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Original article

Interleukin--37 Expression in Primary Immune Thrombocytopenia in Egyptian Patients

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Abstract:

Background: Immune thrombocytopenia (ITP) is a bleeding disorder mediated by the immune system in which anti-platelet antibodies prematurely destroy platelets, resulting in a decreased platelet count. One of the IL-1 cytokines; IL-37, has an effect against inflammation in cancer, inflammatory and autoimmune diseases by regulating both innate and acquired immunity. **Aim:** Here, we evaluated the IL-37 gene both serum levels and gene expression in ITP children patients and correlated it with the disease severity. **Patients & Methods:** Forty newly diagnosed ITP patients and forty healthy, age- and sex-matched controls participated in a case-control study. Blood IL-37 gene mRNA expression and serum IL-37 level were both identified using ELISA and RT-qPCR, respectively. **Results:** ITP patients had significantly elevated serum IL-37 (P= 0.006) and IL-37 gene expression (P= 0.001) than controls. Positive correlations were found between the severity of ITP and a significantly lower platelet count, as well as significantly lower serum and gene expression levels of IL-37. **Conclusion:** IL-37 mRNA and serum expressions are promising biomarkers for ITP diagnosis and predictors for disease severity.

Keywords:

ITP; thrombocytopenia; IL-37; expression; biomarker.

Introduction:

Decreased number of platelets as a result of accelerated platelet breakdown and reduced platelet synthesis characterizes immune thrombocytopenia (ITP), an autoimmune disorder that can result in various bleeding manifestations, fatigue due to possible

associated anemia, and reduced quality of life [1]. All ages, genders, and races are impacted by ITP [2]. ITP affects a comparable number of adults as children overall with childhood incidence about 4–5 /100,000 person/year in Europeans and 8 /100,000 person/year in North Americans. ITP incidence peak occurs between 1 and 5 years with a male predominance [3]. Mucocutaneous bleeding that affects the gastrointestinal tract, skin, and oral cavity and presents as purpura is the most typical clinical symptom of ITP along with epistaxis, menorrhagia, gingival and gastrointestinal bleeding [4]. ITP can be idiopathic in its main form or result from another illness. Isolated thrombocytopenia (platelet count 100 10^9/L) without other associated causes or diseases is present in primary ITP. So, primary ITP is diagnosed by exclusion [5]. In most primary ITP patients there is increased expression of CD4+ Th0/Th1 cytokine profile including declined peripheral Th2+ and T regulatory (Treg) cells [7] and IFN-γ and IL-2 [6]. Recent research has revealed that the IL-1 cytokine family member IL-37 regulates both innate and acquired immunity, delays immunological senescence brought on by age, and reduces inflammation in inflammatory, autoimmune, and cancerous conditions [8]. The present investigation sought to assess the IL-37 gene expression and levels of serum in ITP patients and their correlation to the severity of the condition.

Patients and Methods:

Study design

This case-control study was done in Benha University Hospitals between October 2020 and April 2022 on 80 subjects; 40 newly diagnosed ITP children patients and 40 aberrantly healthy age- and sex-matched controls. ITP diagnosis and severity were defined based on published diagnostic criteria [9,10]. According to platelet count, ITP severity was classified into mild (50 – 100 x10^3/µl), moderate (30 – 50 x10^3/µl), severe (10 – 30 x10^3/µl), and very severe (<10 x10^3/µl). Subjects with a history of autoimmune or inflammatory disorders were excluded. The study protocol was authorized by the committee of local ethics for research involving human subjects at the Faculty of Medicine, Benha University, and it was carried out in keeping with the 2004 ethical standards of the Helsinki declaration. Before enrolment, participants' or their legal guardians' informed consent were taken.

Methods:

All participants were subjected to clinical examinations, complete history taking, and standard laboratory tests including:

- Complete blood picture was evaluated by full-automated hematology analyzer ***(New Swelab Alfa Plus; Sweden).***
- IL-37 Serum level by ELISA.
- Relative IL-37 gene expression by RT-PCR.

Sampling:

- Five ml venous blood were collected from each subject by clean venipuncture using disposable plastic syringe. Blood was divided into: 3 ml on plain tube (without anticoagulant) for serum separation to determine serum IL-37 level and 2 ml on ethylene diamine tetra-acetic salt (EDTA) (1.2 mg/mL). EDTA blood was then divided into two equal parts one part was used for CBC and the other one was used for RNA extraction. The extracted RNA was saved in research refrigerator at -80°C so we can use it later for measurement of IL-37 gene expression. Serum was obtained by leaving the plain tube at 25°C for 20 minutes so it can be coagulated, and then put it in a centrifuge at 1500 rpm for 12 minutes. The resultant serum was then saved at -20°C to be used for IL-37 serum level measurement.

Serum IL-37 level

An ELISA (Enzyme-Linked Immune Sorbent Assay) commercial kit for research use (Cat#: 201-12-1948, Sun Red Biotechnology, China) was used to detect serum IL-37. The assay window was 1 – 200 pg/ml and kit sensitivity was 0.513 pg/ml.

