Turk J Gastroenterol *2014; 25 (Suppl.-1): 122-8*

NOD2/CARD15 gene influences disease behaviour but not IBD susceptibility in a Moroccan population

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ABSTRACT

Background/Aims: IBD (Crohn's disease and Ulcerative Colitis) is chronic and multifactorial disease of the gastrointestinal tract. Till now, his pathogenesis remains unclear. It involves innate immunity, environmental component and genetic predisposition. Polymorphisms in NOD2/CARD15 have been implicated in Crohn's disease in several ethnic groups. The purpose of our study was to assess the frequency of the three major variants of this gene (Leu1007fsinsC, R702W, and G908R) in Moroccan IBD patients and to determine a possible effect of these variants on Disease's phenotype and clinical course.

Materials and Methods: A total of 96 Moroccan unrelated IBD patients and 114 healthy controls were genotyped (PCR-RFLP method) for the three main polymorphisms.

Results: In this study, no correlation was found between NOD2/CARD15 polymorphisms and ulcerative colitis or Crohn's disease in our population. Nevertheless, 3020insC (Leu1007fsinsC) variant was associated to a structuring behaviour on CD patients.

Conclusion: These findings suggest that NOD2/CARD15 influences disease behaviour but not susceptibility to crohn's disease in Moroccan IBD patients.

Keywords: Inflammatory bowel disease, Crohn's disease, ulcerative colitis, NOD2/CARD15 gene, polymorphism, moroccan population

INTRODUCTION

IBD is chronic debilitating condition of the gastrointestinal tract, Including Crohn's disease (CD), Ulcerative colitis (UC) and indeterminate colitis. It is characterized by a chronic course in which phases of remission of variable length are interrupted by acute episodes (1). CD and UC typically appear in young adolescents (2nd to 4th decades of life) with no differences in prevalence between males and females (1). Incidence of IBD in adults varies significantly according to geographical and ethnic origins. The highest rates have been reported in the Scandinavian countries and Scotland followed by England and Southern Europe (2). However, it can be stated that IBD is now relatively frequent in most industrialized countries and childhood IBD accounts for nearly 20% of total cases (3). The annual incidence of UC and CD in Western countries is about 10 and 6 per 100.000, respectively (4). IBD, like most common diseases, has a complex aetiology involving multiple genetic and environmental factors. IBD is a multifactorial disorder that involves genetic susceptibility, immunodysfunction, and environmental and microbial factors. In 2001, the first IBD gene, NOD2 (for nucleotide-binding oligomerization domain containing 2, previously known as caspase recruitment domain protein 15/CARD15) was identified through association mapping of one of these linkage regions (5, 6). As a pathogen-recognition molecule for muramyl dipeptide (MDP), NOD2 controls both innate and adaptive immune responses, through the regulation of cytokines, chemokines and antimicrobial peptides production (7). To date, more than 30 variations in this gene have been reported (8). Moreover, the three common CD-associated variants of CARD15, R702W, G908R and Leu1007fsinsC are located in the C-terminal portion (5). For UC, it was shown that several genes, including the interleukin-23 receptor gene (IL23R), the inter-leukin-12B gene (IL12B),

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Received: July 08, 2012 **Accepted:** October 10, 2012
 • Available online at www.turkjgastroenterol.org • DOI: 10.5152/tjg.2014.3870

the protein extracellular matrix 1 gene (ECM1),the actin-related protein 2/3 complex subunit 2 gene (ARPC2) and the interleukin-10 gene (IL10) were significantly associated to this disorder (9, 10, 11).

In this study, we investigated the presence of variants R702W, G908R and 1007fs in the CARD15 (NOD2) gene in the Moroccan population and performed case–control association studies to assess the significance of this gene in the pathogenesis of IBD (both CD and UC) in Moroccan people.

MATERIALS AND METHODS

Subjects

A group of 96 Moroccan unrelated IBD patients were enrolled in this prospective study. They were recruited between February 2010 and January 2011. All the patients were diagnosed and managed at the gastroenterology service of Averroes Hospital in Casablanca, Morocco.

The control group included 114 unrelated Moroccan volunteers (blood donors) with no discernable symptoms suggestive of IBD.

