Study the Incidence of TNF-α-induced Protein 3 Genetic Polymorphisms in Primary Immune Thrombocytopenia Patients

Mohamed Ibrahim Zanaty (zanaty012@psas.bsu.edu.eg)
Beni-Suef University: Beni Suef University  https://orcid.org/0000-0002-5386-8570

Osama Korayem
Beni-Suef University: Beni Suef University

Mohamed Hussien Meabed
Beni-Suef University: Beni Suef University

Doaa El- Demerdash
Cairo University

Wafaa Mohamed Abdelghany
Cairo University

Research Article

Keywords: Primary Immune Thrombocytopenia, Polymorphisms, TNFAIP3, rs2230926, rs5029939

Posted Date: February 18th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-205009/v1

License: © This work is licensed under a Creative Commons Attribution 4.0 International License.  Read Full License
Abstract

Background

Immune Thrombocytopenia (ITP) is a relatively common acquired hematological disorder, affecting 2 to 4/100000 adults. Understanding of the pathogenesis of ITP has been greatly improved with taking into consideration the important role of the genetic variants. This study aimed at investigating the incidence of TNFAIP3 SNPs (rs2230926 and rs5029939) in primary ITP Egyptian patients as well as their response to therapy in addition to the linkage between the two SNPs.

Methods and Results

the study was conducted in 110 ITP patients diagnosed as primary ITP (PITP) selected among cases referred to the Hematology Outpatient Clinic of Kasr El Aini Hospital and 110 matched healthy controls. The polymorphisms were detected by real-time polymerase chain reaction (real-time PCR). Data indicated that there is a significant difference in the allelic distribution between PITP patients and the control group regarding rs2230926 and rs5029939 (p-value <0.05). Regarding LD analysis of the two SNPs, it has been revealed that there was a significant linkage disequilibrium between rs2230926 and rs5029939 among PITP group LD (D' = 0.966, r² = 0.694, p-value < 0.001). On behalf of improvement by treatment, patients with rs2230926 wild genotype showed a more significant response to treatment than mutant type (p-value <0.05).

Conclusion

There was a correlation between TNFAIP3 SNPs (rs2230926 and rs5029939) and the occurrence of primary ITP in the adult Egyptian populations and there was a linkage disequilibrium between them. Moreover, patients with rs2230926 wild genotype showed significant improvement than mutant type.

1. Introduction

Thrombocytopenia is a hematological disorder characterized by decreased platelets production, increased platelets destruction or consumption, or increased capturing of circulating platelets in the spleen and platelets count below 100 x 10⁹/L[1]. Immune thrombocytopenia (ITP) is a type of thrombocytopenia that results from increased autoimmune platelet destruction and/or reducing its production with the absence of the underlying cause[2].

There are two types of ITP; primary ITP which may be occurred due to impaired megakaryocyte (MK) function, T cell-mediated platelet destruction, and pathogenic anti-platelet autoantibodies[3]. On the other hand, Secondary ITP is induced by acquired or inherited diseases such as autoimmune diseases including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), or chronic infections including human immunodeficiency virus (HIV) and Helicobacter pylori[4].

Both genetic and environmental factors play an important role in the pathogenesis of many diseases and also for primary ITP[5]. Variation of alleles may occur due to a single nucleotide change which will results in single nucleotide polymorphisms (SNPs) in the genome sequence. Many of these DNA changes do not result in differences at the protein level. However, some SNPs lead to a noticeable clinically relevant change in phenotype[6].
TNF-α-induced protein 3 (TNFAIP3), known as A20 and existing on the long arm of chromosome 6 (6q23.3) between the oligodendrocyte transcription factor 3 (OLIG3) gene and TP53 apoptosis effector (PERP) gene[7], is a ubiquitin-editing enzyme which proved to be a negative regulator of NF-kB in various signaling pathways[8]. TNFAIP3 is evolved in the normal differentiation, proliferation, and activation of selected subsets of B cells. TNFAIP3 also down-regulates the stimulatory immune properties of the dendritic cells[9].

