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RESEARCH ARTICLE

Detection of Norovirus in association with ATG16L1 T300A genetic variant in Crohn's Disease

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Abstract

In Crohn's disease, the interaction between ATG16L1 genetic variant and Murine Norovirus infection has been proposed. This study aims to estimate the association of Human Norovirus infection with ATG16L1 genetic variant among inflammatory bowel disease patients. This Case-control study involves 35 Crohn's disease (CD) and 40 ulcerative colitis (UC) and 35 normal subjects obtained from 3 gastroenterology centers in Baghdad. Sequence specific primer polymerase chain reaction (SSP-PCR) used for ATG16L1 genotyping. Biopsies were sectioned and Norovirus by indirect immunofluorescence staining. Among Iraqis, T300A genetic variant confirmed as a risk factor in CD than HC (OR=2.57) or UC (OR=2.76). Among IBD patients, 9 (25.7%) CD and 7 (17.5%) UC patients were infected with Norovirus. 6 (50%) of homozygous mutant (GG) have Norovirus and 2 (20%) of heterozygous (GA) while, homozygous wild type have no evidence of Norovirus in their tissue biopsies. Among G allele carriage 38.5% were positive while 9.68% among allele A carriage were Norovirus positive. Among clinical samples the interaction between host genetics and viral infection.

INTRODUCTION

In Crohn's disease (CD), The autophagy gene ATG16L1 is one of susceptibility loci that considered as risk factor for CD (Rioux et al. 2007; Hampe et al. 2007). Despite that, many viruses can impair cellular autophagy as escape mechanism through avoidance of capturing, impairment of initiation of phagosome(Deretic 2010).

A number of potential cofactors such as viruses can confer risk for Crohn's disease development, but the exact etiological agent (s) remains a controversial (Sartor 2008). Noroviruses are positive sense RNA viruses, belongs to the family Caliciviridae. The human pathogens cause at least 95% of nonbacterial gastroenteritis and 50% of all gastroenteritis outbreaks worldwide(Phillips et al. 2010). It can be self-limiting with 1-2 weeks or more than 2 years in immunocompromized individuals. It has been reported that Norovirus infection may be associated with exacerbation of diarrhea. Yet, it's not known whether the virus initiate or associated with the disease (actively or passively) (Khan et al. 2009). The interaction between Norovirus and host ATG16L1 genetic variant produced Crohn's disease. In clinical samples, this study try to document this interaction by testing the association between Norovirus infection and host ATG16L1 genetic variation.

Material 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. 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. 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 allelic frequency were presented in Table 2. The carriage of CD risk allele was statistically significant higher among 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: 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*	-

Detection of Norovirus in tissue among study groups:

Our results found that 9 (25.7%) were positive among 35 CD patients and 7 (17.5%) among 40 cases with UC patients and all healthy controls were negative by immunofluorescence (figure 1).

In CD and UC, Norovirus detected in colonic sections in the crypt lining epithelial cells mostly at the base of crypts where paneth cell found, inflammatory cells between glands and lining mucosa. It has been suggested that localization of Norovirus at the surface epithelium lining mucosa made it as a possible site of entry to underlining tissue (figure 1 A, B, C and D). There is an association with colonic CD disease 7/19 (36.8%) were Norovirus positive (p=0.044) and associated with samples whose patients need for surgery 7/17 (41.2%) p=0.048.

Among patients carrying G allele, there are 38.5% of them were Norovirus positive compared with 9.68% of patients carrying A allele (p=0.006) see table 4.

