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RESEARCH ARTICLE

"Tissue IL-17A expression associated with inflammatory lesions and ATG16L1 T300A allele in Crohn's disease"

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Abstract

Autophagy related 16 like 1 gene (ATG16L1) Thr300Ala genetic variant may be influence innate and adaptive immunity in Crohn's disease. This study aims to determine the association tissue IL-17A with ATG16L1 T300A and clinical disease phenotype. This case control study involved 35 CD, 40 UC and 35 HC. After extraction of DNA from blood samples, ATG16L1 T300A genotyping were done by SSP-PCR. Direct immunofluorescence technique done for localization of lysozyme and IL-17A in tissue samples of all subjects. A highly significant increase in tissue IL-17A found in CD 36.4±7.89 and UC 30.23±5.86. Among CD, significant increase in IL-17A has been shown that CD risk allele carrier 37.97±6.96 compared with patients carrying normal allele 33.42±8.51. In other hand, IL-17A associated with inflammatory lesions 42.13±6.83 compared with stenosing 33.56±9.41 and penetrating 35.28±6.52. The study found that IL-17A associated with inflammatory lesions under the influence of ATG16L1 T300A genetic variant in CD.

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INTRODUCTION

Inflammatory bowel disease such as Crohn's disease and ulcerative colitis characterized by chronic inflammatory relapses of enterocolitis. Activation of T cells and monocytes / dendritic cells are considered as an important factor in disease pathogenesis (Scharl and Rogler 2012).CD is linked to a predominant T helper (Th) cell 1/ Th 17 immune responses and in UC dominate Th2 cytokine effects(Geremia *et al.* 2014),and UC may have common endstage pathways and cytokines, such as IL-17A (Fujino *et al.* 2003).

IL-17 secretion has been evolved in defending strategy against pathogenic bacteria, fungi and others, when innate and adaptive immune systems failure (Weaver *et al.* 2007). IL-17A produced by Th17 under the stimulation of IL-6 and transforming growth factor-betta (TGF-β) (Mangan *et al.* 2006).In addition,IL-17 enhances the proinflammatory responses induced by IL-1β and tumour necrosis factor α (TNF-α).

Th17 cells play an important role in the gut mucosal barrier by affecting the innate and adaptive responses(Blaschitz and Raffatellu 2010). It can regulate the integrity of the physical barrier by epithelial cells, through chemotaxis of neutrophils and macrophages to prevent against invading pathogens(Liu *et al.* 2009). Its effector cytokines further stimulate the tight junction formation in the mucosal epithelial cells, thus providing resistance to bacterial translocation across gastrointestinal mucosa into the bloodstream(Kinugasa *et al.* 2000). Th17 cells also affect the cellular components of the immune system by recruiting neutrophils to the site of inflammation, stimulating antimicrobial peptide production from epithelial cells, increasing matrix metalloproteinase production from fibroblasts, enhancing immunoglobulin production from B cells and regulating different T cells. Thus, Th17 cells contribute to the mucosal barrier by several mechanisms that help the host against invading pathogens(Geddes *et al.* 2011, Raza *et al.* 2012).

Subjects and Methods:

Patients and controls:

Seventy five inflammatory bowel disease patients (35 Crohn's disease and 40 Ulcerative colitis) and thirty five subjects were selected as negative control whom reported as negative for endoscopic picture or histopathologically normal reports. All subjects recruited from the gastroenterology centers in three hospitals in Baghdad: The Gastroenterology and Hepatology Teaching Hospital, Baghdad Teaching Hospital and Al-Emamain Al-Kadhemain medical city as well as private hospitals in the period of March, 2013- June, 2014. Those subjects were either established or newly diagnosed as directed to do colonoscopy for complete their examination or receiving treatments (Inflaximab and/or anti-inflammatory drugs) patients details summarized in Table 1.

Direct immunofluorescence staining for IL-17A:

Slides were deparaffinized and rehydrated, then 20% rabbit serum in Tris Buffered Saline (TBS) was used for blocking. The primary monoclonal rabbit anti- *IL-17A* (Bioss, Germany) antibody was added 100µl on tissue section then incubated at 37˚C for 1 hr. After rinsing with washing buffer, dehydration done. A negative control was performed in all cases by omitting the primary antibody, which in all instances resulted in negative immunoreactivity. Slides were covered by anti-fading media (performed in our laboratory). Then examined under 495 filter of ultra violet light in fluorescent microscope (BH2, Olympus, Japan).

