-test) program and also, the receiver operate characteristic curve (ROC) was used to identify their possibility of diagnostic value.

Results: Serum level of TNF-alpha, TRAF-1 and TRAF-2 were significantly higher in UC patients than those in controls (P values were = 0.000). TNF-alpha, TRAF-1, and TRAF-2 shown to have a display to fabulous value in the differentiation of UC patients and control with the area under the ROC curve (AUR) of 0.698–1.000 (P value < 0.001).

Conclusion: In light of the obtained results in current study, we conclude that the serological levels of TNF-alpha, TRAF-1 and TRAF-2 were increased in active UC patients. It seems that the activation of TNF-alpha, TRAF-1, and TRAF-2 may be first events in the development of UC.

Recommendation: Additional genetic and immunological markers, including standardized lymphocytes and other assays, are needed. These are to determine the pro-inflammatory cytokines and monitor the progress of the disease in order to detect the suitable time of a possible intervention.

Keywords: TNF-alpha, TRAF-1, TRAF-2, Ulcerative Colitis (UC), IBD.

INTRODUCTION:

Ulcerative colitis (UC) and Crohn's disease (CD) are two clinical presentations of inflammatory bowel disease (IBD) that affects the lining of the large intestine, 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]. Although the causes of UC are not fully understood, immune factors are reported to be associated with this disease. Overexpression 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 most important proinflammatory cytokines. TNF-α blocking agent such as infliximab have definite therapeutic effects on immune-mediated inflammatory diseases [3]. Two characterized receptors, TNF receptor 1 (TNF-R1) and TNF-R2, mediate most of the biological functions of TNF-alpha, while TNF-R-associated factors (TRAFs), an intracellular adapter proteins, is involved in TNF-R signaling pathways which are associated with induction of other cytokines, cell survival, proliferation, differentiation or cell death [4]. Stimulation of TNF-R1 leads to formation of two signaling complexes. Complex I – formed at the membrane – comprises TNF-R1, TNF-alpha, TNFR-associated death domain protein (TRADD), Receptor-interacting protein (RIP), TRAF-1, TRAF-2, and probably other, as-yet-unidentified proteins. It was suggested to trigger the nuclear factor kappa enhancer of activated B cells (NF-κB) signaling pathway through activates Jun N-terminal kinase (JNK) and I-κB kinases (IKK) complex recruitment. complex II comprises Fas-Associated protein with Death Domain (FADD), procaspase-8/10 and FADD-like IL-1β-converting enzyme- inhibitory protein (FLIP) are recruited to form the so-called traddosome. In the traddosome, activation of procaspase-8 takes place and it is followed by downstream death signaling activation [5].

TRAFs are a proteins family, which primarily involved in the regulation of inflammation, apoptosis and antiviral responses. Actually, seven TRAF proteins (TRAF1–7) have been characterized in humans [6]. These proteins have no intrinsic enzymatic activity but interact with a series of other proteins [7].

TRAF-1 and TRAF-2 can form a heterodimeric complex that is involved in the TNF-alpha mediated activation of mitogen-activated protein kinase 8 (MAPK8)/c-JNK and NF-κB signaling pathway. The complex interacts with inhibitors of apoptosis (IAP) and thus mediates anti-apoptotic signals from TNF-R [8]. In stressed cells, TRAF-2 interacting with eukaryotic translation initiation factor 4GI (eIF4GI), a scaffold protein, blocks TNF-alpha signaling [9]. NF-κB signaling pathway, which is activated by TRAF-2, protects cells from endoplasmic reticulum (ER) stress-induced apoptosis. In fact, ER stress plays a pivotal role in the inflammation development [4]. As TRAF-1 and TRAF-2 are closely related to cell apoptosis and inflammation, they may play important roles in the development of IBD[4]. In this study, we investigated serum TNF-alpha, TRAF-1 and TRAF-2 levels in active Ulcerative Colitis and also, identified the diagnostic possibility of the studied parameters in diagnosis of UC patients in Iraq.

PATIENTS AND METHOD

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

1. Patients Group: All members of the patients group; 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 endoscopic examination due to gastrointestinal symptoms but were confirmed to have no bowel inflammation. Their age ranges from 18 to 75 with a mean age of 42.67±15.816. Blood samples were collected from both groups and all the diagnoses were confirmed based on clinical, endoscopic, histopathological examinations.