Defect in \textit{TNFAIP3} expression is associated with disturbance in immune hemostasis which results in enhancement of the inflammatory processes. Therefore, several polymorphisms in or near the \textit{TNFAIP3} locus were described as being associated with inflammatory autoimmune disorders, including systemic lupus erythematosus, rheumatoid arthritis, psoriasis, multiple sclerosis, and type 1 diabetes. These outcomes suggested that \textit{TNFAIP3} may play an essential role in ITP[10].

Interestingly enough, this study is the first study conducted on the Egyptian population to investigate the frequency of \textit{TNFAIP3} SNPs (rs2230926 and rs5029939) in primary ITP patients. However, many previous studies based on the genome-wide association studies on genetic material proved that there was a correlation between the \textit{TNFAIP3} gene and human autoimmune pathogenesis, there was a discrepancy in the results which could be due to studying different ethnic groups and regions.

The present study aimed at clarifying the frequency of \textit{TNFAIP3} SNPs (rs2230926 and rs5029939) in primary ITP Egyptian patients as well as their response to therapy.

\section*{2. Materials And Methods}

\subsection*{2.1. Subjects}

The present case-control study included 110 adult patients diagnosed as primary ITP selected among cases referred to the Hematology Outpatient Clinic of Kasr El Aini Hospital, Faculty of Medicine, Cairo University and marked as primary ITP (PITP) group. The control group included 110 age and sex-matched healthy volunteers with platelets counts within the normal range (150-400 x10$^3$/mL)

\subsubsection*{2.1.1. Demographic distribution}

In this study, the primary ITP patients group consists of 93 females and 17 males aged (18-50 years). The healthy control group consisted of 92 females and 18 males, aged (20-55 years).

\subsubsection*{2.1.2. Inclusion and Exclusion Criteria}

All included subjects with primary ITP were selected following the diagnostic features of ITP established by the American Society of Haematology (ASH) guidelines 2011[11]. Exclusion criteria included all patients with Secondary ITP.

\subsection*{2.2. Methods}

\subsubsection*{2.2.1. Clinical examination}

Proper clinical examination was done according to the standard sheet of hematological disorders of hematology Department, Kasr EL-Aini Teaching Hospital, Cairo University. All eligible patients and controls were subjected to
full clinical assessment including bleeding manifestations such as petechiae, ecchymosis, mucous membrane bleeding, or bleeding from other orifices as well as complete history, including age and sex, patient complaint, the onset of symptoms, duration, history of treatment modalities received response to therapy, and history of splenectomy for chronic ITP patients.

2.2.2. Laboratory assessment

2.2.2.1. Routine investigations:

Complete blood picture was done according to Dacie and Lewis (1991)[12] using a cell counter instrument (HeCo SEAC). Reticulocytes count were done manually according to Houte et al. (1994)[13]; erythrocyte sedimentation rate (ESR) was done using Wintrobe Tubes (Sinton,1948)[14]. PTT was done using kits of EGY- CHEM for lab technology and according to the method of Biggs and McFarlane's (1962)[15]. Prothrombin time (PT), prothrombin concentration (PC), and international normalized ratio (INR) were determined according to the method of Wanger and Dati (1998)[16]using kits supplied by Siemens Healthcare Diagnostic (USA). Random blood sugar was done according to Kaplan et al. (1984)[17] using kits purchased from SPINREACT, Spain. Serum liver enzymes (ALT and AST) investigation were done according to Schumann and Klauke (2003)[18] using Kits of Noble Diagnostic, Egypt. As for kidney function test, Serum creatinine was investigated according to Murray and Kaplan (1984)[19] using kits purchased from SPINREACT, Spain. Immunological studies, e.g. anti-nuclear antibody titer (ANA), was investigated according to Emlen and O'Neill's (1997)[20]using kits of Bio-Quant -United States and the automated ELECSYS 1010, Roche instrument. Direct coombs was done according to Theis and Hashmi (2019)[21], Hepatitis B surface antigen (HBs Ag) was performed by enzyme-linked immunosorbent assay according to Wolters et al. (1977)[22] using the instrument Radium, Germany and AXIOM DIAGNOSYIC kits, Germany. Hepatitis C virus (HCV) antibodies were performed with a third-generation ELISA according to Choo et al. (1990)[23] using the instrument READER, 2000 and HCV BIO kits S.A Barcelona. H. Pylori was done using rapid test manufactured by (abbott) china company. Third-generation Human immunodeficiency virus (HIV) antibody was measured using rapid kits manufactured by abon biopharm (hang zhou) Co., Ltd. company, China.