Genotyping of ATG16L1 T300A by Sequence Specific Primer-Polymerase Chain Reaction (SSP-PCR):

DNA was extracted from 300µl peripheral blood EDTA containing tubes using DNA isolation kit (Wizard®, Promega, USA) following manufacturer information with some modifications. Substitution mutations of Adinin with Guanine result in substitution of Alanin by Thrionin (dbSNP: rs2241880) of ATG16L1 gene in the chromosome 2 at the position 37.1. Allelic discrimination were checked by SSP-PCR. DNA from study groups individuals were amplified by using two sequence specific primers as well as two internal control-primers in two separated reaction mixtures, to give a PCR products of 201bp in positive reaction for allele A and allele G, allowing discrimination of homozygous or heterozygous alleles (Štaffová 2011). The sequence of primers customized as Forward allele A: $^{5'}$CCCCAGGACAATGTGGATA'^{3}, Forward allele G $^{5'}$CCCCAGGACAATGTGGATG'^{3} and common reverse $^{5'}$AGGTGGAAAGGCTTGATATAAG'^{3} the sequence of internal control (β-globin) are Forward primer $^{5`}$ACACAACTGTGTTCACTAGC'$^{3}$ and reverse primer $^{5`}$GAAAATAGACCAATAGGCAG'^{3}. For each reaction for allele A or G or internal control 0.3 µl of each primer (forward and reverse) added to pre-mix PCR tube (Promega, USA) and 0.5-3 µl of genomic DNA and complete reaction volume to 20 µl by DNAse free water.

PCR reaction tubes were transferred into thermal cycler (eppendroff-thermal cycler, Germany), that was programmed as following in (separated PCR-runs-for each allele): 96ºC for 1minutes (X1), (96°C 20s, 72°C) for 1min 10s (X5), 96°C for 25s, 69°C for 50s, 72°C for 30s (X21), 96°C for30s, 59°C for 1min and 72°C for 1 min and 30s (X4) then PCR products were electrophoresed in 2% agarose gel.

Statistical analysis:

All statistical analysis were done by using Statistical Package for Social Sciences (SPSS version 20). Crosstab model used to estimate association of allelic variant among study groups and ORs and corresponding 95% CIs were estimated. ANOVA test were used to compare means of numerical variables among more than two groups.

Results:

ATG16L1 Thr300Ala allelic variant associated with CD susceptibility:

The carriage of CD risk allele was statistically significant higher among CD (55.71%) compared with 32.8% in healthy controls and it was associated with the increased risk for CD (p=0.010, OR=2.57, CI=1.3-5.1). The

risk of developing CD was significantly specific associated with G allele when compared with 31.25% in UC patients (p=0.003 OR=2.76, CI=1.4-5.4) Table2.

Elevation of Il-17A protein expression among IBD:

To evaluate expression of IL-17 protein in the mucosa, biopsies were stained with fluorescein labelled anti-IL-17A antibodies. There was low IL-17A protein expression in normal colonic mucosa (3.14±2.93). In contrast, a marked increase in IL-17A was seen in biopsies of CD (36.4±7.89) (p<0.001) and UC (30.23±5.86) (p<0.001) patients. CD patients have higher percentage than those of UC (p<0.001). The cellular localization of IL-17A producing cells mainly between crypts along with mucosal layer (Figure 1A, C and D) and dense (high intensity) of paneth cells (at the distal third of crypts) (Figure 1-A)
According to Montreal disease classification (Figure 2), there are no statistical significant difference in the mean of expression of IL-17A according to age at diagnosis, location of CD or UC, even according to the presence of extra-intestinal manifestations or need for surgery. Notably, CD patients with inflammatory type of disease have higher mean of tissue IL-17A expression than stenoting (p=0.002) or penetrating disease (p=0.002).

IL-17A protein expression influenced by CD risk allele:

We investigated the influence of IL17A tissue expression by genotypic or allelic variant by comparison of each defined allelic groups. However, the results of comparisons ofpatients carrying allele G have significant higher (37.97±6.96) tissue expression of IL-17A protein than those carrying allele A (33.42±8.51) (p=0.048).

Table 1: summary of demographic and clinical description for study groups.

Study groups	CD (n=35)	UC (n=40)	HC (n=35)
Gender type			
Female (%)	21 (60.00%)	26 (65.00%)	20 (57.14%)
Age (year)			
Mean±SE*	38.26±1.49	34.00±1.80	37.11±1.24
Median	38.00	31.00	37.00
Range	32.00	42.00	34.00
ASCA positivity	27 (77.14%)	10 (25%)	4 (11.43%)
pANCA positivity	11 (31.43%)	31 (77.5%)	1 (2.86%)
Age at diagnosis			
A1: Younger than 16	3 (8.57%)		
A2: 17-40 years old	23 (65.71%)		
A3: Older than 40	9 (25.71%)		
Disease behavior			
B1: Inflammatory	8 (22.86%)		
B2: Stenosing	9 (25.71%)		
B3: Penetrating	18 (51.43%)		
Disease location (CD)			
L1: Ileal	4 (11.43%)		
L2: Colonic	19 (54.29%)		
L3: Ileocolonic	12 (34.29%)		
Disease location (UC)			
E1: ulcerative proctitis		11 (27.5%)	
E2: Left sided (UC)		19 (47.5%)	
E3: Extensive		10 (25%)	

colitis	
Presence of extra-intestinal manifestations	
No	17 (48.6%)
Yes	18 (51.4%)
Need for surgery	
No	18 (51.4%)
Yes	17 (48.6%)

Table 2: Allelic Frequencies of rs2241880 ATG16L1 Polymorphism in Iraqi CD, UC Patients and Controls.

