Genotyping of Tumor Necrosis Factor-*α* in Inflammatory Bowel Iraqi Patients

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Abstract

The *TNF-a* gene considered as strong candidate for immune modulator and pro inflammatory cytokine responsible for genetic susceptibility of chronic disease such as the initiation and development of inflammatory bowel disease (IBD).The aim of this study is to investigate the genetic polymorphisms of -1031 in *TNF-a* gene with susceptibility of Iraqi IBD patients

Method: The total number of this study 95 blood samples (75 Iraqi IBD patients and 20 from healthy individuals as a control group). The genetic polymorphisms in *TNF-a*-1031 gene was investigated using restriction fragment length polymorphism (RFLP) and Sanger sequences techniques.

Results: The genotype allele frequency of -1031 polymorphism was significantly higher in CD Iraqi patients (*P*=0.042 , Chi 4.612. OR= 0.72-1.64) ,C allele may be have protective role , whereas the T allele may increase susceptibility to IBD.

In conclusion: The *TNF-a* -1031 gene polymorphisms in promoter region have an important role in the occurrence of inflammatory bowel disease of Iraqi population especially in CD patients although some results didn't give us a significant differences.It's possible that C allele may be have protective role , whereas the T allele may increase susceptibility to IBD.

Key words: *TNF-a -1031 Tumor necrosis factor , inflammatory bowel disease, ulcerative colitis and Crohn 's disease.*

Introduction

IBD include both Crohn's disease (CD) and ulcerative colitis (UC) ,patients with IBD suffer from some common symptoms such as acute diarrhea ,abdominal pain ,fatigue and weight loss [(1)].The location of the two type of inflammation is different ,its affects entire gastrointestinal tract in the CD where the UC affects the ileum and colon [(2)]. Environmental factors ,genetic and immune regulation play a key role in development and progression of IBD which characterized by an irregular immune response of the mucous layer in the intestine to bacterial antigens within the intestinal lumen [(3)]. Regulation of cytokines such as TNF- α and IL-6 play a key role in activation of T helper cells (type 1 and 17) which causes inflammation disease [(4)].TNF-α is the important cytokine for inflammation as it participates in immune response to IBD [(5)].TNF-α characterized with a wide range of inflammatory activity it is usually produce by macrophage and monocyte although there are other types of cells that are produced but in limited quantities [(6)]. Inflamed mucous layer of the intestine in IBD patients have increased gene expression of *TNF-a* gene [(7)] .TNF-α not only stimulates the acute stage of inflammation but plays role for the occurrence apoptosis ,proliferation and differentiation of cells and several immune disorders [(8)].

There is a correlation between the genetic polymorphisms of the encoded gene of TNF-α and the susceptibility to IBD [(9)]. The *TNF*-α - gene have shown several polymorphisms in its four exons. However, most of the common reported polymorphisms are identified in the promoter region of this gene [(10)] . The *TNF*-α promoter region include various a single nucleotides

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polymorphisms that have shown a significant association with IBD [11],like *TNF*- α (-308G/A) [12] and *TNF*-α -857 polymorphism [13]. The response to anti TNF- α agents is increased and reduced by the effect of genetic factors of individuals [14].

The aim of this study is to detect the *TNF*-α -1031 gene polymorphisms as its important factor in IBD development and treatment.

Materials and Method

Patients:

Blood samples were obtained from 75 Iraqi patients suffering of IBD who were attending the Gastroenterology and Hepatology Disease Center in Baghdad between September–Desember,2018 .Patients samples were selected after the diagnosis was made by the specialist doctor, in addition to 20 blood samples were collected from apparently healthy individuals. Ethical permission to conduct the research was obtained from this hospital and from all participants in this study.

DNA Extraction

Genomic DNA was isolated from blood sample according to the instructions of ReliaPrep™ Blood gDNA Miniprep System kit (Promega, USA). .

