The potential association of tumor necrosis factor-β (252 G/A) cytokine gene polymorphism with immune thrombocytopenic purpura among Egyptian children

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ABSTRACT

Objectives: The main objective of this study was to study tumor necrosis factor beta (TNFB) + 252G/A gene polymorphism, known to be related to autoimmunity, in immune thrombocytopenia (ITP) patients. We also aimed to investigate the association between TNFB + 252G/A polymorphism and susceptibility to develop persistent/chronic ITP.

Methods: One hundred pediatric ITP patients, as well as 50 age- and sex-matched healthy Egyptian subjects, were included. Genotyping of TNF-β gene (G252A) was done using the PCR-RFLP method.

Results: TNFB allele B2 frequency was (64%, 64/100) in controls, (69%, 138/200) in ITP patients, while the frequency of the variant allele B1 was 36% (36/100) in controls, (31%, 62/200) in ITP patients. TNFB genotype frequency in ITP patients showed equal frequency for B2B2 and B1B2 genotypes, (46%, 46/100), while B1B1 frequency was 8% (8/100). Among controls, frequencies of B2B2, B1B2 and B1B1 genotypes were 36% (18/50), 56% (28/50), and 8% (4/50), respectively. Odds ratio for the risk of developing ITP revealed no statistically significant risk, associated with any allele or genotype. No association was encountered between different genotypes and age, hematological parameters, gender, stage of the disease or response to treatment.

Discussion: Comparison between ITP patients and controls as regards TNFB allele and genotype frequencies showed no statistically significant difference. No increased risk for developing ITP was associated with any allele/genotype.

Conclusion: The risk of developing ITP was not related to the studied polymorphism.

KEYWORDS
ITP; TNF; TNFB+252G/A polymorphism

INTRODUCTION

Immune thrombocytopenia (ITP), formerly known as idiopathic or immune thrombocytopenic purpura, is an acquired bleeding disorder due to premature platelet destruction, reduced platelet production, or a combination of both [1,2].

Idiopathic thrombocytopenic purpura (ITP) is associated with dysregulation of the cytokine network. Cytokine gene polymorphisms have been associated with different autoimmune diseases including ITP [3].

The tumor necrosis factor (TNF) cytokine superfamily plays a crucial role in variant vital processes such as apoptosis, immune cell functions, and also in autoimmune diseases and cancer. TNF family entails 19 ligands, among which TNF-α, TNF-β (lymphotoxin-alpha), and Fasl are of great interest in different autoimmune diseases. TNF gene is located on chromosome 6 in the class III region of the major histocompatibility complex (MHC) [4].

Different studies have reported the association of the SNP at tumor necrosis factor beta (TNFB) + 252G/A with various autoimmune diseases, for example, G allele was associated with systemic lupus erythematosus [5], Graves’s disease [6], and A allele with systemic sclerosis [7].

The TNFB + 252G/A polymorphism is located in the first intron and it correlates with the level of TNFB protein production by lymphocytes [8]. Individuals having TNFB + 252 G (B1) allele are high producers of TNFB, while those possessing the A allele (B2) are low producers. TNFB is produced by activated T cells and is involved in the maturation and activation of B-cells [9,10].

The main objective of this study was to study TNFB + 252G/A gene polymorphism, known to be related to autoimmunity and inflammation, in ITP patients. We also aimed to investigate the association between TNFB + 252G/A polymorphism and susceptibility to develop persistent/chronic ITP.

PATIENTS AND METHODS

In this study, we enrolled 100 pediatric ITP patients, as well as 50 age- and sex-matched healthy Egyptian children.
subjects, who were included as a control group. Informed consent was obtained from each participant’s guardian before being enrolled in this study. The study was approved by the ethics committee of Beni-Suef University and conducted according to the World Medical Association Declaration of Helsinki.

This study is a conjoint work between the Pediatric Department, Clinical and Chemical Pathology Department, Faculty of Medicine, Beni-Suef University together with Pediatric Department and the Clinical and Chemical Pathology Department, Kasr Al Aini Hospitals, Faculty of Medicine, Cairo University. Patients being followed up in the Hematology Clinics of the pediatric departments of both university hospitals between June 2016 and August 2017. All cases were subjected to careful history taking and comprehensive clinical examination. ITP was diagnosed in accordance with the guidelines of the American Society of Hematology [11].

The detailed demographic, clinical and laboratory data of the patients group are summarized in Table 1. Among the studied controls, the age ranged from 4 to 16 with a median age of 9.0 years, 26 (52%) were males and 24 (48%) were females.