The diagnosis of CD or UC was based on established clinical, radiologic, endoscopic, and histopathologic criteria.

Demographic and clinical characteristics were obtained from participants through a detailed questionnaire. CD phenotype was classified by age at diagnosis, location, and behavior of disease according to the Montreal classification.

In patients with UC, anatomic location was also subgrouped using the Paris classification as being ulcerative proctitis (E1), left-sided UC (E2), and extensive UC (E3).

Differences in the frequency of disease characteristics such as age at diagnosis, gender, extra-intestinal manifestations, similar familial cases, and antecedents like appendectomy and smoking were also assessed.

The study was approved by the local ethics committee of our institution and written informed consent was obtained from all participants or their guardians.

Both IBD patients and control group come from the different regions of Morocco and confirmed the Moroccan origin of their parents and grandparents.

Genotyping methods

The extraction of Genomic DNA from whole blood samples was carried out using salt method (12). DNA amount and quality were measured for each sample by spectrophotometry. Both Patients and control's DNA were genotyped for the three variants R702W, G908R, and p.L1007fsinsC of the NOD2 gene using PCR-RFLP method.

PCR products for R702W, G908R, and L1007fs were digested respectively by Msp I, Hha I and Apa I as previously reported (13).

Statistical analysis

Statistical analysis was performed using MedCalc statistical software version 11.6. The Hardy-Weinberg equilibrium test was performed separately for cases and controls to measure the distribution of polymorphism. Allele and genotype frequencies in patients with IBD and in controls were determined. The relation between IBD (CD and UC) and NOD2 genotypes (R702W, G908R, L1007fs) was determined by using Fisher's exact test (Odds Ratio with Confidence interval (CI) at 95%). The genotype-phenotype correlations were assessed with $\chi 2$ test. The P value (<0.05) was considered statistically significant in all variables.

Table 1. Allelic and Genotypic frequencies (%) of three common variants of NOD2/CARD15 in IBD patients and Controls

NOD2 Variant	CD Patients % N=69	Controls N=114	OR (95% CI)	P value (P<0.05)
G25386C	GG	65 (94.2)	112 (98.2)	Réf
GC	4 (5.8)	2 (1.8)	3.45 (0.61-19.34)	0.16
G	134 (97.1)	226 (99.1)	Réf	
C	4 (2.9)	2 (0.9)	3.37 (0.61-18.67)	0.16
C14772T	CC	66 (95.7)	105 (92.1)	Réf
CT	3 (4.3)	8 (7)	0.60 (0.15-2.33)	0.46
TT	0	1 (0.9)	0.52 (0.02-13.17)	0.70
C	135 (97.8)	218 (95.6)	Réf	
T	3 (2.2)	10 (4.4)	0.48 (0.13-1.79)	0.28
32629insC				
Wt/wt	67 (97.1)	113 (99.1)	Réf	
I/wt	2 (2.9)	1 (0.9)	3.37 (0.30-37.91)	0.32
wt	136 (98.6)	227 (99.6)	Ref	
I	2 (1.4)	1 (0.4)	0.34 (0.30-37.16)	0.33
	UC Patients % N=27	**Controls N=114**	**OR (95% CI)**	**P value (P<0.05)**
C14772T				
CC	26 (96.3)	105 (92.1)	Réf	
CT	1 (3.7)	8 (7)	0.50 (0.06-4.21)	0.53
TT	0	1 (0.9)	1.33 (0.05-33.51)	0.86
C	53 (98.1)	218 (95.6)	Réf	
T	1 (1.9)	10 (4.4)	0.4 (0.05-3.28)	0.40

OR: Odd Ratio

No significant difference in frequency of the G908R (2.9 vs 0.9 %, P = 0.16), R702W (2.2 vs 4.4%, P = 0.28) and 3020insC (1.4 vs 0.4, P= 0.33) alleles was found between CD patients and controls, respectively. Similarly, there was no significant difference in the genotype frequency.

For UC patients, only the C14772T mutant allele was found. There was no significant difference of R702W allele frequency between UC patients and controls (1.9 vs 4.4, P= 0.40).

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RESULTS

Our study included a total of 96 IBD patients (69 with CD and 27 with UC) and 114 controls.

