International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 12 (2014) pp. 633-642**
http://www.ijcmas.com

Original Research Article

Clinical Significance of ATG16L1 Thr300Ala Genetic Variants in Patients with Crohn's Disease and Ulcerative Colitis

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A B S T R A C T

Autophagy related 16 like 1 gene (ATG16L1) Thr300Ala genetic variant confer an increased risk for the development of Crohn's disease. According to Montreal disease classification, this study aims to determine the clinical significance of ATG16L1 T300A genetic variant among those patients. 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. Immunohistological techniques done for localization of ATG16L1, LC3C, Lysozyme, IL-17A, Norovirus and *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%. Clinically, patients with CD risk allele had non-specific clinical phenotype, but they tend to have an extra-intestinal manifestations (75%) and the need for surgical intervention (76.4%). This study concluded that ATG16L1 T300A genetic variant (CD risk allele) is a risk factor for CD development rather than UC.

Keywords

ATG16L1 T300A, Montreal , Inflammatory bowel disease

Introduction

It is becoming increasingly clear that ATG16L1 T300Aasan interacting autophagy pathway, as many of the genes encoded by these risk loci mediate common cellular functions, such as microbial recognition, autophagy, cytokine signaling, epithelial barrier maintenance, lymphocyte activation, metabolism, and endoplasmic reticulum stress responses(Cho and Brant 2011, Graham and Xavier 2013).

ATG16L1 encodes a key autophagy protein, which functions as part of a complex with autophagy proteins Atg5 and Atg12, and is responsible for the proper subcellular localization of the autophagy machinery(Kuma *et al.* 2002, Fujita *et al.* 2008). In 2007, genome-wide association scans (GWAS) identified a single nucleotide polymorphism (SNP) in the coding region of the ATG16L1 gene associated with an

increased risk of developing CD (rs2241880) (Hampe *et al.* 2007). The disease-associated ATG16L1 variant results in a threonine-to-alanine amino acid change at position 300 (T300A) within the evolutionarily conserved WD-repeat region. This finding has been replicated in a number of studies across Europe and the United States.(Consortium 2007, Prescott *et al.* 2007, Rioux *et al.* 2007, Glas *et al.* 2008, Van Limbergen *et al.* 2008, Franke *et al.* 2010).

The loss of function mutations result in reduced antimicrobial peptides production and increase pathogenic microbial invasion intestinal epithelial cell and reduced activation of NF-kB in monocyte and dendritic cell responses (Smith *et al.* 2009, Homer *et al.* 2010, Brain *et al.* 2012). Other studies suggest that the loss of function of NOD2 may result in the lack of inhibition of TLR2 stimulation, leading to activation of inflammatory pathways and excessive Th-1 and Th-17 responses(Watanabe *et al.* 2004).

A number of studies have now endeavored to correlate CD phenotypes with autophagy gene risk variants in an attempt to translate knowledge of the autophagy pathway to clinical application. The most replicated finding in this respect is the positive association of ATG16L1 variants with ileal disease phenotype. Along with Prescott et al. who initially reported this finding for ATG16L1 in a cohort from the United Kingdom(Prescott *et al.* 2007),

Similarly carriage of the ATG16L1 risk variant has been shown to correlate with a reduction in extra-intestinal manifestations; however the small patient numbers in this report may have been a confounding factor (Lauriola *et al.* 2011). Another reported positive association was with IRGM variants and the need for ileocolectomy (a surgical procedure to remove the affected distal small bowel and proximal large bowel), however the number of patients in the study again make this difficult to interpret(Sehgal *et al.* 2012).

Materials and Methods

Thirty five Crohn's disease patients, 40 ulcerative colitis and 35 subjects were selected as negative control whom reported as negative for endoscopic picture or histopathologically normal reports were enrolled in this case controlled study.

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 Norovirus

After overnight packing at 65˚C, tissue sections were deparaffinized in xylene and rehydrated in ascending grades of alcohol. 20% rabbit serum in Tris Buffered Saline (TBS) was used for blocking. The primary monoclonal rabbit anti-Capsid Protein (VP1) (ABIN965745, Bioss, Germany) antibody was added 100µl on tissue section then incubated at 37˚C for 1 hr.

After rinsing with washing buffer, then Secondary Fluorescien 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 by dipping of slides in ascending dilution of alcohol. 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, K. and Mrázek, F., 2011).

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.

Result and Discussion

Association between ATG16L1 Thr300Ala genotypic and allelic variant and disease susceptibility

All cases were investigated for ATG16L1 Thr300Ala polymorphism by SSP-PCR. The genotypic and allelic frequency were presented in Table 2. When we compared the frequency of genotypic possibility (Ala300Ala, Thr300Ala and Thr300Thr) from CD patients with those from HC group and UC group, there were statistical significant difference (p=0.039 and p=0.045) respectively.