IL-37 gene mRNA expression analysis

Real-time quantitative PCR (RT-qPCR) was utilized to analyze the IL-37 gene's mRNA expression via a 3-steps procedure. The first step was total RNA (including mRNA) extraction using A.B.T.^{TM} Total RNA Mini Extraction Kit (Spin Column) (Cat# ABT002, Turkey). Nanodrop 2000$^{®}$ spectrophotometer was used to detect extracted RNA purity and concentration. Complementary DNA (cDNA) was created in the second stage using a High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Cat# 4374966, Thermo Fisher Scientific, USA) with the reaction settings; 10 minutes at 25°C, 120 minutes at 37°C, 5 minutes at 85°C, and hold at 4°C. The third step was the RT-qPCR using Step One Plus® real-time PCR System (Applied Biosystems, USA) and QuantiNova SYBR Green PCR Kit (Cat# 208052, QIAGEN, Germany). IL-37 and the endogenous control (β actin) genes primers were used [IL-37 F: 5′-GCTGAAGAAGGAGAAACTGATGAAG-3′, IL-37 R: 5′-TTATCTGTCACCCCAACAGGC-3′, β-actin F: 5′-

TCCACCTTCCAGCAGATGTG-3′ and β-actin R: 5′-AGCATTTGCGGTGGACGAT-3′]. The following real-time cycler settings were used; 45 cycles of denaturation (95°C for 5 sec) and combined annealing/extension (60°C for 10 sec) are used in the initial activation stage of the PCR (95°C for 2 min). The melting curve of PCR products was analyzed to verify their specificity and identity. For accurate and reproducible results of mRNA quantification, a standard endogenous reference gene (as β-actin) should be used to normalize the amount of target mRNA. IL-37 gene mRNA detection and quantification were expressed as relative mRNA expression in comparison to the endogenous reference gene (β-actin) according to the $2^{-\triangle\triangle ct}$ method.

Statistical Analysis

Using SPSS version 28, data management and statistical analysis were conducted (IBM, Armonk, New York, United States). The Shapiro-Wilk test and methods for direct data visualization were utilized to establish the normality of the quantitative data. According to normality, quantitative data were summarized using means, standard deviations, or medians and ranges. Numbers and percentages were used to represent a categorical set of data. Depending on whether the quantitative data were normally or non-normally distributed, the independent t-test or Mann-Whitney U test was used to compare the quantitative data between the research groups. We compared categorical data using the Chi-square test. Serum IL-37 and IL-37 mRNA expression underwent ROC analysis to distinguish between patients and controls. For each, the optimal cutoff point, diagnostic indices, and Area Under Curve (AUC) with a 95% confidence interval were calculated. Spearman's correlation was utilized to perform the correlations. Using the Mann-Whitney U test, serum IL-37 and IL-37 expression were compared based on several criteria. Multivariate logistic regression analysis was performed to predict thrombocytopenia. Calculated were odds ratios and 95% confidence intervals. Each statistical test has two sides. Significant P values were considered to be those of 0.05 or less.

Results

Demographic, laboratory, and clinical data of studied groups

In the current case-control study, 40 patients with a recent ITP diagnosis were included (male/female, 24/16; age range 2–18 years) and 40 matched healthy controls (male/female, 20/20; age range 1–18 years). Data from all patients' and healthy controls'

lab tests and demographics are shown in (Table 1), which demonstrates that the only statistically significant data between patients and controls is the platelet count.

Clinical and bleeding characteristics:

All enrolled cases had childhood onset of ITP and were newly diagnosed at the time of the study. The median disease duration was 2 weeks. Half of the patients had very severe disease and most of them had petechiae and ecchymosis as bleeding manifestation. Clinical and bleeding manifestations of all patients are demonstrated in (Table 2). **Correlation between IL-37 serum and expression levels of studied groups:** Both serum and expression levels of IL-37 demonstrate statistical significance between patient and control groups as shown in (Table 3)**Correlation between serum and gene expression levels of IL-37 and other parameters:** Table 4 shows that serum and gene expression level of IL-37 have significant negative correlation with platelet count and significant positive correlation with disease severity in ITP cases. No significant correlations were reported with age, disease duration, hemoglobin, and WBCs.

Performance of IL-37 in ITP diagnosis

ROC analysis was performed to assess the performance of IL-37 in ITP diagnosis. serum IL-37 was significant in ITP diagnosis with 60% sensitivity and 100% specificity at cutoff > 93.8 pg/ml (P 0.006). (Figure 1)

Also, IL-37 gene expression was a significant marker in diagnosing ITP with 87.5% sensitivity and 100% specificity at cutoff > 0.008-fold (P < 0.001) (Figure 2).

Assessment of severity in ITP patients

The severity of ITP is identified with platelet count. Thus, to assess the predictors of thrombocytopenia in ITP patients, multivariate logistic regression was used. A substantial independent predictor for thrombocytopenia was found to be serum IL-37 after controlling the effect of age and gender (P < 0.001). One unit increase in serum IL-37 level increases the risk of thrombocytopenia by 2% (Table 5).