The average age was 27 years old for CD patients and 38 years old for UC, the sex ratio was 1,59 (59 man/37 women). 3 of the UC patients and 4 of CD patients had a positive familial history for IBD and one patient had a relative with colon cancer history.

The control group included 114 unrelated Moroccan volunteers (blood donors) with no discernable symptoms suggestive of IBD. There were 62 women and 52 men, aged from 17 to 87 years old. The NOD2/CARD15 allele frequencies were in Hardy-Weinberg equilibrium (G25386C (G908R) (x^2 x^2 =1.04, p=0.31; x^2 x^2 =0.29, p=0.59), C14772T (R702W) (x^2 x^2 =0.26, p=0.61; x^2 x^2=0.90, p=0.64) and 3020insC (Leu1007fs) (x^2x^2=0.11, p=0.74; x^2x^2=0.02, P=0.90)) in all patients and in control subjects.

Table 2. Genotype- Phenotype Correlations in CD patients

	G25386C				32629insC				C14772T			
	N	GG %	GC%	P	N	wt/wt	I/wt	P	N	CC	CT	P
Onset age(years)	69			0.53	69			0.73	69			0.62
<17	11	11(100)	-		11	10(100)	0		11	11(100)	-	
17-40	53	49(92.5)	4(7.5)		53	51(96.2)	2(3.8)		53	50 (94.3)	3(5.7)	
>40	5	5(100)	-		5	5(100)	-		5	5(100)	-	
Sexe	69			0.31	69			0.74	69			0.61
Femme	25	25(100)	0		25	25(100)	-		25	23(92.0)	2(8.0)	
Homme	44	40(90.9)	4(9.1)		44	42(95.5)	2(4.5)		44	43(97.7)	1(2.3)	
Type	69			0.38	69			0.04	69			0.68
Fistulizing	28	25(89.3)	3(10.7)		27	25(100)	-		27	25(92.6)	2(7.4)	
Not Fistulizing Not stricturing	22	22(100)	-		22	22(100)	-		22	21(95.5)	1(4.5)	
Stricturing	13	12(92.3)	1(7.7)		14	12(85.7)	2(4.3)		14	14(100)	-	
Fistulizing and stricturing	6	6(100)	-		6	6(100)	-		6	6(100)	-	
Localisation	69			0.63	69			0.83	69			0.64
L1	21	19(90.5)	2(9.5)		21	20(95.2)	1(4.8)		21	19(90.5)	2(9.5)	
L2	22	20(90.9)	2(9.1)		22	22(100)	-		22	21(95.5)	1(4.5)	
L3	19	19(100)	-		19	18(94.7)	1(5.3)		19	19(100)	-	
L4	3	3(100)	-		3	3(100)	-		3	3(100)	-	
L4+L2	4	4(100)	-		4	4(100)	-		4	4(100)	-	
FSC	69			0.55	69			0.24	69			0.41
yes	4	4(100)	-		4	4(100)	-		4	4(100)	-	
no	65	61(93.8)	4(6.2)		65	63(96.9)	2(3.1)		65	62(95.4)	3(4.6)	
Smoking	69			0.36	69			0.65	69			0.39
yes	28	25(89.3)	3(10.7)		28	27(96.4)	1(3.6)		28	28(100)	-	
no	41	40(97.6)	1(2.4)		41	40(97.6)	1(2.4)		41	38(92.7)	3(7.3)	
Appendicectomy	69			0.13	69			0.61	69			0.85
yes	9	7(77.8)	2(12.2)		9	9(100)	-		9	8(88.9)	1(11.1)	
no	60	58(96.7)	2(3.3)		60	58(96.7)	2(3.3)		60	58(96.7)	2(3.3)	
EIM	69			0.43	69			0.36	69			0.15
yes	39	38(97.4)	1(2.6)		39	39(100)	-		39	39(100)	-	
no	30	27(90.0)	3(10.0)		30	28(93.3)	2(6.7)		30	27(90.0)	3(10.0)	

(FSC : Familial similar cases, EIM : extra intestinal manifestations)

No genotype-phenotype correlation was found between NOD2 variants and UC or CD group except for the 32629insC mutation that was associated with a structuring behaviour on CD patients (P value: 0.04)

The genotype and allele frequencies of the three major CARD15 polymorphisms in IBD patients and healthy controls are shown in Table 1. No significant difference in frequency of the G908R (2.9 vs 0.9 %, p=0.16), R702W (2.2 vs 4.4%, p=0.28) and 3020insC (1.4 vs 0.4, p=0.33) alleles was found between CD patients and controls, respectively. Similarly, there was no significant difference in the genotype frequency.