Polymerase chain reaction (PCR)

PCR was done for *TNF*-α-1031 gene amplification, each 20 µl mixture of PCR reaction include 10 µl of master mix , 1 for each primer and 5 distilled water and 3 of genomic DNA. The forward and the reverse primers were 5`-TATGTGATGGACTCACCAGGT-3` and 5`-CCTCTACATGGCCCTGTCTT-3` respectively [15].

PCR amplifications were achieved in Thermal cycler (Applied Biosystem 96). PCR reactions were started through initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 30 s., annealing at 55 °C for 30 s. and extension at 72 °C for 30 s. followed by a final extension at 72 °C for 7 min. then hold at 4 °C.

Restriction Fragment Length Polymorphism (RFLP):

PCR products were digested with restriction enzyme, 1µl from BbsI enzyme (Biolabs, England) was added to 5µl of PCR product for each sample. RFLP was performed using Thermal Cycler (Bio Rad, USA) with the following temperature program: 37 °C for 3 hour, enzyme inactivation at 65°C for 5 minutes followed by 10 min incubation at 4°C to stop the reactions. The restriction enzymes fragments were separated on 2% agarose gel stained with ethidium bromide stain.

Sequencing

PCR products were send to Macrogen Corporation – Korea for Sanger sequencing using (ABI3730XL, automated DNA Sequencer).The results were analyzed using genious software.

Statistical analysis

The Hardy-Weinberg equilibrium was used by the chi-square test to evaluate the frequency of genotypes and correlation of the TNF-α -1031genotypes or alleles between IBD patients and controls group. Calculated Odds ratio (OR) with 95% confidence interval (95% CI) for assessing the correlation strength. All data were analyzed using SPSS (2012). A p value of <0.05 was considered significant [16].

Results and Discussion:

Clinical Characteristic of Patients

The total number of this study was 95 blood samples including 75 blood samples from IBD Iraqi patients in addition to 20 blood samples from healthy individuals as a control group. The IBD patients whose enrolled in this study divided into two groups: the first group 47 UC patients and 28 CD patients. Table 1 refer to the clinical characteristics of IBD patients , the ages where ranged between (19-57) years. High rate of patients less than 50 years (81%) with IBD were found in this study while the older age group or more than 50 years was (19%) with high significant association ($P<0.01$) . This result was agreed with [17] they stated that the peak occurrence of the IBD occurs in the second or third decade of life. High incidence of IBD in females, it was (57%) compared with (43%) in males, significant association ($P<0.05$), was appear depend on patients gender. Statistically significant differences were observed between IBD patients and controls depend on type of disease, patients with UC were (60%) higher than patients with CD (40%), these results consist with other Iraqi study from Erbil city [18], this data may be indicate to that UC has more prevalent than CD in Iraqi population.

Table 1: Clinical Characteristics of IBD Patients

Clinical characteristics	Total No. and Percentage %	Chi-Square
	75(100%)	
Average age		
50 ≥	14(19%)	13.208 **
50 <	61(81%)	
Gender		
Male	32(43%)	5.017 *
Female	43(57%)	
Type of disease		
UC	45(60%)	7.250 **
CD	30(40%)	

Genotyping of *TNF-a* - 1031 gene and Alleles frequency in IBD patients

The genetic polymorphisms in *TNF-a* gene considered as strong candidate for immune modulator and pro inflammatory cytokine responsible for genetic susceptibility of chronic disease and the initiation and expansion of IBD [19].

The genetic polymorphisms in the -1031 (T→C) of *TNF*-α gene promoter (db SNP accession number 1rs1799964) was investigated in Iraqi patients with IBD by PCR-RFLP technique and Sanger sequences to determine the genotypes/allele frequency, at this site there are three genotype of *TNF*-α T-1031C in promoter was found , TT with band sizes 251bp and other small band 13 pb that emerge with primer dimer while TC and CC that have band sizes (251/180/ 71) pb and (180/71) pb respectively Figure 1. The sequences analysis of *TNF-a* – 1031T>C showed in Figure 2 .