Exclusion criteria were:

(i) Previously diagnosed IBD.

(ii) Pregnancy.

(iii) Age < 18 years.

(iv) Antibiotic use when patients were admitted to the hospital and previous history of medical treatment.

(v) Previous history of colectomy.

(vi) Other concurrent infection, chronic diseases or cancer.

Laboratory Analysis: Antecubital venous blood (3 ml) was drawn from each subject of the two groups and was put in plain tubes. Sera were separated by centrifugation at 3000 rpm for 10 minutes and part of the separated sera was used immediately for Epstein-Barr Virus (EBV)-agglutination Test to exclude cases with EBV infection, whereas the remaining amount was stored in three separated plain tubes at -20˚C before further testing. All sera tubes were allowed to thaw once (repeated thawing is avoided).

All stored sera were tested with:

1. Tumor necrosis factor-alpha (TNF-alpha) ELISA kit (Code: ab46087) which is a quantitative ELISA test for detection of circulating TNF-alpha (supplied by Abcam, USA).
2. TNF receptor-associated factor 1 (TRAF-1) ELISA kit (Code: CSB-E13601h) which is a quantitative ELISA test for detection of circulating TRAF-1 (supplied by Cusabio.Inc, China).
3. TNF receptor-associated factor 2 (TRAF-2) ELISA kit (Code: CSB-EL024147HU) which is a quantitative ELISA test for detection of circulating TRAF-1 (supplied by Cusabio.Inc, China).

Statistical Analysis: T-Test was used for statistical analysis to show if there is any significant differences between results and also, Receiver Operating Characteristic (ROC) curve was used for calculation of Area Under ROC curve to evaluate the cutoff values of studied parameters.

RESULTS:

Table (1): Comparison between means of serum TNF-alphalevelsin Studied Groups.

Variables	Groups	Count	Mean ± SD	T-test P-value	Sig.
TNF-alpha (pg/ml)	Patients	56	17.78 ± 11.62	t = 6.132 P = 0.000	(HS)
	Control	30	5.16 ± 7.38		

t-Test (In comparison between the two groups).
t:t-Test,   HS: High Sig. at P< 0.05.

The table (1) shows that serum TNF-alpha level was found to be 5.16 ± 7.38 ng/L in control group and 17.78 ± 11.62 ng/L in patients with UC. The results were significantly higher in the UC patients than those in control (p value =0.000).

Table (2): Comparison between means of serum TRAF-1, and TRAF-2 levels in Studied Groups.

Variables	Groups	Count	Mean ± SD	T-test P-value	Sig.
TRAF-1 (ng/ml)	Patients	56	5.48 ± 0.54	t = 23.641 P = 0.000	(HS)
	Control	30	1.25 ± 0.89		
TRAF-2 (ng/ml)	Patients	56	3.23 ± 0.66	t = 14.827 P =0.000	(HS)
	Control	30	1.06 ± 0.62		

t-Test (In comparison between the two groups).
t:t-Test, HS: High Sig. at P< 0.05.

The table (2) shows that serum TRAF-1 level obtained were 1.25 ± 0.89 ng/ml in the control group and 5.48 ± 0.54 ng/ml in patients with UC and also, serum TRAF-2 levels were 1.06 ± 0.62 ng/ml in the control group and 3.23 ± 0.66 ng/ml in patients with UC. The results were significantly higher in UC patients than those in controls (all p values = 0.000).

Table (3). Cut-off values, sensitivity, specificity and the area under the receiver operating characteristic curve (AUROC) of TNF-alpha, TRAF-1, and TRAF-2 in the differentiation of ulcerative colitis (UC) patients from control.

Proteins	Cut-off value (ng/ml)	Sensitivity (%)	Specificity (%)	AUROC (95% CI)	P-value
TNF-alpha	7.926	100.0	52.0	0.799	0.000
TRAF-1	1.617	100.0	100.0	1.000	0.000
TRAF-2	1.434	100.0	36.0	0.698	0.000

CI: Confidence Interval

Table (3) shows the cut-off values, sensitivity, specificity and the area under ROC curve (AUROC) of TNF-alpha, TRAF-1, and TRAF-2. In UC patients, the TNF-alpha cut-off value of 7.926 ng/L had a sensitivity of 100% and specificity of 52%, with the AUROC of 0.799 (P =

0.000); while the TRAF-1 cut-off value of 1.617 ng/mL had a sensitivity of 100% and specificity of 100%, with the AUROC of 1.000 (P = 0.000).