While, there was no statistical significant difference in genotype frequency between UC patients and HC (p=0.856).

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).

The association of ATG16L1 Thr300Ala genotypic and allelic variants with Montreal disease classification and clinical variables

Among CD patients, no significant association between age at diagnosis with the genotypic variant (p=0.207) or allele variant frequency (p=0.179), the results showed a higher frequency of genetic variant and allelic variant in both early onset of disease (younger than 16 years) 66.67% and 66.67% respectively and late onset of disease (older than 40 years) 55.56% and 72.22% respectively in comparison with adult onset of disease (17-40 years) 21.74% and 47.83% respectively.

While, the lack of association of disease behavior with genotypic variation (p=0.242) and allelic variation (p=0.848), only allelic variation showed higher frequency in three sub-phenotypes (inflammatory=56.25%, stenosing=61.11% and penetrating=51.78%) Table 3. This increase due to higher frequency of heterozygous genotype (Thr300Ala) in inflammatory subtype (62.5%) and penetrating subtype (50%).

According to disease location, CD patients with ileal involvement have higher frequency (62.5%) for allelic variant followed by colonic disease (57.89%) then ileocolonic (50%). However, neither genotypic nor allelic variant give an association with CD location (p=0.680 and 0.763). In ulcerative colitis disease, patients with left sided colitis didn't have homozygous GG genotype. While, 27.27% of ulcerative proctitis and 30% of extensive colitis have this genotype. There are no association between genotype or allelic variant and disease location in UC patients. The presence of extra-intestinal manifestations have higher frequency of both genetic variant (61.11%) and allelic variant (75%) than its absence. CD patients whom need surgery have significantly higher frequency for genetic variant (64.71%) and allelic variant (76.47%) than don't need, see Table 3).

The principal job of the intestinal immune system is to maintain a balance between the recognition and elimination of pathogens while keeping the commensal bacteria and food antigens. Single cell intestinal epithelial barrier that separate intestinal lumen and host tissues. However, ATG16L1"in particular" plays a pivotal role in this mechanism through autophagy process (Randall-Demllo *et al.* 2013).

ATG16L1 participate in the initiation and elongation of autophagosome surrounding microbes(Cadwell *et al.* 2008). The first Genome Wide Association studies in 2006 increased knowledge of genetics in the CD, the ATG16L1 rs2241880 has been identified as a risk factor among CD but not UC(Hampe *et al.* 2007).

ATG16L1 represents a key molecule within the autophagy network being responsible for subcellular localization of the autophagy machinery [15]. The SNP rs2241880 within the gene encoding ATG16L1 causes a switch from A to G allele at position 300. Presence of the disease-associated genotype GG results in substitution of threonine by alanine (T300A). To the best of our knowledge, we believe that this study is the first in Iraq concerning the risk of autophagy related gene 16 like 1 T300A SNP in inflammatory bowel diseases. According to the results in Table 2, ATG16L1 (T300A) allelic variant associated with risk of CD development.

Table.1 Summary of demographic and clinical description for study groups

Study groups	CD (n=35)	UC (n=40)	HC (n=35)
Female (%)	21 (60)	26 (65)	20 (57.1)
Age (year) Mean±SE*	38.26±1.49	34.00±1.80	37.11±1.24
ASCA (%)	27 (77.14)	10 (25)	4 (11.43)
pANCA (%)	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 Genotypic and Allelic Frequencies of rs2241880 ATG16L1 Polymorphism in Iraqi CD, UC Patients and Controls

		HC	CD	UC
ATG16L1 genotype	AA	18 (51.43%)	8 (22.86%)	19 (47.50%)
	GA	11 (31.43%)	15 (42.86%)	15 (37.50%)
	GG	6 (17.14%)	12 (34.29%)	6 (15.00%)
	Total	35 (100%)	35 (100%)	40 (100%)
P value	vs control		0.039*	0.856^{NS}
	vs CD			0.045*
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= No statistical significant difference ($p>0.05$); *= statistical significant difference ($p\leq0.05$).