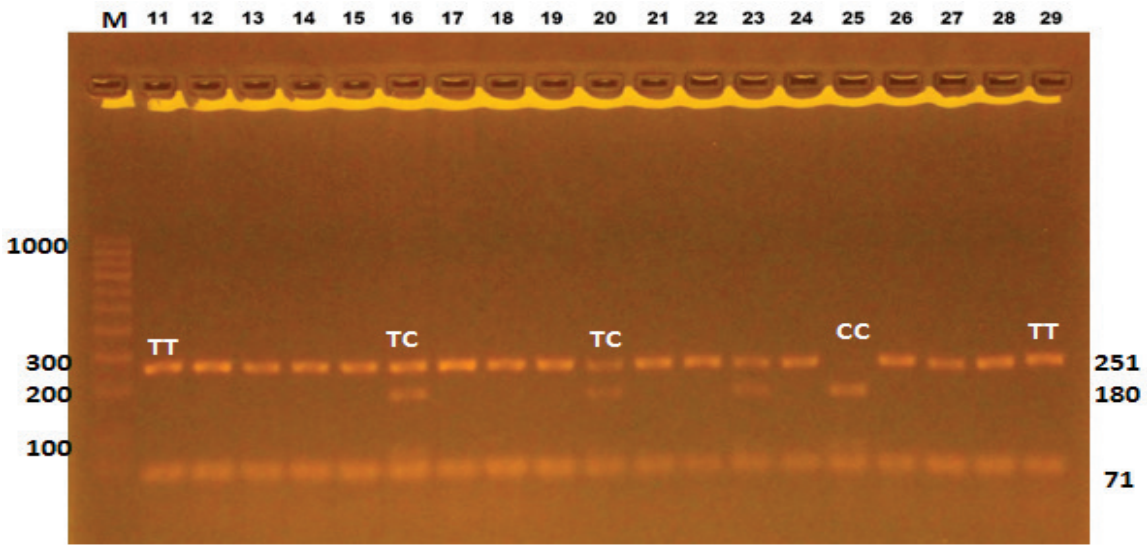

Figure 1: Genetic polymorphism in T -1031 C of TNF-α gene on 2% agarose gel with ethidium bromide dye after digestion with Bbsl enzyme, M : DNA molecular size marker (100bp) , Lane (11) TT genotype (251/13) bp , Lanes (16,20) TC genotype (251/180/ 71) bp and line (25) CC genotype (180,71)bp.

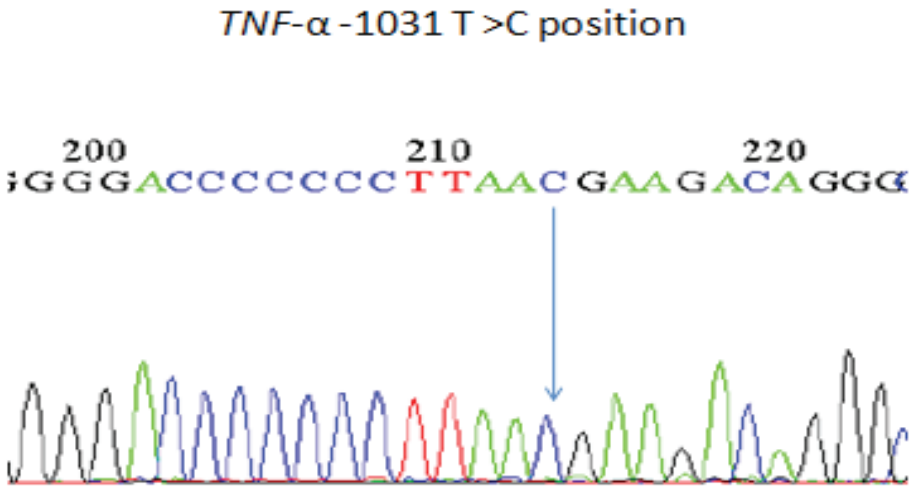

Figure 2 : Sequences analysis of *TNF-a* – 1031T>C using forward primer.

