Role of tumor necrosis factor-alpha–308 G/A and interleukin-10 promotor–592 C/A polymorphisms in adult immune thrombocytopenic purpura

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Abstract

Introduction: Immune thrombocytopenia (ITP) pathogenesis has been related to cytokine imbalance, which is controlled genetically with gene polymorphisms. The correlation of the interleukin (IL)-10 gene and tumor necrosis factor alpha (TNF-α) polymorphisms with ITP susceptibility has been previously investigated, but the association with clinical and prognostic parameters remains unclear.

Material and methods: To investigate the relation between IL-10-592 C/A and TNF-α-308 G/A gene polymorphisms and their clinical significance in adult patients with ITP.

This study was conducted on 40 ITP patients and 40 control individuals. The IL-10-592 C/A polymorphism was genotyped by the polymerase chain reaction-restriction fragment length polymorphism method and the TNF-α-308 G/A polymorphism by amplification refractory mutation system analysis.

Results: The TNF-α-308 G/A polymorphism was significantly associated with low platelet count, wet purpura, higher bleeding score, higher incidence of complications, and lack of response to steroid therapy. The IL-10-592 C/A polymorphism was not significantly associated with any of these parameters.

Conclusion: We found a significant association between the TNF-α-308 G/A polymorphism and several clinical parameters, which suggests a probable role in the prognosis among adult ITP patients.

Key words: immune thrombocytopenic purpura, polymorphism, interleukin 10 promotor gene, tumor necrosis factor-alpha gene, prognosis

Introduction

Immune thrombocytopenia (ITP) is an acquired thrombocytopenia characterized by a platelet count of fewer than \(100 \times 10^9/L\), and it is caused by elevated destruction of antibody-sensitized platelets [1, 2]. This disorder occurs in both adults and children, showing a bimodal incidence with a peak in childhood and another peak in young adults. The pathology in childhood and adult ITP may be basically different, as evidenced by the incidence of chronic ITP in adults [3]. Most children have a self-limited disease, whereas ITP is mostly a chronic disorder in adults.
Recently, the pathophysiology of this disease has become better understood. The human immune system is known to play an important role, including antibodies, cytokines, antigen presenting cells, as well as T and B lymphocytes [4]. Understanding the important role of genetic factors in autoimmune diseases has led to the conduction of many studies. Consequently, many single-nucleotide polymorphisms (SNPs) with potential clinical significance have been identified. Some immune-related genes have been studied as candidates for the genes susceptible to ITP, including the polymorphisms of inflammatory cytokine genes which have been correlated with ITP [5]. These cytokines include tumor necrosis factor alpha (TNF-α), beta (TNF-β), interferon (IFN)-alpha (IFN-α), beta (IFN-β), gamma (IFN-γ), interleukin (IL)-1α, β, and IL-10 based on the presence of well-defined SNPs associated with autoimmune diseases.

It should be noted that risk susceptibility studies cannot be anticipated from one ethnic group to another due to the popular variability of the relative frequency of different alleles among different populations [6]. Hence, to evaluate the possible role of cytokine genes in addition to their polymorphisms in adult ITP, we investigated the allelic and genotypic frequencies of cytokine gene polymorphisms known to be correlated to autoimmunity and inflammation (IL-10-592 and TNF-α-308) in Egyptian patients with ITP and healthy controls. Additionally, we evaluated the possible role of these polymorphisms in relation to the risk of ITP development, disease prognosis, and response to steroid therapy.

TNF gene locus is located within the class III region of the human major histocompatibility complex (MHC) on chromosome 6 (6p21.31) [7]. Several SNPs have been identified in the human TNF-α gene promoter region, such as TNF-α 308 (G/A) polymorphism. TNF-α is a pleiotropic cytokine produced mainly by macrophages and T-cells which has a range of inflammatory and immunomodulatory activities [8]. The polymorphisms of TNF-α promoter are correlated with high levels of TNF-α and have been evaluated as a risk factor for the development of numerous diseases [8–10].