For UC patients, the G25386C and 32629insC mutant alleles were absent. Only the C14772T mutant allele was found. There was no significant difference of R702W (C14772T) allele frequency between UC patients and controls (1.9 vs 4.4, p=0.40).

In both CD and UC groups, there was no homozygous status. No patient had compound heterozygous. In control group, there was one patient with homozygous R702W polymorphism.

We also evaluated whether the CARD15 SNPs (R702W, G908R and L1007fs) could have an influence on specific disease phenotypes such as gender, disease location and behavior, resective surgery, family history, smoking habit, extra-intestinal manifestations, appendectomy and surgery. No genotype-phenotype correlation was found between NOD2 variants and UC or CD group except for the 3020insC polymorphism that was associated with a structuring behaviour on CD patients (p=0.02) (Table 2 and 3).

DISCUSSION

Till now, the etiology of IBD is still poorly understood, but it is more likely admitted that their pathogenesis involves a dysregulation in the immune response against the commensal flora in a context of genetically susceptible individual.

In the last decade, the major discovery in the field of IBD pathogenesis had been the identification of NOD2/CARD15 polymorphisms in Caucasian populations suffering from Crohn's disease. Three major variants were described in the C-terminal end encoding the LRR region of the NOD2 protein (5,6,14).

The role of NOD2/CARD15 gene variants was controversial in patients with UC.

Since that, several studies have been conducted to establish the relation between these polymorphisms and the IBD patients in numerous ethnics (Table 4).

Approximately, 27-50% of Caucasian CD patients were carriers of at least one susceptibility allele of NOD2 gene (15). By cons, in Scandinavian countries generally characterized by more homogeneous populations, there are much lower frequencies than expected in European populations (16,17,18). Furthermore, no one of the NOD2 variants was found in some Asiatic ethnics (China, Japan and Korea) (19,20,21).

Thus, these finding suggest that ethnic and geographic variations may partially have an impact on the differences in the frequency of developping CD and the presence of NOD2/CARD15 gene variants in different world populations (22).

In this study, we tried to establish an association between inflammatory bowel disease (CD and UC) and the three main variants in the coding regions of the NOD2/CARD15 gene in a Moroccan population.

When comparing our results in CD with those in other Moroccan study (23), we found that the frequencies of R702W and L1007fs variants were slightly higher (2.2% vs 0.49% and & 1.4 % vs 0.99% respectively). By cons, the G908R variant was found in much lower frequency (2.9% vs. 6.43%). It is likely that this variation can reflect the relatively small sample size in our cohort (69 vs. 101 CD patients). Also, it could be explained by the difference in sample's origin. Given that both study were undertaken in different areas and (Casablanca vs. Rabat). Thus, Patients could be from different ethnics (Arab, Berber or sub Saharan).

Table 3. Genotype- Phenotype correlations between Disease Behaviour and NOD2 variants in CD patients

	32629insC Fistulizing N=27			
	Patients %	**Controls**	**OR (95%)**	**p**
Wt/wt	27 (100)	113 (99.1)	Réf	
I/wt	0 (0)	1 (0.9)	1.38 (0.05-34.70)	0.85
wt	54 (100)	227 (99.6)	Ref	
I	0 (0)	1 (0.4)	1.39 (0.06-34.63)	0.84
	32629insC Not Fistulizing Not Stricturing N=22			
	Cas%	**Témoins**	**OR (95%)**	**p**
Wt/wt	22 (100)	113 (99.1)	Réf	
I/wt	0 (0)	1 (0.9)	1.68 (0.07-42.61)	0.75
wt	44 (100)	227 (99.6)	Réf	
I	0 (0)	1 (0.4)	1.70 (0.07-42.51)	0.75
	32629insC Stricturing (N=14)			
	Cas%	**Témoins**	**OR (95%)**	**p**
Wt/wt	12(85.7)	113 (99.1)	Réf	
I/wt	2(4.3)	1 (0.9)	18.83 (1.59-223.36)	0.02
wt	26 (92.9)	227 (99.6)	Réf	
I	2 (7.1)	1 (0.4)	17.46 (1.53-199.25)	0.02
	32629insC Fistulizing and Stricturing N=6			
	Cas%	**Témoins**	**OR (95%)**	**p**
Wt/wt	6 (100)	113 (99.1)	Réf	
I/wt	0(0)	1 (0.9)	5.82 (0.22-157.31)	0.30
wt	12 (100)	227 (99.6)	Réf	
I	0	1 (0.4)	6.07 (0.24-156.61)	0.28