The frequency distribution of genotypes and alleles of *TNF*-α-1031

in IBD patients and control groups are summarized in Tables 2 .The Present results showed 62.7% vs 70 % (*p*=0.061,Chi=3.091 NS,OR=0.377) for homozygous T allele in IBD patients and control group, the homozygous CC genotype was (5.33% vs 5%) in IBD patients and control group , (p=0.872,Chi=0.055 NS ,OR=0.0162) The T>C heterozygous genotype in IBD patients (32%) was higher ratio than control(25%) (p=0.069,Chi=2.983,OR=0.352) the data showed no significant difference was found in correlation to the three genotypes, our results agreed with Iranian population [20].

Alleles frequency for T and C alleles in IBD patients and control group were (0.97, 0.83) and (0.21, 0.17) respectively. The results revealed that there was no significant association regarding genetic polymorphism in *TNF*-α (-1031T/C) polymorphism in IBD patients , statistical analysis indicated that *TNF*-α (-1031T/C) was not a risk factor to IBD.

Table 2: Genotyping of *TNF-a* - 1031 gene in IBD patients

Genotype	Patients IBD N (%) 75(100%)	**Control N (%) 20 (100%)**	***P* -Value**	**Chi-Square**	**OR (CI)**	**EF**	**PF**
TT	47 (62.67%)	14 (70.00)	0.061	3.091 NS	0.377 (0.78-1.49)	0.511	***
TC	24 (32.00%)	5 (25.00)	0.069	2.983 NS	0.352 (0.82-1.55)	***	0.376
CC	4 (5.33%)	1 (5.00)	0.872	0.055 NS	0.0162 (0.69-1.58)	***	0.152
Allele Frequency							
T	0.79	0.83	--	--	--	--	--
C	0.21	0.17	--	--	--	--	--
NS: Non-Significant.							

OR = Odd Ratio CI = Confidence Interval EF = Etiology fraction PF= Preventive fraction

Our study demonstrate that the TT genotype has clearly indicates an etiology for IBD, as it had an OR of 0.377 and Etiologic Fraction (EF) of 0.511 (Table 3), in contrast, the TC and CC genotype have rather preventive role as it had Protective Fraction (PF) of .376 and 0.152 respectively with low OR (0.352 and 0.0162) .With the possibility of C allele may be have protective role , whereas the T allele may increase susceptibility to IBD.

Genetic polymorphisms of *TNF-a* gene in UC patients

Genotype of the *TNF*-α genes (-1031) polymorphisms in 45 UC patients (Table 3) .The data revealed that homozygous TT genotype was found 63.83% vs 70% in UC patients and control group respectively (p=0.067,Chi=3.055 NS,OR=0.362) while homozygous CC genotype in UC patients and control group 6.38% and 5% respectively .Heterozygous T>C genotype in UC patients(29%) was higher than control group (25%) however , the three genotypes in current work didn't give significant differences . The heterozygous T>C genotype in this study similar with [21].

Our results also similar to data by Asghar and his colleagues [22] ,they investigated the possible association between five single nucleotide polymorphism (SNPs) in TNF-a gene promoter polymorphisms including -1031T/C in a Japanese population with endometriosis, their results revealed that –1031 (65.1%) TT , (31.7%) TC, (2.8%) CC, (81.4%)T (18.6%)C, the -1031C polymorphism with no significant difference in the frequency of in the *TNF*-a-1031 gene promoter .

