Genetic Association between tumor necrosis factor (TNF-alpha and TNF-beta) gene polymorphisms and inflammatory bowel disease

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Abstract

Inflammatory bowel disease (IBD) is associated with chronic inflammation of the endothelial lining of the gut. Although, the exact aetiology is not completely understood, combined genetic and immunological factors appear to promote disease initiation and progression. Several studies associated the illness with single nucleotide polymorphism (SNP) on major immunological cytokines such as TNF-alpha and TNF-beta. Thus, the present study aims to investigate TNF-α and TNF-β genes polymorphisms in cases diagnosed with IBD. Subjects and methods: Genomic DNA isolation was performed on isolated buffy coat layers from peripheral blood of 75 individuals. Candidate SNP locations on TNF-α and TNF-β coding sequences were amplified by PCR and sequenced for SNP identification. Results: Genetic examination of TNF-α and TNF-β allele polymorphism revealed significant association with IBD prevalence and disease manifestation, (p=0.002) and (p<0.001) respectively. GA haplotype frequencies were higher in IBD patients when compared to healthy control, being 29(58%) in TNF-α, and 26(52%) in TNF-β of the studied alleles. Similarly, both GG and AA haplotypes of TNF-α showed a strong association with cases diagnosed with ulcerative colitis but not with Crohn’s disease (p=0.007). Additionally, none of the studied haplotypes of both cytokines showed any association with gender or age groups of the included individuals. Conclusion: TNF-α (-308G/A) and TNF-β (+252A/G) sequence analysis revealed that cytokines heterogeneities are associated with IBD susceptibility. Early genetic screening for individuals with familial history could provide a better predictive value for IBD initiation and progression, that would essentially help in early diagnosis, management and prevention.

Keywords: Inflammatory bowel disease (IBD), TNF-alpha and TNF-beta, single nucleotide polymorphism (SNP)

Introduction:

Inflammatory bowel disease (IBD) is a generalized term applied to define associated bowel disorders that include chronic inflammation of the digestive tract. Ulcerative colitis (UC) and Crohn’s disease (CD) considered as the two major inflammatory disorders of IBD[1, 2]. Ulcerative colitis affects the entire epithelial lining of the colon and the rectum, whereas Crohn’s disease characterises by patchy areas of inflammation that may extend to any area of the gut[3]. The exact cause of IBD is still to be identified. However, several genetic, environmental, and immunological factors
were found to increase the disease prevalence and the overall pathogenesis of the UC and CD in patients\cite{4, 5}. For instance, epidemiological studies revealed that the highest incidence rate with IBD has been found in developed western countries, suggests important genetic factors that are highly likely to be associated with the development of the chronic disease\cite{6, 7}. Additional, environmental impact on societies of these countries post the industrial revolution were found to associated with increasing the risk factors for IBD. Such factors could have severe and direct impact on the gut microbiome, and on the integrity of the intestinal barrier that which is essential in maintaining a healthy mucosal lining of the gut\cite{8-10}.

The intestinal single-celled epithelia provide a protective layer that separates the gut immune system from the luminal contents. Damaging any part of this protective barrier would trigger series of immunological responses that are essential to maintain the integrity of the gut\cite{11}. Thus, IBD is thought to arise from inappropriate immune responses upon exposure to a specific invader that results in recruiting the immune-mediated disease\cite{12, 13}. Although, the mechanism that is responsible for initiating and maintaining IDB may differ accordingly, studies showed that both UC and CD could be triggered by a dysfunctional property of the antigen-presenting cell in those patients\cite{14, 15}. These types of immune cells are responsible for stimulating a cascade of pro-inflammatory and inflammatory signals that trigger cellular differentiation and direct stimulation to multitude subsets of local and circulating lymphocytes to migrate to the site of inflammation. It was found that profuse production of inflammatory cytokines and cellular apoptosis damage the intestinal barrier continuity allowing microbiome to penetrate the gut beyond the lamina propria causing additional inflammatory responses that in turn ensures endothelial microvascular permeability\cite{9, 16, 17}. Tumor necrosis factor (TNF) superfamily proteins perform a multitude of functionality in processes of normal physiology or illnesses. As one of the main pro-inflammatory mediators, TNF is produced mainly as a cytokine by activated macrophages and many other immune-mediated cell types such as, CD4+ lymphocytes, neutrophils, and natural killer cells\cite{18}. TNF regulates wide range of biological responses including enhancement of T-cells responses, stimulate production of adhesion molecules (E-selectin) from leukocytes, and neutrophils recruitment to the site of inflammation\cite{19, 20}. In patients with UC, TNF is mainly detected in the macrophages of the intestinal subepithelial, whereas in intestinal tissue of CD patients, TNF-positive cells were found to be located in the layers of lamina propria and the submucosa\cite{21}. The high transcriptional properties of TNF post inflammation in monocytes, T-cells, macrophages, and adipocytes of these tissues, result in increasing of soluble circulating levels of TNF in blood\cite{22}. All these responses have been shown to critically orchestrate IBD pathogenesis and disease progression.