Furthermore, the TRAF-2 cut-off value of 1.434 ng/mL had a sensitivity of 100% and specificity of 36% with AUROC of 0.698 (P < 0.000).
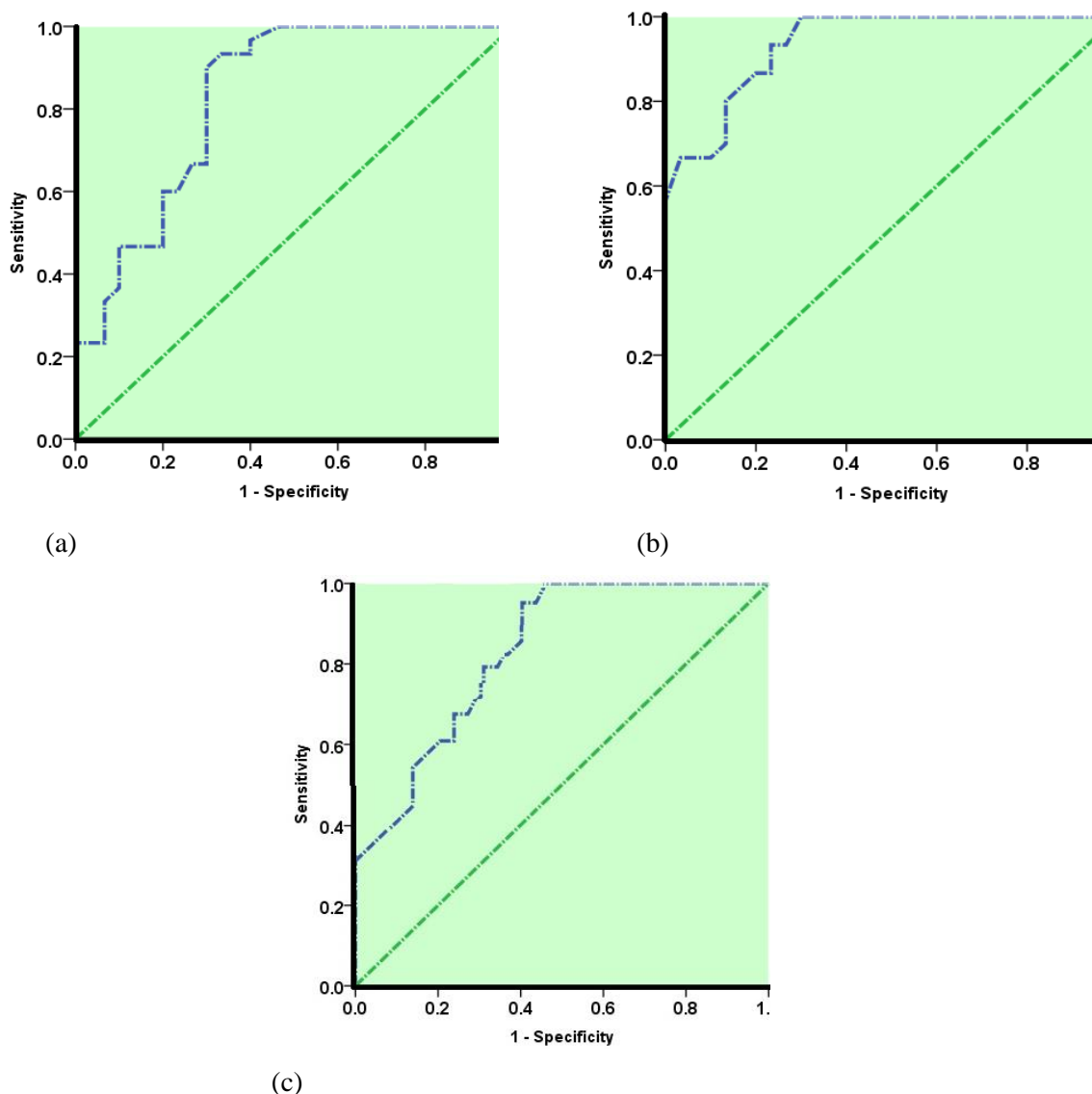

(a)

(b)

(c)

Figure (1): Receiver operating characteristic curve of (a) TNF-alpha (b) TRAF-1 (c) and TRAF-2.