The T allele in promoter region of *TNF*- α gene at -1031 site gave a significant risk for development in Turkish and Iranian IBD especially UC patients and also found that C allele was very low in patients and could have a protective role ,the variation in *TNF*α-1031 T allele may increase the risk for developing UC , single nucleotide polymorphism -1031 T > C could have important effect in pathogencity of IBD that lead to increase TNF-α levels in IBD patients [23]. Increased expression of TNF-α , high serum levels have been documented in intestinal tissues and IBD patients [24].

Table 3: Genotyping of *TNF-α*-1031 gene in UC patients

Genotype	UC Patients N (%) 47(100%)	Control N (%) 20(100%)	*P*-Value	Chi-Square	OR (CI)	EF	PF
TT	30 (63.83)	14 (70.00)	0.067	3.055 NS	0.362 (0.84-1.58)	0.269	***
TC	14 (29.79)	5 (25.00)	0.080	2.741 NS	0.279 (0.78-1.62)	***	0.084
CC	3 (6.38)	1 (5.00)	0.706	0.051 NS	0.0158 (0.83-1.62)	***	0.081
Allele Frequency							
T	0.79	0.83	--	--	--	--	--
C	0.21	0.17	--	--	--	--	--
NS: Non-Significant.							

Conclusion

This paper suggests that *TNF-a* -1031 gene polymorphisms in promoter region has an important role in the occurrence of inflammatory bowel disease of IBD patients in Iraqi population especially in CD patients although some results didn't give us a significant differences. It's possible that C allele may be having protective role, whereas the T allele may increase susceptibility to IBD. Other studies are needed with

large number of IBD patients to support our results.

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

Conflict of Interest: The authors declare that they have no conflict of interest.

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References

1- Schoultz I ,Keita A. Cellular and Molecular Therapeutic Targets in Inflammatory Bowel Disease—Focusing on Intestinal Barrier Function. Cells. 2019 ; 8(2): 193.

2- de Lange KM , Barrett JC. Understanding inflammatory bowel disease via immunogenetics. Journal of autoimmunity. 2015 ;64: 91-100.

3- Baumgart D C , Carding S R. Inflammatory bowel disease: cause and immunobiology. The Lancet.2007 ; 369 (9573):1627-1640.

4- Prieto-Pérez R, Almoguera B, Cabaleiro T, Hakonarson H , Abad-Santos F. Association between genetic polymorphisms and response to anti-TNFs in patients with inflammatory bowel disease. International journal of molecular sciences .2016; 17(2):225.

5- Waters J P, Pober JS , Bradley JR. Tumour necrosis factor and cancer. The Journal of pathology. 2013; 230(3):241-248.

6- Wilson AG, Symons JA, McDowell, TL, McDevitt, HO , Duff G W. Effects of a polymorphism in the human tumor necrosis factor α promoter on transcriptional activation. Proceedings of the National Academy of Sciences. 1997; 94(7) :3195-3199.

7- Pierik M, Vermeire S, Steen K V, Joossens S, Claessens G, Vlietinck R , Rutgeerts P. Tumour necrosis factor-α receptor 1 and 2 polymorphisms in inflammatory bowel disease and their association with response to infliximab. Alimentary pharmacology & therapeutics. 2004 ;20(3): 303-310.

8- Aggarwal BB, Gupta SC ,Kim JH. Historical perspectives on tumor necrosis factor and its superfamily: 25 years later, a golden journey. Blood.2012; 119(3): 651-665.

9- Al-Meghaiseeb E S, Al-Robayan A A, Al-Otaibi M M, Arfin M, Al-Asmari A K. Association of tumor necrosis factor-α and -β gene polymorphisms in inflammatory bowel disease. Journal of inflammation research. 2016; 9, 133–140.

10- Hajeer AH, Hutchinson LV. TNF-α gene polymorphism: clinical and biological implications. Microsc Res Tech. 2000 ;50:216–28.

11- Neuman MG , Nanau RM. Single–nucleotide polymorphisms in inflammatory bowel disease. Transl Res 2012; 160(1): 45–64.