The most predominant isoforms of the TNF superfamily are the TNF-α and TNF-β (also known as Lymphotoxin alpha (LTα)) isoforms, both were found highly expressed in intestinal biopsies and serum of patients with IBD as described by several studies\cite{23, 24}. A strong association has been reported between increased levels of both cytokines and excessive production of interferon-gamma and IL-2 in mononuclear cells from intestinal mucosa of patients with UC and CD\cite{25}. The coding sequence for TNF-α and TNF-β are located in class III locus of the MHC that comprises multiple single nucleotide polymorphisms (SNPs)\cite{26}, and some of these SNPs have been linked with alterations in TNF-α or TNF-β production and IBD susceptibility\cite{23, 27, 28}. Therefore, to understand the adverse effects of SNPs formation on the potential role of these pro- and anti-inflammatory cytokines in cases with IBD,
this study investigated the association between TNF-α (−308G/A) and TNF-β (+252G/A) polymorphisms with the clinical manifestation of UC and CD. Findings here would provide a great insight into the autoimmune mechanism that contribute to increase IBD prevalence and progression, which that would provide better approaches in the future for early diagnosis, management and prevention.

Subjects and methods:

The included subjects in this case-control study were consisted of 75 individuals of either sex, 50 of those were patients with IBD, breakdown as 31 patients with UC, and 19 with CD (constitute the study group) recruited from, whereas the control group included 25 healthy those sex and age matched individuals. A written consent was approved from the included individuals had been taken and the study has been approved by the Iraqi Council for Medical Specializations. Differential diagnosis of patients with UC and CD were performed according to the clinical endoscopic, radiologic and histopathological samples. Genomic DNA isolation was performed on the isolated buffy coat layer from the peripheral blood by utilising TRI-reagent method (Sigma: T9424) according to the manufacturer’s instruction. Candidate SNP locations on TNF-α and TNF-β coding sequences were amplified by PCR-Taq DNA Polymerase with Standard Taq Buffer (New England Biolabs-UK) with the appropriate primers set for each location as described by Naderi et al. [27], Bouma et al. [29]. The resulting PCR products were then confirmed on 2% agarose gel electrophoresis provided with 0.2ug/ml ethidium bromide DNA stain. Once confirmed positive PCR reaction, 5ul of the PCR products were sent for sanger sequencing and forward primers were provided for each location. SnapGene software were utilised to align sequencing files and SNPs were identified by single nucleotide substitution. The SNP frequencies from UC and CD patients were calculated and analysed with the standard χ² test with the aid of Microsoft Excel and SPSS software (24v, USA). P<0.05 was considered significant.

Table 1: Details of SNPs identification code, primers set, and PCR products size.

| SNP             | Primers set                                    | PCR product |
|-----------------|-----------------------------------------------|-------------|
| TNF-α (-308G/A) | 5'-GAGGCAATAGGT TTGAGGGCCAT-3' 5'-GGGACACACAAGC ATCAAG-3' | 147bp       |
| TNF-β (+252A/G) | 5'-CCGTGCTTCGTGCTTGGACTA -3' 5'-AGAGCTGGTGATGGACATGCTG -3' | 740bp       |

Results:

