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Original Research Article

The Association of ATG16L1 Thr300Ala Allelic Variant with *Helicobacter pylori* Infection among Inflammatory Bowel Disease Patients

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Keywords

Helicobacter pylori, Autophagy and Crohn's disease

A B S T R A C T

Autophagy related 16 like 1 gene (ATG16L1) Thr300Ala genetic variant may be influence *Helicobacter pylori* infection in Crohn's disease. This study aims to determine the significance of ATG16L1 T300A genetic variant on *H. pylori infection.* 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. Indirect immunofluorescence technique done for localization of *H. pylori* in tissue samples of all subjects. In this study, we observed an association of ATG16L1 Thr300Ala genetic variants with CD (55.71%) conferring higher risk for the disease development (OR=2.76, 95% CI= 1.3-5.1), rather than UC, the genetic variation was 31.25% showed no association with disease development (OR=0. 93, 95% CI= 0.4-1.8) when compared with HC 32.8%. A protective role for *H. pylori* has been reported in CD (OR=0. 28, 95% CI= 0.079-0.99) and UC (OR=0. 24, 95% CI= 0.07-0.85) when compared with HC, without any clinical nor immunological association among CD patients. The study found that there was no interaction between ATG16L1 T300A genetic variant and *H. pylori* infection in CD.

Introduction

Helicobacter pylori infection is one of the most common bacterial infections worldwide affecting over half of the whole human population (Höcker & Hohenberger 2003). It can cause gastritis, peptic ulcer (Marshall & Warren 1984), mucosa-associated lymphoid tissue (MALT) lymphoma (Parsonnet et al. 1994), intestinal metaplasia (Leung et al. 2004) and gastric cancer (Watanabe et al. 1998; Parsonnet et al. 1991; Forman et al. 1991).

The precise mechanisms by which *H. pylori* exploits host cell machineries for intracellular survival are poorly understood. Over the last decade, several research groups have independently reported that infection by *H. pylori* can induce macroautophagy (Y.-H. Wang et al. 2009; Wang et al. 2010). Although mostly targeting intracellular bacteria, macroautophagy can also act against those bacteria regarded as being extracellular

when they manage to enter the eukaryotic cell(Cemma & Brumell 2012). It has been proposed that *H. pylori*, once internalized and sequestered in double-membraned autophagosomes, can use these compartments as a replicative niche(Y.-H. Wang et al. 2009; Wang et al. 2010; Chu et al. 2010). It has been reported to evade the autophagic machinery by down regulating the expression of autophagic proteins(Tang et al. 2012).

H. pylori also excellent colonizers of the gastrointestinal surface for their microaerophilic metabolism, spiral shape, and peculiar motility(Y. H. Wang et al. 2009). Considering the immune regulation, the capacity for colonization, and the nature of autoimmune-related damage in CD, it is theoretically plausible that *H. pylori* infection may take part in the pathogenesis of CD(Friswell et al. 2010). Linking with ATG16L1 T300A genetic variant, that the resultant protein reduce autophagic responses to vaculating cytotoxin A (VacA) and increased susceptibility to infection with an *H. pylori* Vac-A suggesting that it facilitates chronic inflammation (Deen et al. 2013; Raju, Seamus Hussey, et al. 2012).

Materials 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).

In-direct immunofluorescence staining for *H. pylori*

Slides were deparaffinized and rehydrated, then 20% rabbit serum in Tris Buffered Saline (TBS) was used for blocking. The primary monoclonal rabbit anti-*H. pylori* (Dako). Antibody was added 100µl on tissue section then incubated at 37˚C for 2 hr. After rinsing with washing buffer, then Secondary Fluorescein labeled anti-rabbit antibody (ABIN1512917, 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 immuno reactivity. 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: $^{5'}$CCCCAGGACA ATGTGGAT A'^{3}, Forward allele G $^{5'}$CCCCAGGA CAATGTGGATG'^{3} and common reverse $^{5'}$AGGTGGAAAGG CTTGATATAAG'^{3} the sequence of internal control (β-globin) are Forward primer $^{5`}$ACACAACTGT GTTCACTAGC'$^{3}$ and reverse primer $^{5`}$GAAAATAGACCA ATAGGCAG'^{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 and Discussion

ATG16L1 Thr300Ala allelic variant associated with CD susceptibility

The carriage of 300G/G allele was statistically significant higher in CD (55.71%) compared with 32.8% in healthy controls (p=0.010, OR=2.57, CI=1.3-5.1) and it was associated with the increased risk for CD. 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) Table 2.