12- Sykora J, Subrt I,Didek P,Schwarz J , Machalva V..Cytokine tumor necrosis factor-alpha A promoter gene polymorphism at position -308 G-->A and pediatric inflammatory bowel disease: implications in ulcerative colitis and Crohn's disease. J Pediater Gastroentrol Nutr. 2006; 42(5): 479-87.

13- Rahbar Kafshboran H, Bonyadi M, Miri H, Haghi M, Nikravesh A, Abdolmohammadi R, Khoshbaten M. Association of TNF-α -857 Polymorphism with Inflammatory Bowel Disease in a Group of Iranian Azeri Individuals. Middle East journal of digestive diseases. 2014; 6(1), 28–31.

14- Dubinsky MC, Mei L, Friedman M, Dhere T, Haritunians T, Hakonarson H, Kim C, Glessner J, Targan SR, McGovern DP , Taylor KD. Genome wide association (GWA) predictors of anti-TNFα therapeutic responsiveness in pediatric inflammatory bowel disease. Inflammatory bowel diseases .2009;16(8):1357-1366.

15- Naderi N, Farnood A, Dadaei T, Habibi M, Balaii H, Firouzi F, Mahban A, Soltani M ,Zali M. Association of tumor necrosis factor alpha gene polymorphisms with inflammatory bowel disease in Iran. Iranian journal of public health .2014;43(5):630.

16- SAS. 2012. Statistical Analysis System, User's Guide. Statistical.Version 9.1th ed. SAS. Inst. Inc. Cary. N.C. USA .

17- Coppell KJ, Galts CPC, Huizing FY, Norton JK, Gray AR, Schultz K, Hobbs CE, Aluzaite K , Schultz M. Annual Incidence and Phenotypic Presentation of IBD in Southern New Zealand: An 18-Year Epidemiological Analysis. Inflammatory Intestinal Diseases.2018; *3*(1):32-39.

18- Hassan JT , Delmany AS.Epidemiological and clinical characteristics of patients with inflammatory bowel disease in Erbil City Madical Journal of Babylon. . 2018 ;18(4):281285.

19- Al-Mohaya M A, Al-Harthi F, Arfin M, , Al-Asmari A. TNF-α, TNF-β and IL-10 gene polymorphism and association with oral lichen planus risk in Saudi patients. Journal of applied oral science : revista FOB. 2015; 23(3):295–301.

20- Nourian M, Asgharian AM , Asadzadeh Aghdaei H. Lack of association between tumor necrosis factor alpha (TNFα) gene1031C/T polymorphisms and susceptibility to inflammatory bowel disease (IBD). Arak Univ Med Sci J. 2016 ;19:71-79.

21 – Tremelling M, Waller S, Bredin F, Greenfield S , Parkes M. Genetic variants in TNF-α but not DLG5 are associated with inflammatory bowel disease in a large United Kingdom cohort. Inflammatory bowel diseases. 2006 ; 12(3):178-184.

22- Asghar T, Yoshida S, Kennedy S, Negoro K, Zhuo W, Hamana S. The tumor necrosis factor-α promoter -1031C polymorphism is associated with decreased risk of endometriosis in a Japanese population. Human Reproduction . 2004 ;19(11): 2509–2514.

23- Bonyadi M, Abdolmohammadi R, Jahanafrooz Z, Somy MH ,Khoshbaten M. TNF-alpha gene polymorphisms in Iranian Azari Turkish patients with inflammatory bowel diseases. Saudi journal of gastroenterology: official journal of the Saudi Gastroenterology Association. 2014; 20(2): 108.

24- Komatsu M, Kobayashi D, Saito K,Daisuke K, Kaori S, Furuya D, Yagihashi A, Araake H, Tsuji N , Sakamaki S, Niitsu Y , Watanabe N.Tumor necrosis factor-alpha in serum of patients with inflammatory bowel disease as measured by a highly sensitive immuno-PCR. Clin Chem. 2001; 47:1297–1301.