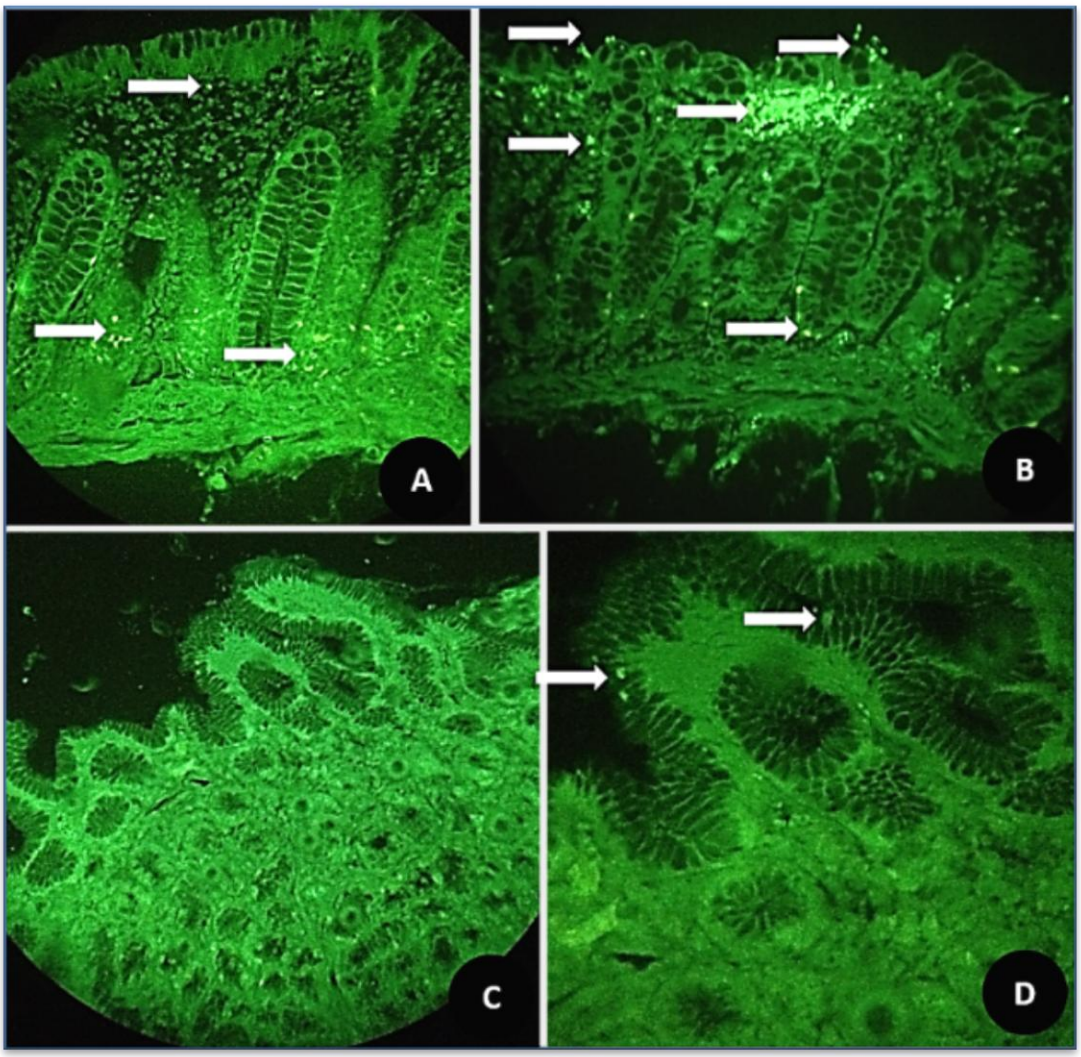

Figure 1: Indirect immunofluorescence detection of Norovirus capsid protein (VP1) in formalin-fixed, paraffin-embedded tissue section of IBD patients. A and B: CD colonic sections showing viral antigen (arrows) in the crypt lining epithelial cells (A&B) mostly at the base of crypts and inflammatory cells between glands were stained with green fluorescence. B: localization of Norovirus at the surface epithelium lining mucosa (viral aggregates can be seen just beneath epithelium in inflammatory cells). Original magnification, x200. C. Colonic tissue section of UC patient (Norovirus positive). D. Higher magnification (X400) of (C) showing viral localization at the surface epithelium (arrows).

Table 3: Association of Norovirus detection with Montreal disease classification and clinical variables among CD.

		Norovirus					p value
		Negative	%	Positive	%	Total	
Age at diagnosis	A1: Younger than 16	2	66.67%	1	33.33%	3	0.256
	A2: 17-40 years old	16	69.57%	7	30.43%	23	
	A3: Older than 40	8	88.89%	1	11.11%	9	
Total		26	74.29%	9	25.71%	35	
Disease behavior	B2: Stenosing	8	88.89%	1	11.11%	9	0.211
	B3: Penetrating	12	66.67%	6	33.33%	18	
	B1: Inflammatory	6	75.00%	2	25.00%	8	
Total		26	74.29%	9	25.71%	35	
Disease location	L1: Ileal	4	100.00%	0	0.00%	4	0.044
	L2: Colonic	12	63.16%	7	36.84%	19	
	L3: Ileocolonic	10	83.33%	2	16.67%	12	
Total		26	74.29%	9	25.71%	35	
Extra intestinal manifestation	No	14	82.35%	3	17.65%	17	0.443
	Yes	12	66.67%	6	33.33%	18	
Total		26	74.29%	9	25.71%	35	
Surgery	No	16	88.89%	2	11.11%	18	0.048
	Yes	10	58.82%	7	41.18%	17	
Total		26	74.29%	9	25.71%	35	

Table 4: Distribution of Norovirus results according to ATG16L1 (Thr300Ala) genotypic and allelic variation among CD.