The histological detection of *H.pylori* indicates the predominance among healthy control subjects, 31.4% (11/40) were positive for immunostaining compared with lower incidence 11.4% (4/35) among the CD group and 10 % (4/40) among UC group odd ratio = 0.28 and 0.24 respectively Table 2.

In most of HC tissue biopsies, *H. pylori* observed as an extracellular curved bacterium mostly found beneath the mucus layer in close relation to the luminal surface of epithelial cells. Sometimes, *H. pylori* detected in the cytoplasm (intracellular) epithelial cells and inflammatory infiltrate between glands and sub-mucosal area (Figure 1).

The association of *H. pylori* infection with IBD phenotypes

According to disease phenotypes and clinical presentations of IBD patients (Table 3), there are no statistical significant association between prevalence of H. pylori and age at diagnosis (p=0.847), disease behavior (p=0.355), CD location (p=0.709), UC disease location (0.450), or presence of extra-intestinal manifestations (p=0.949) and patients need for surgery (p=0.949).

An association between *H. pylori* and ATG16L1 Genotypic and allelic variation has been summarized in Table 4.The results showed that there were no statistical association between *H. pylori* prevalence and genotypic and allelic variants p=0.512 and 0.681 respectively.

H. pylori infection is known to be colonized in gastrodeudenal epithelia causing various disease(Parsonnet et al. 1991; Hirasawa et al. 1999; Honda et al. 1998; Malfertheiner & Peitz 2005; Wilcox 1997). It has been suggested that CD risk allele increase susceptibility to infection with *H. pylori*(Raju, Séamus Hussey, et al. 2012)

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 colitis		10 (25%)	
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*	-

Table.2.Relative percentage of H. pylori incidence in tissue samples among study groups

			Study groups		
			HC	CD	UC
H. pylori	Negative	Count	24	31	36
		%	68.6%	88.6%	90.0%
	Positive	Count	11	4	4
		%	31.4%	11.4%	10.0%
Total		Count	35	35	40
		%	100.0%	100.0%	100.0%
OR (CI)				0.28 (0.079-0.99)	0.24 (0.07-0.85)
p value				0.041*	0.020*

* =Statistical significant difference ($p \leq 0.05$)

Table.3 Association of H. pylori incidence with Montreal classification among CD patients

		H. pylori				Total	p value
		Negative		Positive			
		Count	%	Count	%		
Age at diagnosis	A1: Younger than 16	3	100.0%	0	0.0%	3	0.847^{NS}
	A2: 17-40 years old	20	87.0%	3	13.0%	23	
	A3: Older than 40	8	88.9%	1	11.1%	9	
Total		31	88.6%	4	11.4%	35	
Disease behavior	B2: Stenosing	8	88.9%	1	11.1%	9	0.355^{NS}
	B3: Penetrating	17	94.4%	1	5.6%	18	
	B1: Inflammatory	6	75.0%	2	25.0%	8	
Total		31	88.6%	4	11.4%	35	
Disease location (CD)	L1: Ileal	3	75.0%	1	25.0%	4	0.709^{NS}
	L2: Colonic	16	84.2%	3	15.8%	19	
	L3: Ileocolonic	12	100.0%	0	0.0%	12	
Total		31	88.6%	4	11.4%	35	
Disease location (UC)	E1: ulcerative proctitis	10	90.9%	1	9.1%	11	0.450^{NS}
	E2: Left sided (UC)	18	94.7%	1	5.3%	19	
	E3: Extensive colitis	8	80.0%	2	20.0%	10	
Total		36	102.9%	4	11.4%	40	
Extra intestinal manifestation	No	15	88.2%	2	11.8%	17	0.949^{NS}
	Yes	16	88.9%	2	11.1%	18	
Total		31	88.6%	4	11.4%	35	
Surgery	No	16	88.9%	2	11.1%	18	0.949^{NS}
	Yes	15	88.2%	2	11.8%	17	
Total		31	88.6%	4	11.4%	35	

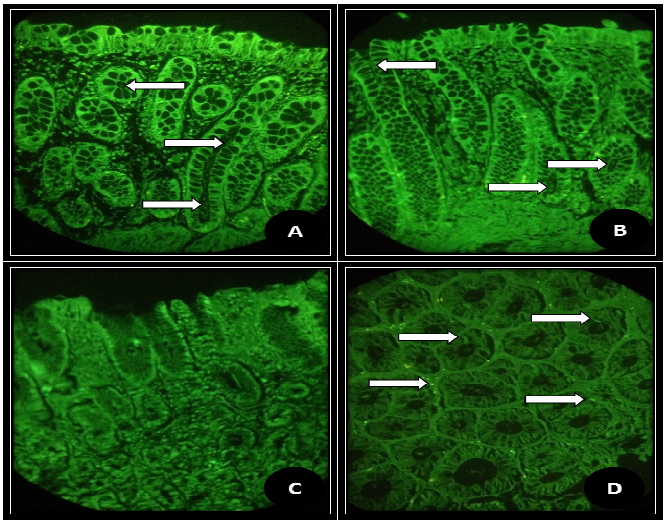
NS= not statistically significant ($p>0.05$)