To the best of our knowledge, the role of the TNF-α-308 G/A polymorphism in the development and progression of ITP has not been previously studied among adult populations. Many polymorphism studies have demonstrated a relation between IL-10 and various immunological disorders [11, 12]. According to previous studies, IL-10 SNPs may have an important effect on the immune function through regulating the activities of natural killer (NK) T-cells, and macrophages, thus affecting disease progression [13]. Several SNPs of IL-10, including −1081, −819, and −592, may be linked to ITP [14].

The aim of our study was to investigate the role of the SNPs of IL-10-592 and TNF-α-308 genes on the susceptibility, severity, and outcomes of ITP. In addition, we compared our data to other published data.

Materials and methods

Our study comprised 40 adult ITP patients and 40 age- and sex-matched healthy Egyptian subjects (control group). This study was jointly conducted by the Hematology Department at the Medical Research Institute, and the Clinical and Chemical Pathology Department at the Faculty of Medicine, Alexandria University, Alexandria, Egypt. Patients with primary ITP and aged 18 and older were included in the study. Patients under 18 and those with proven secondary ITP (as cases initiated by or associated with infections due to human immunodeficiency virus (HIV-associated) or hepatitis C virus associated secondary ITP) were excluded. Moreover, patients with accompanying autoimmune disorders such as systemic lupus erythematosus (SLE) were excluded. Relatives or members from consanguineous families were excluded as patients or controls to ensure more accurate allele segregation frequency. The study protocol was approved by the Ethical Committee of Alexandria University. All patients enrolled in the study signed informed written consent after an explanation of the nature, steps and aim of the study before enrolment.

ITP diagnosis was made on the basis of history, detailed physical examination, complete blood count to determine the presence of thrombocytopenia (<100 × 10^9/L) with otherwise normal red cells and leukocytes, and examination of the peripheral smear which should exclude other causes of thrombocytopenia [15]. Other tests were done if there was a suspicion of a disease other than ITP. Serological tests for infectious causes and autoimmune diseases were performed for antinuclear antibodies, HIV, hepatitis C virus (HCV), and Helicobacter pylori (H. pylori) infection. A detailed questionnaire was carried out including demographic characteristics, duration of disease, bleeding symptoms, life-threatening bleeding if patients had clinically significant symptoms of severe bleeding such as intracranial bleeding, internal hemorrhage, and gastrointestinal or genitourinary bleeding, history of preceding viral infection, and recent medication use. This questionnaire was distributed among patients and answered during history taking. Physical examination included an examination for the presence of lymph nodes as well as the examination of abdominal organs. Severity of bleeding was also assessed using a specific bleeding score from grade 1 to grade 4 [16]. Newly diagnosed patients received either prednisone at 1 mg/kg bodyweight daily for 4 weeks to the maximum and then tapered [17], or a high-dose of dexamethasone (40 mg orally per day for 4 days) with no taper [18]. For the majority of included patients, we recommended a high dose of dexamethasone to avoid prolonged exposures to oral prednisone (which can be associated with significant toxicities). The response to immune thrombocytopenia treatment was evaluated [19] as follows: Complete response (CR): any platelet count of...
at least $100 \times 10^9$/L and absence of bleeding; response (R): any platelet count of at least $30 \times 10^9$/L and at least a two-fold increase in the baseline count and absence of bleeding. No response: any platelet count less than $30 \times 10^9$/L or less than a two-fold increase in the baseline platelet count or bleeding; Loss of CR: platelet count below $100 \times 10^9$/L or bleeding; Loss of R: platelet count below $30 \times 10^9$/L or less than a two-fold increase in the baseline platelet count or bleeding. Time to response: the duration between starting the treatment and the achievement of CR or R.

**Investigations**

Full blood count was done using an ADVIA 2120 automated cell counter (Siemens Healthcare Diagnostics, USA).

Genomic DNA was extracted from all K2EDTA peripheral blood samples by the column method using a DNA extraction kit (Pure Link Genomic DNA Mini Kit, Cat no K182001, Invitrogen™ by Thermo Fisher Scientific).