Figure (1), the receiver operating characteristic (ROC) curve showed that TNF-alpha, TRAF-1 and TRAF-2 could differentiate UC patients from control (P < 0.001).

DISCUSSION:

TNF-alpha, TRAF-1, and TRAF-2 levels were significantly higher in the UC group than these observed in the normal subjects (P-value < 0.0001). The results of previous researches of the TNF-alpha and other pro-inflammatory cytokines (such as IL-1β and IL-8) level in serum, stool and mucosa of intestinal tract in UC patients were contradictory [10]. Some authors reported a significant elevation of serum TNF-alpha level in UC patients, and the others did not find any statistically significant difference in the serum TNF-alpha concentration between UC patients and healthy controls [10]. The results of current study showed that the serum TNF-alpha level was

significantly higher in UC patients compare with the healthy control. The variation of the results (heterogeneity of circulating TNF-alpha) may be duo to many factors:

1- Serum storage Conditions. Unexpectedly, conditions of serum storage prior to measurement of cytokine were not described for all publications (full text). About 40% of authors did not mention the serum storage conditions at all. Serum storage conditions are critical and may affect on cytokine concentration. It is known, for instance, that the level tends to decrease over time and after repetitive cycles of freeze/thaw [11]. Also, a recent study showed that serum samples storage at -80°C and =< 130°C for up to ninety days does not lead to essential changes, while storage at 4°C and -20°C induces essential decreases in cytokine level [12]. Thus, the data on storage conditions should be included; otherwise comparison between cytokine level performed after blood sampling collection and after freezing and thawing might be inutile.

2- Type of Manufacturer and assay. The limitations on manufacturer or assay kind markedly decrease the observations number for each cytokine. Until now, we chose ELISA for TNF-alpha. However, the increase in TNF-alpha levels was revealed or not revealed in different articles without any specific results.

3- Treatment taken at time of blood sample collection. The medications that taken by UC patients should not be mixed or compared with persons that without any medications at all [13].

The observed TNF-alpha serum level was lower than the results of a recent study which reported significant difference of serum TNF-alpha level in patients with UC compared to the healthy control, in which Serum TNF-alpha level was found between healthy control 28.86 (28.74 – 29.19 pg/ml) and UC patients 29.34 (29.14 – 29.71 pg/ml) (p < 0.05) [14].

In addition, another study found that serum concentration of TNF-alpha significantly increase in UC than control (22.4 ± 22.34 and 7.33 ± 8.21 respectively with p value <0.0001) and it concluded that TNF-alpha may provide simple way to monitor disease activity in UC [15].The present results are agreement with results of Murch S. H.*et al*. [16] who observed a significant increase in serum TNF-alpha concentration above control values in Active UC patients.

In UC patients, TNF-alpha level was measured not only in serum but also in stool, intestinal specimens and isolated lamina propria mononuclear cells [10].

Braegger*et al*. [10] reported that children with UC have a significant increase in stool TNF-alpha concentration compared to healthy children. They also found significantly higher concentrations of TNF-alpha in the stool of children in the active phase of UC compared to the control group, which is in accordance with present results related to serum TNF-alpha. The stool TNF-alpha concentration in inactive phase of the disease, due to surgery or treatment with steroids, felt down to the control level. These results suggest that measurements of stool TNF-alpha concentration may provide a simple way to monitor the disease activity in UC.

In spite of numerous experimental and clinical studies, the etiology and pathogenesis of the UC remains unknown. Whether the trigger for the development of UC, is bacterial, viral, dietary or environmental, there is general agreement that immune mechanisms are of probable importance [17]. UC is characterized by an influx of inflammatory and immune cells in to the diseased mucosa and local production of soluble mediators of inflammation occurs [17]. These mediators include cytokines, arachidonic acid metabolites, reactive oxygen intermediates and growth factors that have important regulatory and effector activities relevant to intestinal inflammation [18]. Cytokines have autocrine, paracrine and endocrine activities that mediate the local and systemic manifestation of intestinal inflammation. These molecules regulate and amplify the immune response, induce tissue injury and mediate complications of inflammatory response. These also have a critical role in suppressing inflammation and mediating repair and healing [18].*In vivo* and *in vitro* exposure to cytokines, particularly TNF-alpha, can produce many of the universal features of intestinal inflammation, including activation of immune, mesenchymal, endothelial and epithelial

cells, diarrhea; recruitment of circulating inflammatory cells, tissue damage (villous atrophy, crypt hyperplasia, and ulceration) and fibrosis [19].