2.2.2.2. Molecular studies:

Detection of single nucleotide polymorphisms (SNPs) of TNFAIP3: (rs2230926 and rs5029939) using Applied Biosystems Fast Real-Time PCR apparatus with serial No.275014873.

2.2.2.2.1 Extraction of genomic DNA

About 3 ml of the venous blood sample was collected on 5% ethylene diamine tetra-acetic acid (EDTA) by sterile venipuncture using a sterile vacutainer tube from both PITP and control groups. Samples were stored at -20° C until DNA extraction.

Genomic DNA was extracted from a peripheral blood sample by using GeneJET Whole Blood Genomic DNA Purification Mini Kit (Cat. No. K0781) and done according to the manufacturer's protocol.

2.2.2.2.2. Genotyping of TNFAIP3 polymorphism

The two SNPs (rs2230926 and rs5029939) of the TNFAIP3 gene were analyzed using TaqMan ready-made assay supplied by Thermo Fisher Scientific Company (Cat. No. 4351379). The rs2230926 SNP was identified by its
Context Sequence [VIC/FAM] GACTTGGTACTGAGGAAGGCGCTGT [G/T] CAGCACGCTCAAGGAAACAGACACA.

Also, the rs5029939 SNP was identified by its Context Sequence [VIC/FAM] GTCACCTAAACTAGTTAGGAGCAGA [C/G] TTAAGCTAGAACCAAGGTCCCTGG.

For DNA amplification TaqMan® Genotyping master mix was provided by Thermo Fisher Scientific Company (Cat. No. 4371353). PCR reactions were carried out in 20 μL of final volume using 3 μL extracted DNA, 10 μL Genotyping master mix, 6.5 μL distilled water, and 0.5 μL SNP Amplification included 40 cycles with the following program: denaturation at 94 °C for 30 s, annealing at 67 °C for 30 s, and extension at 72 °C for 1 min. For validations of polymorphism sequences and primers, TaqMan® Genotyping master mix has been tested across all types of TaqMan® SNP Genotyping Assays. Besides, the master mix is validated with the Applied Biosystems® thermal cyclers and real-time PCR systems. Moreover, the study used a standard procedure to run PCR on serial dilutions of our sample (DNA). Each time a new set of primers is got to ensure that the primers are of good quality, and to find the optimal primers annealing temperature. Also, qPCR protocol is double-checked to ensure adding the right reagents at each step.

2.3. Response to Therapy:

Treatment was initiated in the presence of bleeding symptoms or when the platelet count was less than 30 × 10⁹ /L. Prednisolone was administered with a dosage of 1mg/Kg/day for 2-3 weeks then gradually tapered after that.

- Complete response: Platelet count ≥ 100 × 10⁹/L and absence of bleeding.
- Response: Platelet count ≥ 30 × 10⁹/L and at least 2-fold increase the baseline count and absence of bleeding.
- No response: Platelet count < 30 × 10⁹/L or less than a 2-fold increase of baseline platelet count or bleeding[24].

Patients who didn't respond to steroids after 3 months of therapy receive 2nd line therapies which include: 1-immunosuppressive: azathioprine, cyclosporine. 2- anti-CD20: rituximab. 3- TPO receptor agonist: Romiplostim and Eltrombopag. 4-Splenectomy) according to the choice of the physician & protocol of the department.

ITP disease consists of three different stages: newly diagnosed (diagnosis to 3 months), persistent (3-12 months), and chronic (> 12 months) which is presented by the International Working Group (IWG) specialized for ITP[25].

2.4. Statistical analysis

The collected data were statistically analyzed using IBM SPSS statistics software version 22.0. Descriptive statistics were done for quantitative data as minimum & maximum of the range as well as mean ± SD (standard deviation) for quantitative normally distributed data, median and 1st & 3rd inter-quartile range for quantitative non-normally distributed data. As for qualitative data, it was done as number and percentage. In qualitative data, inferential analyses for independent variables were done using the Chi square test for differences between proportions and Fisher’s Exact test for variables with small expected numbers. The odds ratio (OR) and 95% confidence interval (CI) were also calculated. The level of significance was taken at P value < 0.05. LD was
calculated according to methods introduced by reference[26]. Hardy-Weinberg equilibrium was calculated for controls and ITP patients.