Table.4.Association of H. pylori incidence with ATG16L1 genotypic and allelic variants among CD patients

		H. pylori				P value
		Negative	%	Positive	%	
ATG16L1 genotype	AA	7	22.6%	1	25.0%	0.512^{NS}
	GA	14	45.2%	1	25.0%	
	GG	10	32.3%	2	50.0%	
Allele	A	28	45.2%	3	37.5%	0.681^{NS}
	G	34	54.8%	5	62.5%	

NS= not statistically significant (p>0.05)

Figure.1 Indirect immunofluorescence detection of H. pylori antigen in formalin-fixed, paraffin-embedded tissue section of IBD patients and control. A and D: UC. B: CD.A&B: showing H. pylori (arrows) in the lumen of crypt as an extra-cellular curved bacteria D: localization of H. pylori at the base of crypt cells and inflammatory cells between glands were stained with green fluorescence. Original magnification, x200. C. Colonic tissue section of UC patient negatively stained (without adding specifying antibody).



H. pylori prevalence in each study groups was estimated by indirect immunofluorescence staining method. We reported lower prevalence of *H. pylori* among IBD patients (CD =4/35 and UC=4/40) when compared with HC 11/40, suggesting a protective role of *H. pylori* against colitis OR=0.28, p=0.041 for CD and OR=0.24, p=0.020 for UC. this finding agreed with several clinical studies mentioned the lower association between *H. pylori* among IBD patients compared with HC(Luther et al. 2010). This possible protective benefit of *H. pylori* against colitis is not expecting. Rad, et al demonstrated that H. pylori infected individuals express a higher level of Foxp3 (T- cell regulatory transcriptional factor) (Rad et al. 2006). Furthermore, the importance of Tregs in the pathogenesis of IBD can be illustrated by the termination of spontaneous colitis in mice(Leach et al. 1999). Alsothe adoptive transfer of Tregs inhibits the development of experimental colitis in several experimental models(De Winter et al. 1999; Izcue et al. 2006),suggesting an essential role of T-reg in prevention of colitis development (Haribhai et al. 2009). Furthermore, it has

been shown that *H. pylori* DNA has the ability to down regulate pro-inflammatory response from DC(Luther et al. 2011).

These may reflect an unexpected role of *H. pylori* in the influencing small intestinal mucosa. Its plausible that increasing pH of small intestine thus may protect intestinal mucosal damage, *H. pylori* protect small intestinal mucosa against damage, this may establish a stable relationship with the intestinal bacterial flora(Maeda et al. 2013). Studies showed that after eradication of *H. pylori* there is an exacerbation of NSAID induced small intestinal injury due to dysbiosis. It shifts a significant reduction in the jejuna *Actinobacteria* and *Bifidobacterium spp*. and the administration of Bifidobacteria enriched commensal bacteria during treatment with omeprazole and naproxen prevents intestinal damage and bleeding.

An important question rising here, what are the tissue factors that leads to reducing in *H. pylori* among IBD in comparison with control subjects? The hypothesis stated that the lower prevalence of *H. pylori* infection among IBD had led to the notion that there is protective role of *H. pylori* against IBD (Matricon et al. 2010). We repeated that, our results are in concordance with this hypothesis. There is no direct answer for the above question. We thought that it may be related to the state of dendritic cell polarization. It was reported previously that infection with *H. pylori* alters the dendritic cell-polarized from Th17 (pro-inflammatory) to Treg (regulatory or anti-inflammatory) which may influence the immunological response in the intestine(Kao et al. 2010; Zhang et al. 2010). An abnormal dendritic cell polarization among IBD patients towards Th17 with an elevated IL-17A tissue expression could explain the lower prevalence of *H. pylori* (Otani et al. 2009). Another study reporting an increased interleukin-10 expression in the mesenteric lymph nodes of mice infected with *H. pylori*, alters the immunological environment of the lower gastrointestinal tract, providing mechanistic support for the epidemiological observation of a negative association between *H. pylori* status and the risk of IBD(Higgins et al. 2011).We thought that *H. pylori* actively found in intestinal tissue and impair autophagy by its virulence factor VAC-A and increased intracellular survival (Raju, Seamus Hussey, et al. 2012) despite that increased il-17A may have a greater impact on elimination of *H. pylori* among IBD patients.

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