Newly diagnosed ITP (acute) is defined as thrombocytopenia up to 3 months since diagnosis; persistent ITP is defined as thrombocytopenia lasting from 3 to 12 months since diagnosis, while chronic ITP is persistent thrombocytopenia for more than 12 months of duration [12].

The follow-up for ≥1 year to judge for chronicity was available for 39 out of the 100 patients (39%). The severity at the time of sampling was assessed according to the absence or presence of significant bleeding, using specific bleeding score [13]. Defining the disease state at the time of sampling according to Zhou et al., 68 of our patients (68%) were in activity (platelet count <100 × 10^9/l in newly diagnosed cases or persistent ITP with relapse following termination of therapy within 3–12 months from the diagnosis), and 32 patients (32%) were in remission (platelet count ≥100 × 10^9/l either spontaneously or following therapy) [14].

Assessment of laboratory response to the applied line of management was applied in 89 ITP patients who were eligible for follow-up for at least 3 months according to Rodeghiero et al., non-responders (platelets count <30 × 10^9/l or less than doubling of the baseline count), partial responders (platelets count between 30 and 100 × 10^9/l or at least doubling of the baseline count), and complete responders (platelets count ≥100 × 10^9/l) [12].

Genotyping of TNF-β gene (G252A) was done using the PCR-RFLP method as described before [15]. Genomic DNA was purified from leukocytes using the thermo scientific Gene JET whole blood Genomic DNA purification kit (Cat. #K0781, #K0782, Fermentas Life Sciences). The polymorphic sequence, corresponding to the TNF-β alleles, was amplified using the following primers: 5′-CCGTTCTTGGGTGTTTGG ACT-3′ and 5′-AGAGGG GT G GATGCTTGGGTTC-3′. PCR amplification protocol was performed according to Yapijakis et al., to generate a 782 bp PCR product, in brief, the PCR amplification consisted of an initial denaturation step at 95°C, followed by 35 cycles of 94°C for 50 s, 68°C for 1 min and 72°C for 55 s, as well as a final step at 72°C for 4 min. The amplified PCR product of TNF-β gene was digested using Nco I restriction enzyme resulting in two fragments of 586 and 196 bp if the B1 allele was present and a single 782 bp fragment if the B2 allele was present. The restriction products were applied to gel electrophoresis in a 2% agarose gel and were visualized upon staining with ethidium bromide in reference to a molecular weight marker. Ten per cent of the samples were amplified twice for verification of genotyping results.

All statistical calculations were done using computer programs Microsoft Excel (Microsoft Corporation, NY, and USA) and SPSS (Statistical package for the social science) statistical programs (SPSS, Inc., Chicago, IL, USA).

Table 1. Clinical and laboratory data of the 100 studied ITP patients.

| Variable                        | Value (number = 100) |
|---------------------------------|----------------------|
| Sex, number (%)                 |                      |
| Male                            | 57 (57)              |
| Female                          | 43 (43)              |
| Age at sampling, years          |                      |
| Mean ± SD, range                | 6.4 ± 3.6 years (1.2–15) |
| Platelet at sampling, ×10^9/l   | 13.3 ± 12.0 (0–68)   |
| Hemoglobin at sampling, g/dl    | 11.1 ± 1.4 (7.9–14.2) |
| TLC at sampling, ×10^9/µl       | 10.4 ± 4.3 (3.4–25)  |
| Phase, n (%)                    |                      |
| Acute (newly diagnosed)         | 44 (44)              |
| Persistent                      | 17 (17)              |
| Chronic**                       | 39 (39)              |
| Disease state at sampling, n (%)|                      |
| Activity                        | 68 (68%)             |
| Remission                       | 32 (32%)             |
| Grades of bleeding severity, n (%)|                    |
| Grade 1                         | 3 (3)                |
| Grade 2                         | 16 (16)              |
| Grade 1/2                       | 35 (35)              |
| Grade 3                         | 46 (46)              |
| Treatment, n (%)                |                      |
| Observation                     | 9 (9)                |
| Pulse steroids                  | 41 (41)              |
| Oral steroids                   | 92 (92)              |
| IV Ig                            | 9 (9)                |
| Cyclosporine                    | 8 (8)                |
| Azathioprine                    | 22 (22)              |
| Splenectomy                     | 0 (0)                |
| Platelet transfusion            | 11 (11)              |
| Anti D                          | 1 (1)                |
| Quality of laboratory response at FU*, n (%) |          |
| Complete response               | 28/100 (28)          |
| Partial response                | 57/100 (57)          |
| No response                     | 4/100 (4)            |
| No available data               | 11/100 (11)          |

SD, standard deviation.