3. Results

3.1. The comparison between PITP patients and control groups regarding demographic characteristics.

In this study, PITP group consists of 93 females (84.5%) and 17 males (15.5%) aged (18-50 years) with (mean age 33.79 ± 14.41 years). For the healthy control group, there were 92 (83.6%) females and 18 males (16.4%) aged (20-55 years) with (mean age 30.75 ± 11.64 years). And there was a non-significant difference regarding age and sex between PITP and control groups (p-value 0.086 and 0.854 respectively).

Duration of disease ranged between 1 month and 180 months where the median duration was 8.5 months. All patients had unremarkable physical examination except 8 (7%) patients had a scar of splenectomy. The median platelet count at the time of diagnosis was $15 \times 10^9$/L where 32 patients (29.1%) had a platelet count < $10 \times 10^9$/L.

PITP cases were selected at different phases of the disease, 35 newly diagnosed (31.8%), 33 persistent (30%), and 42 chronic (38%) data are indicated in the table (1). Follow-up of the patients for one year revealed complete recovery occurred in 19 (17.3%) of the cases).

3.2. The comparison between PITP and control groups regarding clinical findings.

By analyzing the relation of genotype to clinical findings we observed that there were non-significant differences between mutant and wild genotypes of both rs2230926 and rs5029939 regarding age at presentation and duration of disease. However, there was a notable increase in non-cutaneous bleeding manifestations and bleeding gum in mutant genotypes as compared with wild genotypes of both rs2230926 and rs5029939. Data are indicated in Table (1).

At presentation, all the 110 selected patients (34 patients with mutant type and 76 patients with wild type) were having platelet count less than 30 x10^3/mL. After treatment, 3 patients (8.8%) with mutant type and 4 patients (5.3%) with wild type were non-responsive; 31 patients (91.2%) with mutant type and 72 patients (94.7%) with wild type were responsive.

In the present study, all PITP patients started treatment with corticosteroids as the first-line therapy for ITP. Furthermore, complete recovery was reported with a notable degree in the wild genotype of rs2230926 when compared to the mutant genotype of the same SNP with p-value <0.05 as indicated by table (3).

The mortality rates were with non-significance value because in this study only (1) patient (1.3%) in wild rs2230926 genotype and (1) patient (2.2%) for mutant rs5029939 genotype were reported.

3.3. Allelic and genotypic frequencies of rs2230926 and rs5029939 SNPs in PITP patients and controls regarding sex.

Genotype distributions in patients and controls were consistent with the Hardy–Weinberg equilibrium (p-value >0.05). In the present study, it is observed that the frequency of rs2230926 G polymorphism is more common in
PITP female patients (p-value < 0.001). Moreover, a female has an 8.16-fold risk to PITP than a male (OR = 8.16, 95% CI = 3.47-19.16, p = <0.001). However, the frequency of rs5029939 G between male and female showed a non-significant association as indicated by table (2).

3.4 Genotyping and Allelic distributions

Results revealed that there was a significant expression of GG&TG for rs2230926 genotypes in the PITP group compared to the control group. Moreover, there was a significant expression of GG&CG for rs5029939 genotypes in the PITP group as compared to the control group as represented in Figures I & II.

The allelic distributions between PITP and control groups regarding rs2230926 and rs5029939 were also significantly expressed (p-value <0.05). Data indicated in table 4.

3.5. Linkage disequilibrium of the two SNPs

LD analysis of the two SNPs showed a significant linkage disequilibrium between rs5029939 and rs2230926 among study groups LD (D' = 0.966, r^2 = 0.694) and a complete linkage disequilibrium among controls (D' = 1.0, r^2 = 0.844) (Table 5).

4. Discussion

Immune thrombocytopenic purpura is defined as an acquired autoimmune disorder characterized by immune-mediated platelet destruction due to the binding of immunoglobulin IgG autoantibodies with platelet glycoproteins [27].