Table.3 Association of ATG16L1 T300A genotypic and allelic variants with clinical parameters

	ATG16L1 genotype			Allele	
	AA	**GA**	**GG**	**A**	**G**
Age at diagnosis (35)					
A1: Younger than 16 (3)	1 33.33%	0 0.00%	2 66.67%	2 33.33%	4 66.67%
A2: 17-40 years old (23)	6 26.09%	12 52.17%	5 21.74%	24 52.17%	22 47.83%
A3: Older than 40 (9)	1 11.11%	3 33.33%	5 55.56%	5 27.78%	13 72.22%
P value	0.207 ^{NS}			0.179 ^{NS}	
Disease behavior (35)					
B1: Inflammatory (8)	1 12.50%	5 62.50%	2 25.00%	7 43.75%	9 56.25%
B2: Stenosing (9)	3 33.33%	1 11.11%	5 55.56%	7 38.89%	11 61.11%
B3: Penetrating (18)	4 22.22%	9 50.00%	5 27.78%	17 47.22%	19 52.78%
P value	0.242 ^{NS}			0.844 ^{NS}	
Disease location (35)					
L1: Ileal (4)	1 25.00%	1 25.00%	2 50.00%	3 37.50%	5 62.50%
L2: Colonic (19)	3 15.79%	10 52.63%	6 31.58%	16 42.11%	22 57.89%
L3: Ileocolonic (12)	4 33.33%	4 33.33%	4 33.33%	12 50.00%	12 50.00%
P value	0.680 ^{NS}			0.763 ^{NS}	
Disease location (40)					
E1: ulcerative proctitis (11)	6 54.55%	2 18.18%	3 27.27%	14 63.64%	8 36.36%
E2: Left sided (UC) (19)	10 52.63%	9 47.37%	0 0.00%	29 76.32%	9 23.68%
E3: Extensive colitis (10)	3 30.00%	4 40.00%	3 30.00%	10 50.00%	10 50.00%
P value	0.094 ^{NS}			0.125 ^{NS}	
Extra intestinal manifestation (35)					
No (17)	6 35.29%	10 58.82%	1 5.88%	22 64.71%	12 35.29%
Yes (13)	2 11.11%	5 27.78%	11** 61.11%	9 25.00%	27** 75.00%
P value	<0.001**			0.001**	
Need for surgery (35)					
No (18)	6 33.33%	11 61.11%	1 5.56%	23 63.89%	13 36.11%
Yes (17)	2 11.76%	4 23.53%	11** 64.71%	8 23.53%	26** 76.47%
P value	0.001**			0.001**	

NS: Not significant (p>0.05), *= significant difference (p≤0.05), **= highly significant difference (p≤0.001).

Our finding was similar to several genome wide association studies that mentioned the same statement The T300A variation is present in 58.1 % of CD patients (vs. 51.3 % of non-IBD control patients) and has been strongly associated with increased risk for developing CD, but not UC (Hampe *et al.* 2007, Rioux *et al.* 2007).

The lack of association with ulcerative colitis and rs2241880 suggest that the biological processes may be Crohn's disease specific. This was agreed by several studies globally that mentioned this association(Consortium 2007, Hampe *et al.* 2007, Rioux *et al.* 2007). But, T300A is not associated with CD in Indian nor Japanese(Walker *et al.* 2011).

In this study, we found an association between the need for surgical intervention, this suggest that CD risk allele may play a role in the aggressiveness of the disease such as penetrating behavior or severe inflammation due to deeper tissue localization and severe inflammatory responses by the host. This suggestion had been argued by a study done by Rankin et al. they demonstrate the presence of Human Cytomegalovirus in intestinal tissue lead to life threatening bleeding that ultimately need surgical operation(Rankin *et al.* 2009).

So, the exact functional consequences of rs2241880 partially explored recently. The human ATG16L1 protein has an N-terminal ATG16 domain consisting of coiled coils and eight C-terminal WD repeats(Parkhouse *et al.* 2013). Substitution mutation at the position 300 results in an amino acid exchange of threonine (polar) to alanine (nonpolar) at N terminus of the WD-repeat domain. The resultant protein (mutant protein) has lower affinity for binding to ATG12-ATG5 complex(Fujita *et al.* 2009). Furthermore, Fujita, et al. showed that Atg16L1 T300A mutant have little impact on canonical autophagy and autophagy against *Salmonellaenterica* serovar *Typhimurium*. This prospect points to a potential key role for antibacterial autophagy in CD pathogenesis.

The role of the microbiome in the pathogenesis of CD has become more prominent in recent years(Mukhopadhya *et al.* 2012), a small number of studies have aimed to determine the relationship between autophagy risk-variants and microbial components.

Acknowledgment

This research is a part of Ph.D. thesis. It was funded by post-graduate research funding programme from the Iraqi Ministry of Higher Education and Scientific Research and the Medical College of Al-Nahrain University.We thank lecturer M.M.A. College of Medicine/Al-Basra University for kindly providing ELISA kits (ASCA, pANCA and hCRP).

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