The TNF-alpha in serum is probably an overspill from that produced locally by activated macrophages in the colon and is therefore presumably an underestimate of local production. In support of this, a similar variation between local and systemic TNF-alpha concentration has been shown by Nadal*et al* [20], who found concentrations in the cerebrospinal fluid of 57-20000 pg/ml in 11 children with bacterial meningitis, with serum TNF-alpha detectable in only one.

Most TNF-alpha is produced in activated macrophages and monocytes, although it is produced to some extent by hepatic Kupffer cells, cerebral astrocytes, and microglial cells [21]. Activated macrophages and T cells are abundant in intestinal mucosa in inflammatory bowel disease [22] and in an experiments, it has been shown that production of TNF-alpha is increased at the single cell level [23], thus showing formally that at least some of the TNF-alpha detected in serum in inflammatory bowel disease is gut derived.

These results suggest that current findings of increased serum TNF-alpha in colonic but not small bowel disease may reflect fundamental differences between the populations of macrophages in these sites. TNF-alpha has been shown in animals to have a cytopathic effect on bowel mucosa, probably mediated in part by platelet activating factor, [24] raising the possibility that it may be involved in the pathogenesis of inflammatory bowel disease.

Moreover, this study show that The Serum TRAF-1 levels obtained were 1.25 ± 0.89 ng/ml in the control group and 5.48 ± 0.54 ng/ml in patients with UC. Serum TRAF-2 levels were 1.06 ± 0.62 ng/ml in the control group and 3.23 ± 0.66 ng/ml in patients with UC.

TRAF-1, and TRAF-2 levels were significantly higher in the UC group than these observed in the normal subjects (P-value < 0.0001). The current results were agreement with a recent study that conclude both TRAF-1 and TRAF-2 were significantly higher in UC patients than control group (p value <0.001) [4].

In the present study, we demonstrated that both TRAF-1 and TRAF-2 were activated in UC patients. Although TRAFs have similar overall structural characteristics including a leucine-zipper domain and a carboxyl terminal receptor-binding domain, the structural difference of TRAFs molecules leads to distinctive interaction with related receptors [25].

In the last reports about understanding of TRAF functions increased more rapidly for TRAF-1 than for other TRAFs family members. Early overexpression studies clearly determined that TRAF-2 plays important role in TNF-mediated activation of NF-κB and other signaling proteins. NF-κB activity has been upregulated in LP cells and in epithelium of the inflamed colon in UC patients.TRAF-1 have been identified to indirectly act with TNF-R2 while TRAF-2 have been identify to directly act with TNF-R2. It is upregulated in B-lymphocytes following trigger signal of TNF-R2 [4]. This may explain present result that TRAF-1 level were significantly different in UC patients compared to controls.

However, the present study could not involve laboratory tests such as CRP or ESR or assess the disease activity which may lead to the potential bias of association on endoscopic assessment.

Furthermore, in an attempt to discriminate between patients group and control group, we built the generalized linear model based on the studied variables. Using these parameters, the predicted probability was calculated for patients. An area under the curve (AUC) value of 0.936 means that accuracy of the model is excellent.

TNF-alpha, TRAF-1 and TRAF-2 were highly expressed in the serum of UC patients. ROC analysis in the current study indicated that TRAF-1 was an excellent marker for the differentiation of UC and control (AUROC 1.000). However, the AUROC of TNF-alpha and TRAF-2 in the differentiation between UC patients and control was slightly lower than that of TRAF-1.

CONCLUSION

In this study, we obtained increased circulating levels of TNF-alpha, TRAF-1 and TRAF-2 in active UC patients. Cytokine level and signaling proteins concentrations have potential to serve as objective markers of inflammatory process.

RECOMMENDATION

1. Additional immunological and genetic markers, including standardized T-cell and other assays, are required. These are to identify the pro-inflammatory cytokines and monitor the progression of the disease in order to determine the appropriate timing of a possible intervention.
2. It is also recommended to do further studies to find out the variation of the studied parameters according to the gender and ethnicity.

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