We observed an association between the 32629insC mutation and disease behavior (P value= 0.02).

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Furthermore, we observed a high frequency of the R702W polymorphism in controls in comparison with patients and no statically significant differences were observed for the three main variants between patients and controls. This result is in harmony with other series (23-27) of Morocco, Tunisia, Turkey, South-Africa and Israeli Arab respectively.

The three main variants of NOD2/CARD15 gene were implicated in Caucasian (5,28-32) with high frequencies rates. In contrast, other Asian populations exhibit no variants of this gene in CD patients (19,20,21,33).

In our context, due to the lack of association between CD and NOD2 polymorphisms, the variants frequencies were intermediate between Caucasian and Asian populations. Moroccan population is mainly characterized by ethnic diversity. There are different ethnics: Arabs, Berbers, Jews and black sub-Saharan Africa (34). This genetic heterogeneity may be reflected in changes of genetic status in relation to susceptibility to IBD, taking into consideration that this disease exhibit epidemiological and genetic variations depending on ethnic and geographical changes.

Table 4. Comparison of allelic Frequencies of NOD2 polymorphisms between Moroccan IBD population and other ethnic groups

					Allele Frequency of NOD2 Variants								
		Nbr of patients			R702W			G908R			1007fs		
Ref /Author	**Country/Region**	**CD**	**UC**	**Controls**	**CD**	**UC**	**C**	**CD**	**UC**	**C**	**CD**	**UC**	**C**
Zouiten-Mekki, L., et al., 2005.	Tunisia	130		90	2		0.6	5		3	1		0
Uyar et al. 2004	Turkey	56		100	0.9		0.5	8		0	1.8		1
S.C. Özen et al 2006	Turkey	70	120	106	1.4	1.7	1.9	2.1	0.8	1	1.4	0	0
Nunez, C et al 2004	Spain	165		165	6.7		5.8	4.5		1.0	4.5		1.0
Hama et al. 2012	Morocco	101		107	0.49		0.46	6.43		2.80	0.99		0.00
Vavassori et al. 2004	Italy	165		125	1.2		0.8	5.2		2.0	11.2		1.2
Hugot et al . 2001	France	453		103	11.5		4.7	3.7		1.6	9.0		4.2
KARBAN ET AL. 2005	Israeli arab	66		122	0.75		0.0	2.3		1.2	0.75		0.0
Tukel et al. 2004	Ashkenazy jews	115		246	6.9		2.8	8.3		4.3	5.6		3.0
Esters et al. 2004	Belgium	570	173	165	12.9	7.8	5.8	6.0	3.2	1.8	8.6	1.4	3.0
Vind et al. 2005	Portugal	29		200	12.1		6.3	1.7		0.5	0		2.9
Leong et al. 2003	China	65	63		0.0	0.0		0.0	0.0		0.0	0.0	
Inoue et al. 2002	Japan	350	272	292	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lee GH et al. 2005	Korea	128	47	200	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ahmad et al. 2002	England	244		349	12.5		5.2	5.2		1.4	9.4		1.6
Vermeire et al. 2002	Cananda	229		71	12.9		4.2	5.2		0.7	10.3		0.7
M.G. Zaahl et al. 2005	South africa	41	35	100	6	1	3	0.0	0.0	0.5	1	4	4
Baptista et al. 2008	Brazil	187		255	9.63		2.75	1.93		1.64	3.48		0.78
Cavanaugh et al.	Australia	267	35	409	11	0.0	5.0	2.0	0.0	1.0	7.0	0.0	1.0
V Medici et al. 2006	Norway	79		236	2.4		2.4	1.0		0.9	3.0		1.5
Arnott et al. 2004	Scotland	252	247	189	7.2	2.6	5.3	1.8	2.0	0.3	4.6	2.8	2.1
Derakhshan et al . 2008	Iran	40	100	100	13	1	2	4	3	2	2	0	2
Oostenbrug et al. 2006	Netherlands	369	207	276	9.0	3.9	NA	4.8	3.0	NA	9.2	4.4	NA
Radlmayer et al 2002	Germany	97	97	120							23.7	4.1	3.3
Gazouli et al. 2005	Greece	120	85	100	10.0	7.1	1.0	14.2	13.5	3.5	17.9	3.5	6
Heliö et al. 2003	Finlande	271	99	300	3.3	1.5	1.8	0.6	0.0	0.0	4.8	3.0	1.7
Kanaanet al. 2012	USA	485	276	435	11	7	3	4	1	1	7	2	1
E Leung et al. 2005	New Zealand	185		187	7.3		5.1	3.5		2.4	8.1		0.8
This Study	Morocco	69	27	114	2.2	1.9	4.4	2.9	0.0	0.9	1.4	0.0	0.4