Genotyping was performed following the genomic DNA amplification by amplification refractory mutation system – polymerase chain reaction (ARMS-PCR) technique for the promoter site TNF-α-308 (G/A) SNP using the following primers:

- **Forward primer F:** 5’CTGCATCCCCGTCTTTCTCC-3’ and;
- **Reverse primer 1 (wild allele):** 5’-ATAGGTTTTGAGGGGCATCG-3’;
- **Reverse primer 2 (mutant allele):** 5’-ATAGGTTTTGAGGGGCATCA-3’;

Genotyping using PCR-restriction fragment length polymorphism (PCR-RFLP) was performed for the IL-10-592 (C/A) SNP using restriction enzyme Rsal. The following primers were used:

- **Forward primer:** 5’ GGT GAG CAC TAC CTG ACT AGC 3’;
- **Reverse primer:** 5’ CCT AGG TCA CAG TGA CGT GG 3’;
- **Amplification was performed using an HVD S24 thermal cycler (Quanta Biotech, UK).**

PCRs were performed in a final volume of 50 µL using approximately 200 ng of template genomic DNA which was done using 10 pmol of each of the primers, 1.5 mmol/L MgCl$_2$, 400 mmol/L of each dNTP, 1 U Taq polymerase, and 1 x PCR buffer. The PCR amplification consisted of an initial denaturation step at 96°C for 3 min, followed by 30 cycles of 94°C for 45 s, 55°C for 2 min (for TNF-α-308 G/A), 62°C for 45 s for IL-10-592-C/A, 72°C for 2 min for TNF-α-308 G/A, and 55 s for IL-10-592-C/A, as well as a final step at 72°C for 3 min. The amplified products were applied to gel electrophoresis in a 2% agarose in reference to a molecular weight marker, and detected using ultraviolet (UV) light (UVP dual intensity trans-illuminator).

RFLP analysis was done using a restriction enzyme Rsal for IL-10-592 C/A genotyping by mixing 10 µL of PCR product with 1 µL of Rsal enzyme, 2 µL green buffer, and 17 µL nuclease free water. This mixture was incubated at 37°C for one day then heated at 65°C for 20 minutes. Then, DNA fragments were detected by 2% agarose gel electrophoresis.

Genotypes were determined as follows: IL-10-592 gene CC [412 base pair (bp)], AC (412, 236, 176 bp), and AA (236, 176 bp) (Figure 1), and TNF-α-308 gene 863bp (Figure 2). Ten percent of the samples were amplified twice for verification of genotyping results.
All statistical calculations were done using SPSS statistical programs (version 24.0 SPSS, Inc., Chicago, IL, USA). Data was statistically described in terms of mean ± standard deviation (± SD) and range or frequencies (number of cases) and percentages when appropriate. Descriptive statistics were done for quantitative data as minimum and maximum of the range as well as mean ± SD for quantitative parametric data, while it was done for qualitative data as number and percentage. Inferential analyses were done for quantitative variables using the unpaired t-test in cases of two dependent groups with parametric data for comparing categorical data, the chi-square test ($\chi^2$) was performed. The Mann-Whitney test was used to compare two unrelated samples. Association was expressed as odds ratios (OR) with 95% confidence intervals (CIs). P values of less than 0.05 were considered statistically significant.

**Table I. Characteristics of studied subjects**

| Parameter                      | Cases (n = 40) | Control (n = 40) | p     |
|--------------------------------|---------------|-----------------|-------|
| Sex                            |               |                 |       |
| Male                           | 12 (30%)      | 10 (25%)        | 0.617 |
| Female                         | 28 (70%)      | 30 (75%)        |       |
| Age (years)                    |               |                 |       |
| Median (min–max)               | 33.5 (18–72)  | 34 (18–70)      | 0.326 |
| Mean ± SD                      | 34.3 ± 12.6   | 36.7 ± 13       |       |
| Hb [mg/dL]                     |               |                 |       |
| Median (min–max)               | 11.7 (5.1–15.8)| 12.6 (11.5–15)  | 0.002*|
| Mean ± SD                      | 11.8 ± 1.7    | 12.8 ± 1.1      |       |
| WBCs [× 10^3/mm^3]             |               |                 |       |
| Median (min–max)               | 7.4 (4.3–12.9)| 7.1 (4.2–10.4)  | 0.533 |
| Mean ± SD                      | 7.8 ± 2.2     | 7.5 ± 1.7       |       |
| PLT [× 10^3/mm^3]              |               |                 |       |
| Median (min–max)               | 19 (3–90)     | 268 (160–400)   | <0.001*|
| Mean ± SD                      | 23.5 ± 19.8   | 270.5 ± 72.5    |       |
| Clinical presentation          |               |                 |       |
| Mucocutaneous bleeding         | 28 (70%)      | –               |       |
| Vaginal                        | 7 (17.5%)     | –               |       |
| Accidental discovery           | 5 (12.5%)     | –               |       |