		Norovirus		P value
		Negative (%)	Positive (%)	
Allele	A	28 (90.32)	3 (9.68)	
	G	24 (61.54)	15 (38.46)	0.006*
Total		52 (74.29)	18 (25.71)	

Discussion:

An interplay between microbe and autophagy pathway had been suggested (Messer et al. 2013; Raju et al. 2012; Cadwell et al. 2011). We test this hypothesis in group of Iraqi IBD patients that human Norovirus can cause inflammatory bowel disease beside association of ATG16L1 T300A SNP with Crohn's disease occurrence.

The principal job of intestinal immune system is to maintain a balance between the recognition and elimination of pathogens while keeping the commensal bacteria and food antigens. However, Xenophagy in particular play a pivotal role in this mechanism through autophagy proteins (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 CD, the ATG16L1 rs2241880 has been identified as a risk factor among CD but not UC(Hampe et al. 2007).

In line with this, our results reported the association of rs2241880 T300A with Crohn's disease but not with Ulcerative colitis. This agreed by several studies globally that mentioned this association (Rioux et al. 2007; Hampe 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 and the presence of Norovirus in tissue biopsies of those patients, this suggest that virus may play a role in the aggressiveness of disease such as penetrating behavior or sever inflammation due to deeper tissue localization and sever inflammatory responses by host. This suggestion had been argued by 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).

This study identify human Norovirus in the tissue obtained from 9 CD (25.7%) and 7 UC (17.5%) and not in the normal controls. Superior to other researches, tissue localization of Norovirus has been achieved in this study. It has

been identified at the surface of intestinal epithelium, inflammatory cells and at the base of crypts (Figure 1). This suggests that the portal entry of Norovirus was intestinal epithelium in order to invade deeper tissues (glandular epithelium and inflammatory cells). Our results supported by the evidence that The C-terminal region of the major capsid protein VP1 binds with histo-blood group antigens (HBGA) sites (site I, 325– 331; site II, 340–346; site III, 373–381)(Harrington et al. 2004; White et al. 1996). Furthermore, recent study by Murakami et al., based on incubation of viral particles with fresh pieces of human intestine specimens. They reported that Norovirus particles binds and internalized to epithelial and goblet cells(Murakami et al. 2013). Another study found that in vitro culture of Norovirus with cell culture had revealed tropism for dendritic cells and microphages(Wobus et al. 2004).

Based on clinical samples obtained from IBD patients, this study is first report the association between the ATG16L1 T300A genotype and the presence of Norovirus in tissue obtained from those patients. Furthermore, 50% from CD carrying GG genotype were infected by Norovirus and 20% of GA genotype (data not shown). This result support the hypothesis of association between host genetics and viral infection highlighting the importance of autophagy process in elimination of pathogens. It's difficult to identify single infectious agent responsible for IBDs. Many bacteria and viruses can cause gastroenteritis. Interestingly, the Crohn's disease associated gene NOD2 may recognize viral RNA in addition to bacterial peptidoglycan (Homer et al. 2010; Cadwell et al. 2011), raising the possibility that a viral infection could be influenced by functional consequences of T300A genetic variation.

Competent autophagy process mediate Interferon- gamma production in response to viral infection (Kudchodkar & Levine 2009). IFN-γ had degradative effect and play a key role in control of sindbis virus infection (Orvedahl et al. 2010), herpes simplex virus(Pei et al. 2011) and human immunodeficiency virus (Kyei et al. 2009). In fact, the degradative activity of autophagy likely contribute to anti-pathogen (s) activity of autophagy proteins such as ATG16L1 (Conway et al. 2013). So, ATG16L1 T300A variant results in defective autophagy (in particular Xenophagy) and the association of higher prevalence of Norovirus infection among CD patients suggests non-degradative role of autophagy. This suggestion has been argued by Hwang, et al., whom reported that hypomorphic mice for ATG16L1 (mimics mutant ATG16L1 protein) upon conjugation with their ATG5 and ATG12 complex, they results in non-degradative autophagic vacuole (Hwang et al. 2013). In addition to that, the use of immunosuppressive drugs such as Inflaximab (Tumor necrosis factor-alpha blocking agent), corticosteroids, in the treatment of IBD patients will attenuate body immune system, making a good environment for opportunistic (viral, fungal, bacterial) infections(Toruner et al. 2008). Also, abnormal microbial composition results in increased pathogenic microbes in the gut lumen(Frank et al. 2007).

An association between Norovirus infection and ATG16L1 Genetic variant has been suggested in clinical samples. This association was more frequent in patients whom need for surgery, suggesting their role in the prognosis of disease.

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