*Patients group who were available for short follow-up (FU), (3–6 months after diagnosis).

**Patients group who maintained the follow-up for ≥1 year.
USA). Data were 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 (X²) was performed. The Mann–Whitney test was used to compare two unrelated samples and the Kruskal–Wallis test was used to compare three or more unrelated samples. Association was expressed as odds ratios (OR) with 95% confidence intervals (CIs). P-values less than 0.05 were considered statistically significant.

**Results**

TNFβ allele B2 frequency was 64% (64/100) in the control group and 69% (138/200) in ITP patients, while the frequency of the variant allele B1 was 36% (36/100) in the control group and 31% (62/200) in ITP patients. TNFβ genotype frequency in ITP patients showed exact equal frequency for the B2B2 and the B1B2 genotypes, each of them representing a frequency of 46% (46/100), while the least encountered genotype was the B1B1 showing a frequency of 8% (8/100) of the studied patients. While among controls, frequencies of B2B2, B1B2 and B1B1 genotypes were 36% (18/50), 56% (28/50) and 8% (4/50), respectively.

Comparison between ITP patients and controls as regards TNFB allele and genotype frequencies showed no statistically significant difference (Table 2).

Calculating the OR for the risk of developing ITP in correlation to different TNFB alleles and genotypes, revealed no statistically significant risk for developing ITP associated with any allele or genotype (Table 3).

Comparison between patients with different TNFB genotypes as regards the mean age of disease onset, or hematological data of ITP patients (platelet count, hemoglobin level and total leucocyte count, at diagnosis as well as at the time of sampling), showed no statistically significant difference (Table 4).

**Table 2.** Comparing TNFB genotype frequency between the ITP patients and the control group.

| TNFβ polymorphism, n (%) | Patient (n = 100) | Control (n = 50) | P-value |
|--------------------------|-------------------|-----------------|---------|
| B1B1 (variant)           | 8 (8)             | 4 (8)           | 0.306   |
| B1B2 (heterozygous)      | 46 (46)           | 28 (56)         |         |
| B2B2 (wild)              | 46 (46)           | 18 (36)         |         |
| Allele, n (%)            |                   |                 |         |
| B1                       | 62 (31)           | 36 (36)         | 0.433   |
| B2                       | 138 (69)          | 64 (64)         |         |

No statistically significant difference was encountered in TNFB allele and genotype frequencies when different patients groups were compared in this regard; males versus females; newly diagnosed ITP/persistent ITP/chronic ITP versus controls or between patients with active ITP and those who were in remission (Table 5).

Comparing TNFB allele and genotype frequencies among ITP patients with complete, incomplete and no laboratory response (during the follow-up) showed no statistically significant differences (Table 5).

**Discussion**

ITP is a multifactorial disease in which environmental and genetic factors have been implicated. Primary ITP is manifested by platelet autoantibodies that are responsible not only for destroying platelet by phagocytosis but also for inhibiting their production.

Cytotoxicity and apoptosis are two important pathways through which natural killer (NK) cells destroy autoplatelets. Granzyme and perforin are central functional proteins in cell-mediated toxicity, they are expressed in abnormally activated NK cells of ITP patients and directly destroy the platelets by cytotoxicity, leading to thrombocytopenia [16]. On the one hand, the upregulated expression of FasL on the surface of NK cells in ITP patients is reported to cause apoptosis of autologous platelets through interaction of FasL with their receptors on the surface of platelets [17]. Also NK cells mediate the lysis of platelet and augment the immune response via secretion of TNF-γ and TNF-α [18].

A G/A polymorphism, located at position 252 within the first intron of the TNF-β gene, affects expression of both genes and concentration of TNF-α and TNF-β proteins in the plasma [8,19]. The less common allele B1 (252G) has been correlated with a higher TNF-β expression at the mRNA level and the protein level. On the other hand, the common allele B2 (252A) is associated with increased TNF-α gene expression [8]. In contrast to that, Abraham et al. showed that the B1 allele is associated with higher TNF-α production in lymphoblastoid cell lines [20]. Messer et al. reported that the B2 allele is related to the increase in TNF-β production [8]. The association between the TNF-β gene G252A polymorphism and TNF production has been controversial [21].