*Statistically significant at p < 0.05; p – p value for comparing between studied groups; SD – standard deviation; Hb – hemoglobin; WBCs – white blood cells; PLT – platelets

**Table II. Comparison between studied groups according to tumor necrosis-alpha (TNF-α)-308 and interleukin (IL)-10-592 polymorphisms**

| Polymorphism | Cases (n = 40) | Controls (n = 40) | OR (95%CI) | p     |
|--------------|----------------|-------------------|------------|-------|
| TNF-α-308    |                |                   |            |       |
| GG           | 37 (92.5%)     | 40 (100%)         | 0.17 (0.008–3.34) | 0.077 |
| GA           | 3 (7.5%)       | 0 (0%)            | 5.9 (0.3–119)  | 0.077 |
| AA           | 0 (0%)         | 0 (0%)            | –           | –     |
| Allele       |                |                   |            |       |
| G            | 77 (96.3%)     | 80 (100%)         | –           | 0.080 |
| A            | 3 (3.8%)       | 0 (0%)            | –           |       |
| IL-10-592    |                |                   |            |       |
| CC           | 31 (77.5%)     | 29 (72.5%)        | 1.307 (0.473–3.609) | 0.606 |
| CA           | 8 (20%)        | 10 (25%)          | 0.750 (0.261–2.153) | 0.592 |
| AA           | 1 (2.5%)       | 1 (2.5%)          | 1.000 (0.060–16.56) | 1.000 |
| Allele       |                |                   |            |       |
| C            | 70 (87.5%)     | 68 (85%)          | 1.235 (0.501–3.048) | 0.646 |
| A            | 10 (12.5%)     | 12 (15%)          | 0.810 (0.328–1.997) |       |

R – reference group; p – p value for comparing between studied groups; OR – odds ratio; CI – confidence interval

**Results**

The current study included 40 adults with ITP: 12 males (30%) and 28 females (70%) with a female-to-male ratio of 2.8:1 and ages ranging from 18 to 72 years with a mean of 34.3 ± 12.6. Also, the study included 40 age- and sex-matched healthy controls and adolescents with a mean age of 36.7 ± 12.9 years. Among the 40 ITP cases, it was found that ITP incidence was higher in females than males, especially during the childbearing period. The characteristics of the studied subjects are set out in Table I.

**Distribution of cytokine genotype frequencies in studied population**

A total of 40 adult patients with ITP and 40 hematologically normal volunteers were analyzed for the presence of TNF-α-308 G/A and IL-10-592 (C/A) polymorphisms. The
### Table III. Association between tumor necrosis-alpha-(TNF-α)-308 and interleukin (IL)-10-592 genotypes with different parameters in case group (n = 40)