Ethnic and geographic variations may partially influence differences in the frequency of developping CD and the presence of NOD2 / CARD15 gene variants in different world populations

Ulcerative Colitis is not associated with CARD15 three major variants (35). This was supported by our finding. Thus, allelic frequency for R702W, G908R and L1007fs variants were respectively 1.9%, 0.0% and 0.0%. In our UC cohort, we found only one heterozygous mutant for the C14772T variant.

In addition to our association-study, we carried out a detailed genotype/phenotype analysis in CD patients. We found no association between R702W, G908R and sex, Location, behavior disease, age of onset, familial history or extra intestinal manifestations.

Contrariwise, we observed a correlation between the 3020insC variant and disease behavior (p value=0.04). Several authors had previously reported this association with L1007fs polymorphism (16,30,36-40). Helio et al had shown that NOD2 gene variants were associated with ileal disease as well as stricturing and penetrating forms of the disease. Barreiro et al (22) demonstrated that the need for Crohn's disease-related surgery is higher in carriers of the G908R or 1007fs CARD15 mutation in the Galician population. In addition, the study of Vavassori et al (36) shows that only the Leu1007fsinsC mutation is a risk factor of CD in an Italian population. In the same way, Radlmayer et al observed a positive association of the C-insertion mutation with the fistulising or the fibrostenotic CD subtype, and the necessity of surgical intervention in Crohn's disease, whereas the inflammatory subtype was negatively correlated. All these finding suggest that NOD2 might play an important role in occurrence of CD subtypes which are characterized by fibrostenotic or stricturing behaviour.

In summary, we investigated the impact of the three main variants (R702W, G908R and L1007fs) of NOD2 gene in the Moroccan population with IBD (CD, UC). We were unable to reach a conclusion because of the insufficient size of our sample. Nevertheless, our study suggests no evidence of association of these polymorphisms in Moroccan IBD patients. Moreover, our results show differences in allelic and genotypic frequencies than reported by previous authors (23). Differences in sample size and geographic area could explain these subtle variations between Moroccan CD patients. Nevertheless, in our genotype-phenotype analysis, it seems that Card15 variants confers predisposition to stricturing form in Moroccan CD patients. According to these findings, multicentric studies with larger number of patients are indicated to confirm our results. Also, we suggest that further investigations may help to improve association with other genes susceptibility in Moroccan IBD patients.

Conflict of Interest: No conflict of interest was declared by the authors.

Acknowledgments: We would like thank all the patients and their families for their time and participation. Our gratitude goes also to Dr Maria GAZOULI (Laboratory of Biology, School of Medicine, University of Athens, Athens, Greece) and Pr Severine Vermeire (Division of Gastroenterology, University Hospital Gasthuisberg, Belgium) for providing us with positif controls. Finally, we thank the clinicians and all the staff of gastro-enterology department of CHU Ibn Rochd for their assistance in data and sample collection.

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