In this study, we found no statistically significant difference between ITP patients and controls as regards TNFB allele and genotype frequencies (Table 2). Similar results were demonstrated by Yadav et al. in a study of TNF-β +252 A > G polymorphism in adult Indian patients with primary ITP [22]. However, contradicting results have been illustrated in various studies. Differences between cases and controls were observed by Pavkovic et al. and Satoh et al. Pavkovic
et al. demonstrated significantly different distribution of the TNFβ genotypes in adult patients with ITP, compared to healthy controls. Their data indicated that the B2 allele of TNFβ + 252G/A is more frequent in patients than in controls [23]. Contrastingly, Satoh et al. demonstrated a higher frequency of the TNF-β (B1B1) phenotype in Japanese adult patients with ITP than in healthy controls [24]. Data on pediatric patients with ITP are scanty with the exception of the study done by Foster et al., whose results were different from the current study and showed higher prevalence of B2 allele for TNF-β in Caucasian children with chronic ITP [25]. The reason for these differences is unclear. These differences could be due to the differences in patients' ethnic backgrounds. Another explanation is the possible presence of another susceptibility gene adjacent to the TNF-β genes. A polymorphic allele might be in linkage disequilibrium with the TNF genetic markers located within the major MHC class III region. Previous studies in immune-related diseases have found differences in TNF-β alleles and linkage to HLA-B8-DR3 among European and Japanese patients with systemic lupus erythematosus [26]. Similarly, Ishii et al. concluded that since the TNF-β gene G252A gene polymorphism is a silent mutation, it might have linkage disequilibrium with other functional mutations [21].

In the current study, no statistically significant risk for developing ITP was associated with any allele or genotype (Table 3). No association was encountered between different genotypes and hematological parameters (TLC, Hb, platelets), gender, stage of the disease (newly diagnosed ITP/persistent ITP/chronic ITP) or response to treatment (Tables 4 and 5). Similarly, Satoh et al. showed no difference in gender, age at onset or response to treatment between cases and controls [24]. Moreover, Pavkovic et al. demonstrated no significant differences in genotype distribution and the allele frequencies for TNFB + 252G/A between responders and non-responders [23].

In conclusion, no significant difference in TNFB + 252 genotype or allele frequencies was found

| Genotype, n (%) | Patients (n = 100) | Controls (n = 50) | P-value | OR (95% CI) |
|----------------|-------------------|-----------------|---------|-------------|
| B2B2 (wild)    | 46 (46)           | 18 (36)         | Reference | Reference |
| B1B1 (variant) | 8 (8)             | 4 (8)           | 0.715   | 0.8 (0.20–2.9) |
| B1B2 (heterozygous) | 46 (46)         | 28 (56)         | 0.228   | 0.6 (0.31–1.3) |
| Allele, n (%)  |                   |                 |         |             |
| B2             | 138 (69)          | 64 (64)         | Reference | Reference |
| B1             | 62 (31)           | 36 (36)         | 0.384   | 0.8 (0.48–1.3) |

Table 3. Calculation of the odd ratio for risk of developing ITP, odd’s ratio in association with different alleles.

| Genotype, n (%) | Patients (n = 100) | Controls (n = 50) | P-value | OR (95% CI) |
|----------------|-------------------|-----------------|---------|-------------|
| B1B1 (variant) | 8 (8)             | 4 (8)           | 0.715   | 0.8 (0.20–2.9) |
| B1B2 (heterozygous) | 46 (46)         | 28 (56)         | 0.228   | 0.6 (0.31–1.3) |
| Allele, n (%)  |                   |                 |         |             |
| B2             | 138 (69)          | 64 (64)         | Reference | Reference |
| B1             | 62 (31)           | 36 (36)         | 0.384   | 0.8 (0.48–1.3) |

Table 4. Comparing between patients with different TNFB genotypes as regards the mean age of disease onset and hematological data of ITP patients.

| Genotype, n (%) | Patients (n = 100) | Controls (n = 50) | P-value | OR (95% CI) |
|----------------|-------------------|-----------------|---------|-------------|
| B2B2 (wild)    | 46 (46)           | 18 (36)         | Reference | Reference |
| B1B1 (variant) | 8 (8)             | 4 (8)           | 0.715   | 0.8 (0.20–2.9) |
| B1B2 (heterozygous) | 46 (46)         | 28 (56)         | 0.228   | 0.6 (0.31–1.3) |
| Allele, n (%)  |                   |                 |         |             |
| B2             | 138 (69)          | 64 (64)         | Reference | Reference |
| B1             | 62 (31)           | 36 (36)         | 0.384   | 0.8 (0.48–1.3) |

Table 5. Comparing TNFB genotype and allele frequency between different groups.
between cases and controls. The risk of developing ITP was not related to the studied polymorphism. No association was demonstrated between TNFB +252 genotypes and the age, hematological parameters (TLC, Hb and platelets), gender, stage of the disease (newly diagnosed ITP/persistent ITP/chronic ITP) or response to treatment.

Disclosure statement
No potential conflict of interest was reported by the authors.

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