Examining the mean age of the included individuals in this study showed that the included patients diagnosed with IBD were at 36.30±11.0 years of age, and 35±10.75 years for the control group. There was no statistical difference observed in terms of the mean age differences, nor of the studied haplotypes showed any clear association with age groups (figure-1). Age group categorisation as shown in figure-2 revealed higher incident rate of UC cases among young adults, while adult and elderly group showed significant susceptibility towards developing CD (p=0.005). On the other hand, a significant association was observed between sex-based differences and prevalence of IBD (p=0.022), indicating IBD is more prevalent in males than females.
Genotyping analysis and alleles frequency of TNF-α appeared to be significantly associated with IBD prevalence (p=0.002) and disease manifestation, similar findings were also observed with TNF-β haplotype frequencies (p<0.001). It appears that G/A genotype of both cytokines were the highest genetic predisposing factor in developing the pathogenesis of IBD in general, being 29(58%) in TNF-α, and 26(52%) in TNF-β of the studied alleles. In fact, G/A TNF-α allele mutation were found almost consistently distributed in patients diagnosed individually with either UC or CD, being 13(44.8%) and 16(55.2%) respectively. Nonetheless, UC patients presented with higher frequency of G/G allele variance 10(90.9%), followed by the A/A at 8(80%), whereas the frequency of G/A TNF-β haplotype was noticeably higher by two-third 18(69.2%) in UC compared to individuals with CD (Table-2). Additionally, Spearman correlation test indicated no positive relationship between age groups and specific TNF polymorphism. Age vs TNF-α were at (p=0.389), age vs TNF-β at (p=0.166). Therefore, haplotypes were to some extent distributed evenly among the age groups. Control group has also shown no significance correlation with any of the reported polymorphism.
Table 2: Shows genotypes and alleles distribution of TNF-α (-308G/A) and TNF-β (+252A/G) in patients with IBD.

| Genotype/allele | IBD case n. (%) | Control n. (%) | P     | UC n. (%) | CD n. (%) | P     |
|-----------------|-----------------|----------------|-------|-----------|-----------|-------|
| **TNF-α (-308G/A)** |                 |                |       |           |           |       |
| GA              | 29(58)          | 4(16)          | 0.002 | 13(44.8)  | 16(55.2)  | 0.007 |
| GG              | 11(22)          | 12(48)         |       | 10(90.9)  | 1(9.1)    |       |
| AA              | 10(20)          | 9(36)          |       | 8(80)     | 2(20)     |       |
| **TNF-β (+252A/G)** |                 |                |       |           |           |       |
| GA              | 26(52)          | 1(4)           | <0.001| 18(69.2)  | 8(30.8)   | 0.236 |
| GG              | 4(8)            | 11(44)         |       | 2(50)     | 2(50)     |       |
| AA              | 20(40)          | 13(52)         |       | 12(60)    | 8(40)     |       |

The study has also investigated any possible association between specific allele polymorphism and gender for the included individuals both in study and control. Findings presented that sex-based differences did not correlate positively with the examined SNP distribution between groups, as shown in table 3.

Table 3: Shows alleles distribution according to sex in IBD compared to healthy control group.

| Genotype/allele | IBD | Control |
|-----------------|-----|---------|
|                 | Male n. (%) | Female n. (%) | Male n. (%) | Female n. (%) | P |
| **TNF-α (-308G/A)** |     |         |     |         | 0.263 |
| GA              | 21(72.4) | 8(27.6) | 3(75) | 1(25) |       |
| GG              | 5(45.5)  | 6(54.5) | 7(58.3) | 5(41.6) |       |
| AA              | 7(70)    | 3(30)   | 6(66.6) | 3(33.3) |       |
| **TNF-β (+252A/G)** |     |         |     |         | 0.841 |
| GA              | 18(69.2) | 8(30.8) | 0(0.0) | 1(100) | 0.924 |
| GG              | 3(75)    | 1(25)   | 7(63.6) | 4(36.6) |       |
| AA              | 13(65)   | 7(35)   | 8(61.5) | 5(38.4) |       |