		HC	CD	UC
ATG16L1 allele	A	47 (67.14%)	31 (44.29%)	55 (68.75%)
	G	23 (32.86%)	39 (55.71%)	25 (31.25%)
	Total	70 (100%)	70 (100%)	80 (100%)
Odd ratio (Confidence interval)	vs control		2.57 (1.3-5.1)	0.93 (0.4-1.8)
	vs UC		2.76 (1.41-5.4)	-
P value	vs control		0.010*	0.885^{NS}
	vs UC		0.003*	-

NS= Not statistical significant (p>0.05).
* = Statistical significant difference (p≤0.05).

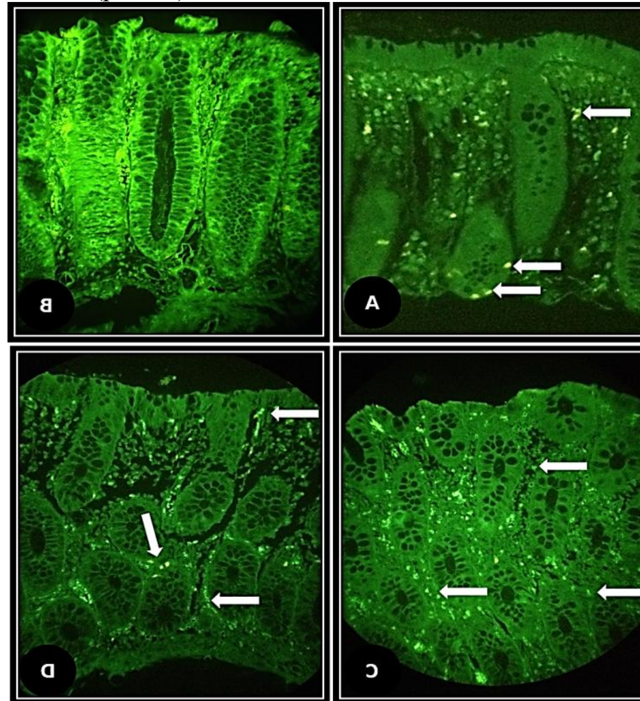

Figure 1: Cellular expression of interleukin 17 A (IL-17A) in Crohn's disease (A–C) and ulcerative colitis (D) patients. Direct immunofluorescence was used to determine IL-17 expression (fluorescein isothiocyanate (FITC), green fluorescence cells showing expression of IL-17. The specificity of antibody was confirmed by negative reactivity without adding antibody on section (B).