| Genotypes   | Sex               | Age (years) | Platelets | Wet purpura | Bleeding score | Fate of ITP patients | Steroid treatment response |
|-------------|-------------------|-------------|-----------|-------------|---------------|----------------------|---------------------------|
|             | Male (n = 37)     | Male (n = 3) | Male (n = 31) | Female (n = 3) | Female (n = 31) | Male (n = 8) | Female (n = 3) | Male (n = 31) | Female (n = 8) | Male (n = 8) | Female (n = 1) |
| GG          | 10 (27%)          | 27 (73%)    | 8 (25.8%) | 3 (37.5%)   | 1 (100%)      |                      |                          |
| GA          | 2 (66.7%)         | 1 (33.3%)   | 23 (74.2%)| 5 (62.5%)   | 0 (0%)        |                      |                          |
| CC          | 3 (37.5%)         | 8 (25.8%)   | 30 (96.8%)| 5 (62.5%)   | 1 (100%)      |                      |                          |
| CA          | 1 (100%)          | 0 (0%)      | 37 (11.5%)| 0 (0%)      | 0 (0%)        |                      |                          |
| AA          | 1 (100%)          | 0 (0%)      | 37 (11.5%)| 0 (0%)      | 0 (0%)        |                      |                          |
|            | χ² (p)            | χ² (p)      | χ² (p)    | χ² (p)      | χ² (p)        | χ² (p)               | χ² (p)                   |
|            | χ² = 2.076 (0.209) | χ² = 2.696 (0.259) | χ² = 0.55 (0.259) | χ² = 0.55 (0.259) | χ² = 2.05 (0.259) | χ² = 0.55 (0.259) | χ² = 0.55 (0.259) |

*Statistically significant at p ≤ 0.05; p — p value for association between different categories; χ² — χ² square test; MC — Monte Carlo; F — ANOVA test; U — Mann-Whitney test; H — Kruskal-Wallis test; SD — standard deviation; ITP — immune thrombocytopenia

Distribution of the TNF-α-308 G/A genotype frequency among the studied population is set out in Table II. Among the included ITP cases, 92.5% showed G/G genotype and 7.5% showed (G/A) genotype. Among controls, 100% showed G/G genotype and none showed (G/A) genotype. Patients with ITP showed an increased frequency of the G/A genotype compared to controls, but this relation was not statistically significant (p = 0.077). Allele A exhibited an increased frequency among cases but was not statistically significant (p = 0.08). Moreover, it was found that none of the patients or the controls had AA genotype of TNF-α-308 (G/A).
The IL-10-592 (C/A) genotype frequency among the studied population is set out in Table II. Among ITP patients, 77.5% showed C/C genotype, 20% showed C/A genotype, and 2.5% showed A/A genotype. Among controls, 72.5% showed C/C genotype, 25% showed C/A genotype, and 2.5% showed A/A genotype. This relation was not statistically significant ($p = 0.893$).

**Relationship between genotypes, clinical parameters, and response to treatment**

There were no significant differences between different TNF-α-308 (G/A) and IL-10-592 (C/A) genotypes in various clinical features including gender and age ($p > 0.5$). Regarding the relation between different genotypes and the hematological profile, no statistically significant difference was detected between the TNF-α-308 (G/A) gene polymorphism and hemoglobin concentration or white blood cell count. However, a statistically significant difference was detected between this polymorphism and platelet count ($U = 15$, $p = 0.035$). In addition, there was no statistically significant association between different IL-10-592 (C/A) genotypes and the hematological profile (Table III).

The three detected ITP cases with TNF-α (G/A) genotype developed wet purpura (100%), while from the seven cases with (G/G) genotype, only six (12.8%) developed wet purpura ($p = 0.004$). Hence, there was a statistically significant association between TNF-α-308 polymorphism and wet purpura. Bleeding score was higher among cases with (G/A) genotype ($p = 0.0001$), showing a statistically significant association between different genotypes of ITP cases regarding the bleeding score. However, no statistical significance was found between IL-10-592 (C/A) polymorphism and wet purpura or the bleeding score ($p > 0.5$) (Table III).

Follow-up on ITP patients revealed that the three cases with TNF-α (G/A) genotype died; two of them (66.7%) developed intracranial hemorrhage up to death, and one (33.3%) developed septic arthritis on top of steroid treatment up to death. The cases with (G/G) genotype showed better survival, and did not develop any life-threatening complications, indicating that mortality was higher among ITP cases with (G/A) genotype than those with (G/G) genotype. There was a statistically significant association between TNF-α-308 (G/A) polymorphism and the incidence of death ($p = 0.001$). Regarding corticosteroid response, two of the cases (66.6%) with (G/A) genotype did not respond to steroids from the beginning, and one case (33.3%) lost complete response after achieving it. However, among the cases with (G/G) genotype, six patients (12.7%) achieved complete response, one (2.1%) achieved response, one (2.1%) did not respond to steroids from the beginning, 19 (40.4%) lost complete response after achieving it, 17 (36.2%) lost response after achieving it, and three (6.4%) did not need treatment in the beginning (Table III).