**Discussion**

IBD is a chronic debilitating disease that arises from dysregulated immune responses towards the gut microflora\(^1,2\). In healthy individuals, the endothelial lining of the gut tube maintains a highly balanced concomitance of immune responses between tissue tolerance to the naturally resides microflora and the defence activities against an invading pathogen\(^30\). This physiological gut adaptation is thought to be disturbed in IBD patients as a result of a predisposing genetic factor or harmful environmental stimulus\(^12,13\). The main pro-inflammatory mediators in these scenarios are the cytokines that are produced from various types of immune cells in response to inflammation-like situations. TNF cytokines were found with an essential role in modulating IBD initiation and progression through time\(^18\). For instance, it has been found that serum levels and intestinal mucosa expression of TNF-α and TNF-β were both elevated in UC and CD patients following routine lab investigation\(^25,29,31\). Targeting these endogenous TNF markers with pharmaceutical inhibitors have proven
effective in achieving complete clinical remission\cite{32}. However, the likelihood of relapse that is always associated with poor prognosis have been discovered in patients with high genetic risk and familial susceptibility to IBD\cite{8-10}. Several genetic loci have been identified within the coding sequence of TNF that were associated with increasing the risk factor, especially when these loci positioned in a promoter segment that regulates TNF expression and secretion from activated immunocytes\cite{23, 33}. Thus, the current study performed stratified analysis on age and gender of patients diagnosed with either UC or CD, and examined the distribution of alleles polymorphism of TNF-\(\alpha\) \((-308G/A)\) and TNF-\(\beta\) \((+252A/G)\) in those patients. The examined age groups did not show any significant correlation with the studied phenotypes of both cytokines and the susceptibility to IBD, as similarly described by Song \textit{et al.} \cite{28}. Interestingly, however, higher incident rate of UC was observed in adult patients below forty years of age. Similar findings were obtained by Stallmach \textit{et al.} \cite{34} who stated that the peak incidence of UC is between age twenty and thirty years. The same author has also detailed the incident rate in patients with CD as the disease is high likely to be diagnosed at both the young adult and the elderly groups, and that what this study detailed in figure-2. On the other hand, the study also showed that gender-based association with IBD revealed a higher incident rates among males than females, similar findings were descried by Greuter \textit{et al.} \cite{35}, Mokhtar \textit{et al.} \cite{36}, Jussila \textit{et al.} \cite{37}, contradicting others findings who stated females have a higher tendency than men towards developing IBD. \cite{6, 38}. The general understanding considers IBD can equally affect male and females\cite{39}, however the fact that several reports stated differently, and the observed higher incident rates among men could be associated with higher contact to environmental predisposing factors\cite{40} or due to racial and ethnic backgrounds. To understand the significantly higher prevalence of IBD in males, the study examined the distribution of TNF-\(\alpha\) \((-308G/A)\) and TNF-\(\beta\) \((+252A/G)\) alleles frequencies in patients and control and did not find any association between allele distribution and gender, this suggests additional factors that needed to be investigated should prone males with higher incidence of IBD. The results of this study also shown that alleles polymorphism of both TNF-\(\alpha\) \((-308G/A)\) and TNF-\(\beta\) \((+252A/G)\) were significantly higher among IBD patients than that of the healthy control. Indicates a strong association between the cytokines’ heterogeneity and IBD susceptibility. Since G/A phenotype was the highest observed frequency among the studies alleles, it could be pathologically responsible for increasing the risk factor and the likelihood of developing IBD in individuals. The findings here are in accordance with Fan \textit{et al.} \cite{41} who associated G/A phenotype with IBD susceptibility in Asians. Moreover, G/A polymorphism did not show any expressing tendency toward increasing susceptibility to either UC or CD. Instead, TNF-\(\alpha\) alleles G/G and A/A showed a significant concentration in patients diagnosed with UC, as previously reported by others in Europeans\cite{41}, New Zealanders\cite{42}, and Brazilians\cite{43}. In contrast, G/A TNF-\(\beta\) haplotype was found higher in two-thirds of the diagnosed cases with UC, however, TNF-\(\beta\) polymorphism did not present any association with increasing the risks of developing either UC or CD, as the differences did not reach to the level of significance. Same findings were reported in studies from several population\cite{28, 44, 45}.

**Conclusion:**
The results of the present study acknowledge the fact that different genetic backgrounds, alleles polymorphism, and gender could influence certain immunopathological responses in individuals susceptible with IBD. TNF-\(\alpha\) \((-308G/A)\)
and TNF-β (+252A/G) sequence analysis revealed that cytokines heterogeneity could provide a predictive value for IBD initiation and progression. In addition, early genetic screening would essentially help clinicians in designing the appropriate therapeutic approaches for individuals presenting susceptibility to IBD.

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