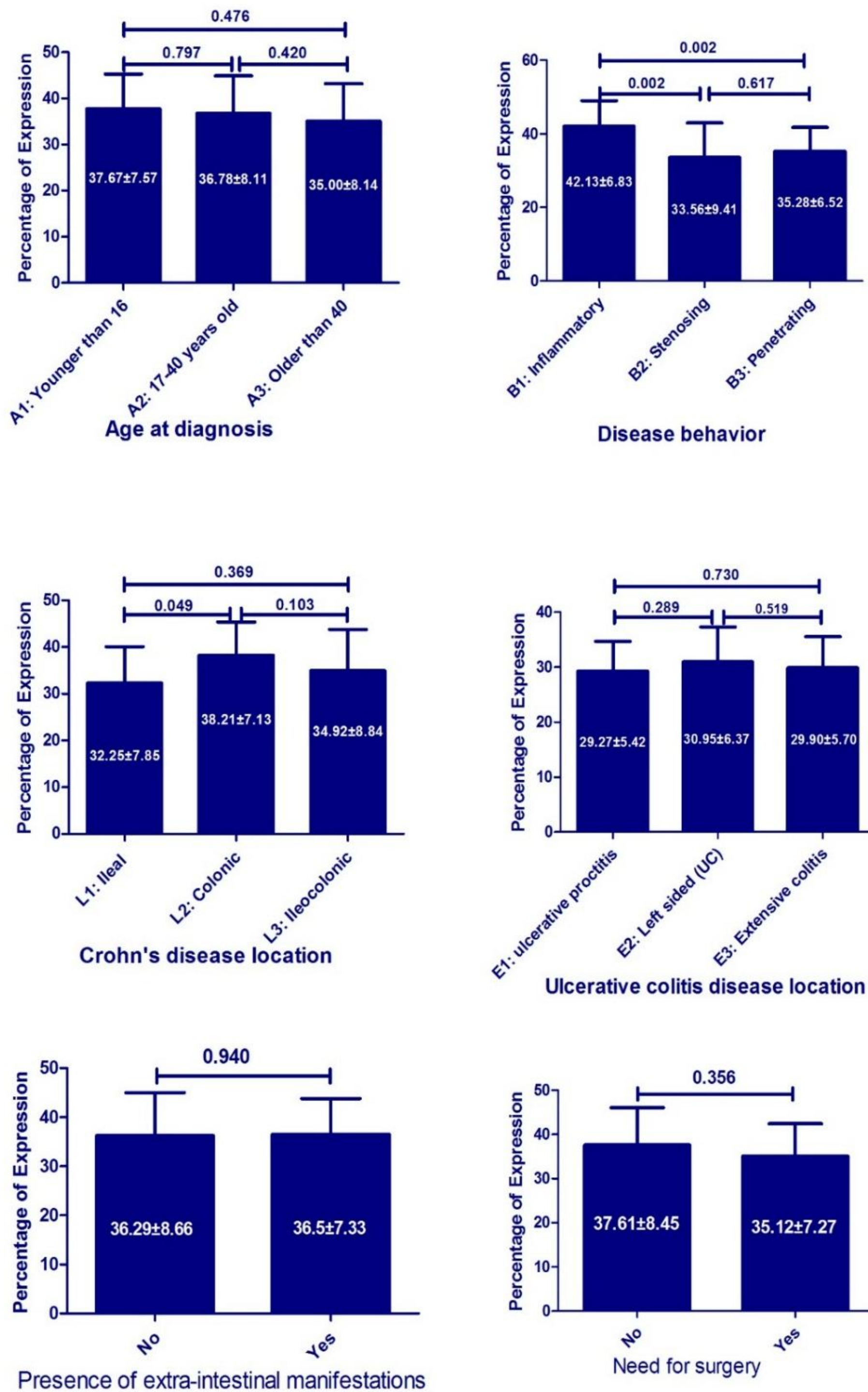

Figure 2: Percentage of IL17A protein expression according to Montreal disease classification and clinical variables.

Discussion:

An impaired autophagy process in the gut mucosa has been proposed in inflammatory bowel diseases. Impaired autophagy may influence disease pathogenesis through alteration of cytokine profile in to proinflammatory state hence that causing tissue damage.

In this study, we reported the association of rs2241880 T300A with Crohn's disease but not with Ulcerative colitis. This agreed by several globalstudies that mentioned this association (Hampe *et al.* 2007, Rioux *et al.* 2007). But, T300A is not associated with CD in Indian nor Japanese (Walker *et al.* 2011).

Several studies focused on the association of il-17Ain of IBD, due to its unique stimulation under TGF-β and IL-6 (Leppkes *et al.* 2009, Monteleone *et al.* 2009). In the other hand emphasizing their role in disease pathogenesis and its anti-bacterial effect(Otani*et al.* 2009, Raza *et al.* 2012). Regarding tissue expression of IL-17A protein, our results reported an increased tissue expression of IL-17A protein in both CD and UC, this result agreed with previously reported findings by Sugihara(Sugihara *et al.* 2010), Fujino(Fujino *et al.* 2003). Its known that IL-17A stimulates expression and production of proinflammatory cytokines in human cells(Fossiez*et al.*1996, Chabaud *et al.* 1998), and the proinflammatory nature of IL-17 depends on activation of the transcription factor nuclear factor κB(NF κB)(Awane*et al.* 1999).

Regarding its association with CD risk allele, the results showed that the increased il-17A protein expressionassociated withthe presence of CD risk variant. This comes in agreement with Morse (Morse 2014) whom linked an elevation of IL-17A with ATG16L1 risk allele. Furthermore, the its elevation may be due to a compensatory aggressive immune response toward defective autophagy (Farache *et al.* 2013).We suggests, the presence of defective autophagy will reduce elimination of intracellular microbes(Sadaghian Sadabad *et al.* 2014). The presence of un-processed microbes in the mucosa will deliver a pro-inflammatory signal mediated by NF κB(Mills *et al.* 2013). In addition to that, an abnormal IL-17A/IL23 axis found among IBD (Kobayashi *et al.* 2008, Brand 2009). It widely reported that genetic polymorphism in autophagy pathway is associated with increasing risk for CD and defective recognition of microbes by TLRs among both CD and UC(Xu*et al.* 2007, Pott *et al.* 2009). IL23R genetic mutation is highly frequent among IBD (Duerr *et al.* 2006, Oliver *et al.* 2007, McGovern *et al.* 2009).

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