Discussion

Like other autoimmune disorders; inflammation is a cornerstone in ITP development [(11)]. The impaired immune homeostasis in ITP led to the auto-destruction of platelets that provokes the inflammatory environment linked with increased pro-inflammatory cytokine

expression and their producing immune cell [12].

One of the IL-1 cytokines known as IL-37 has been known by its anti-inflammatory effect. Patients with autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, and primary Sjögren's syndrome had higher levels of serum IL-37 which were significantly linked with the severity of the disease [13].

In our study, we aimed to assess IL-37 serum level and its gene expression in children ITP patients and compare it with healthy controls. While evaluating IL-37 we discovered that ITP patients' IL-37 mRNA expression and levels of serum were substantially greater than those of healthy controls (median 0.22 vs. 0 RQ, P < 0.001 and median 119.9 vs. 80.18 pg/ml, P= 0.006; respectively).

This was in line with Zhao et al. [14] who found that IL-37 mRNA expression and it's level in plasma was high in a significant way in ITP patients with platelet count less than 30 $x10^3$/µl compared to controls. And this may happen as IL-37 level increases during inflammation as it has an important anti-inflammatory role by activating an anti-inflammatory signaling cascade during which it forms a complex with IL-18 receptor α (IL-18Rα) and IL-1R8 [15]. Thus, IL-37 may be an indicator of inflammation state.

Also Elsaid et al. [16] agreed with our findings as his study demonstrated increased IL-37 level in primary ITP patients more than healthy volunteers.

However, Badr et al. [17] had not observed any important difference between ITP patients and controls as regard IL-37. Therefore he concluded that the serum level of IL 37 may have no important role in ITP development. However, he also mentioned that this may happen as a result of the low number of patients in study groups. Zhang et al. [18] study also agreed with Badr et al. [17]. But he mentioned that more studies with higher subjects' number are needed to be sure about these results, and we may have a higher statistical importance if we did that.

Whereas, Zhan et al. [19] in their study revealed a considerably reduced serum IL-37 level in primary ITP patients without any significant differences by comparing IL-37 mRNA expression to that of healthy controls.

Our study demonstrated important negative correlations between IL-37 mRNA expression and levels of serum with platelets count (r -0.489, P= 0.001 and r -0.730, P <0.001; respectively) and important positive

relations with ITP severity (r 0.594, P <0.001 and r 0.719, P <0.001; respectively). This agreed with Zhao et al. [14] who observed a significant negative correlation between IL-37 plasma concentration and platelet count in patients with ITP (r -0.5502, P= 0.0001), which demonstrates that patients with ITP who have lower platelet counts also have greater plasma levels of IL-37. They mentioned that plasma IL-37 expression was higher in patients who reported symptoms of the skin or oral bleeding and Higher IL-37 levels among those patients were associated with more severe bleeding. Which indicate that IL-37 is a viable factor in detecting the risk of bleeding in ITP patients and an indicator for the disease severity. However, in patients who had a subconjunctival hemorrhage, genitourinary hemorrhage, gynecological hemorrhage, gastrointestinal hemorrhage, and epistaxis, they did not notice any substantial rise in plasma IL-37. This could be due to the rarity of the bleeding symptoms and the relatively limited patients' number in their study.

Su et al. [8] also found that ITP patients had higher IL-37 levels in their serum, which are related to the number of platelets and bleeding severity. While Badr et al. [17] and Zhang et al. [18] had not noted any relation between IL-37 serum level and platelet count among studied patients with ITP. However, Zhan et al. [19] observed an important positive relation between IL-37 serum levels and platelet count in ITP patients (r 0.617, P= 0.008).

The variety of results in these studies may be due to the differences in ethnicity, sample age and size between the studied groups.

As we performed ROC analysis to assess the performance of IL-37 mRNA and serum expressions in diagnosing ITP; we found a significant AUC (0.678) of serum IL-37 at a cut-off >93.8 pg/ml where sensitivity and specificity were 60% and 100%, respectively (P= 0.006). While, for IL-37 mRNA expression level, we detected a significant AUC (0.972) at cut-off >0.008-fold where sensitivity and specificity were 87.5% and 100%, respectively (P <0.001).

Multivariate logistic regression analysis was done to anticipate thrombocytopenia, it revealed that serum IL-37 was a significant predictor for ITP (OR 1.022, P <0.001) after controlling for the effect of age and gender.

This comes along with Zhao et al. [14] who revealed that ITP patients, particularly those who had significant bleeding or a low platelet count, showed greater expression of IL-37. While Badr et al. [17] did not detect a significant role of IL-37 serum level and

mRNA expression in either diagnosis of ITP or prediction of severe disease.

In conclusion, our results support the published data that elucidate the pivotal role of IL-37 as a diagnostic marker for ITP and a potential predictor for disease severity.

Recommendations:

Sample size should be increased to ensure more accuracy of the results.

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