This indicates that the response rate was higher among cases with (G/G) genotype, while the non-response rate was higher among cases with (G/A) genotype. A statistically significant relation was noticed between the TNF-α-308 (G/A) polymorphism and steroid treatment response ($p = 0.034$). However, there was no statistically significant association between the IL-10-592 (C/A) polymorphism and the fate of patients or the steroid treatment response.

The risk of developing ITP was not associated with either the TNF-α-308 G/A (OR 0.7, 95% CI: 0.275–1.981) or the IL-10-592 (C/A) (OR 1.5, 95% CI: 0.396–5.685) polymorphisms.

**Discussion**

Novel genes and loci identified may help to explain the biology of ITP and suggest the possibility of new testing for clinical or prognostic biomarkers of the disease or targets for therapy. Based on our present understanding of the pathogenesis and drug responses, many candidate genes can be studied to confirm, or potentially rule out, postulated contributions [20].

We have examined potential associations between some of the clinical parameters of ITP and SNPs within the genes for the previously mentioned inflammatory cytokines. Cytokine genes are polymorphic, which explains the different levels of cytokine production. Moreover, they are related to the regulation of the immune-mediated inflammatory process. Cytokine gene polymorphisms have become a subject of interest because certain alleles of cytokine genes have been correlated with different immune-inflammatory diseases [21].

IL-10 is the most important anti-inflammatory cytokine in the human immune response. IL-10 is a powerful inhibitor of Th1 cytokines, including both IL-2 and IFN-γ [22]. The gene for IL-10 is located in chromosome region 1q31–q32. It includes multiple polymorphisms associated with multiple immune and inflammatory disorders [23].

Concerning TNF-α, many studies have shown that any SNP at the position -308 G/A is associated with multiple inflammatory conditions [24]. Any genetic variability in the production of TNF-alpha after an infectious stimulus could have a significant influence on the degree of inflammatory response which will eventually influence the clinical outcome [25].

Contrary to our results, a previous study [5] investigated polymorphisms of TNF-α (–308) among chronic adult ITP patients in Turkey. The researchers found that the expression of TNF-α (–308) GA phenotype was significantly increased in patients with ITP compared to normal controls and that the presence of GA genotype increased the risk of ITP by 3.1 times ($p < 0.05$). Also in contrast to our results, an Egyptian case-control study aimed at detecting the frequency of TNF-α (–308) G/A genotype polymorphism revealed that the frequency of TNF-α (–308) A/A homotype in ITP patients...
Our study suggests that TNF-α-308 (G/A) gene polymorphism predicts for some serious complications such as intracranial hemorrhage and non-response to corticosteroid treatment, and other prognostic factors such as the bleeding score and platelet count which may help to identify high-risk patients who should be offered more intensive or alternative lines of treatment from the beginning.

However, the prognostic significance of this polymorphism remains a matter of debate. Larger studies including larger sample size and more sensitive detection techniques will be needed to clarify the prognostic significance of this polymorphism. Our study also has shown no significant association between TNF-α-308 (G/A) and IL-10 (−592) genotypes and alleles frequencies in cases and controls. The risk of developing ITP was not related to the studied polymorphisms. However, the role of other genetic and environmental factors cannot be entirely ruled out. Further studies are required to establish the basis for these observations and their impact on the pathogenesis and progression of, and therapies for, ITP.

Authors’ contributions
The data used and/or analyzed during the current study is available from the corresponding author upon reasonable request.

AS and SI were responsible for creating the study protocol. IM and AB were responsible for ethical approval and patient enrolment. AS, SI, SM and AE were responsible for performing genotype analysis. AE and SM were responsible for data acquisition and statistical analysis. SI and IM were responsible for writing the manuscript. AS, AB and IM were responsible for manuscript revision and proofreading.

Conflict of interest
The authors have no conflict of interest to declare that are relevant to the content of this article.

Financial support
No funding was received for this study.

Ethics
The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments; Uniform Requirements for manuscripts submitted to Biomedical journals.

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