Primary ITP is characterized by isolated thrombocytopenia (peripheral blood platelet count <100 × 10^9/l) with the absence of any other causes or diseases that may be associated with thrombocytopenia[24]. Moreover, primary ITP has a prevalence of up to 9.5/100,000 adults and an incidence of about 3.3/100,000 adults per year[28]. Severe ITP patients are characterized by bleeding symptoms at presentation and require therapeutic intervention[24]. PITP in adults is considered a chronic disease that requires persistent monitoring and treatment intervention[29].

The death rate for patients with ITP is increased compared with the general population due to the severity of the disease[2]. Moreover, the pathophysiology of ITP is indeed becoming more complex. El Ghannam et al. (2105) tried to understand the molecular basis of the incidence of ITP in the Egyptian population and its role in responding to treatment. They concluded that IL-10 promoter polymorphisms are unlikely to affect the development or treatment outcome of chronic adult ITP in the Egyptian population[30]. So, there is an urgent need to understand the molecular concepts related to this disease in the Egyptian population.

The present study is intended to investigate the relation between TNFAIP3 SNPs (rs2230926 and rs5029939) and primary ITP in Egyptian patients as well as their role in responding to therapy.

Our data indicated that the comparison between the genotypes of both SNPs regarding the age of onset, duration of disease, family history, and phase of the disease showed no significant difference.
The study found a statistically significant difference of rs2230926 G allelic frequencies regarding sex which was not found in rs5029939 G distribution. These results were in contrast to the results of Zhou et al. who reported that there was a non-significant difference between male and female regarding rs2230926 polymorphism[10].

The present study indicated that there was a significant difference in the genotyping and allelic distribution between PITP and control groups regarding TNFAIP3 SNPs (rs2230926 and rs5029939). Data showed that there was a significant difference between PITP and the control group regarding rs2230926 wild TT, heterozygous TG, and homozygous GG genotypes. The data expressed were 69.1%, 24.5% and 6.4% and 80%, 20% and 0% for PITP and control group respectively. Furthermore, the rs2230926 GG & TG genotype have a 1.79-fold risk of developing ITP (OR = 1.79, 95% CI = 0.97-3.32, p = 0.063). Moreover, the results proved that there was a significant difference between PITP and control groups regarding rs2230926 allelic distributions. data indicated that the frequency of the T and G alleles among ITP patients were 81.4% and 18.6% respectively versus 90% and 10% in the control group. Also, the G allele showed a 2.06-fold increased risk. Our results agree with Zhou et al. (2015) who reported that, regarding rs2230926 T/G, the frequency of TT and TG genotypes in ITP patients were detected in 76.7% and 23.3% respectively versus 90.2% and 9.8% in the control group. Furthermore, their allelic distribution revealed that T and G alleles among primary ITP patients were 88.4% and 11.6% respectively, and 95.1% and 4.9% in the control group.

Regarding rs5029939, the present study revealed that the frequency of wild CC, heterozygous CG, and homozygous GG genotypes among primary ITP patients were 59.1%, 36.4%, and 4.5% respectively versus 77.3%, 22.7%, and 0% in the control group. The mutant genotypes (GG & CG) have a 2.35-fold risk to primary ITP. Besides, regarding rs5029939 allelic distributions, the present study indicated that the frequency of the C and G alleles among ITP patients were 77.3% and 22.7% respectively versus 88.6% and 11.4% in the control group. G allele showed a 2.29-fold increased risk to primary ITP. This result is matched with the results of Zhou et al. (2015) who found that the C and G alleles among PITP patients were 84.9% and 15.1% respectively and 96.4% and 3.6% in the control group.

Meanwhile, the association between ITP and the immune linked genetic changes has been greatly established[31]. TNFAIP3 is a zinc-finger cytoplasmic protein proving a strong inhibitor of the inflammatory pathway by suppressing NF-κB stimulation in response to various enhancing triggers such as NLR [Nod (nucleotide-binding oligomerization domain)-like receptor], IL (interleukin)-1, TLR (Toll-like receptor) and TNF ligands[32]. Genetic abnormalities of TNFAIP3 genes proved to be related to a wide range of human diseases[33]. The coding SNP rs2230926 changes phenylalanine-to-cysteine at residue 127 of the TNFAIP3 protein having lower capabilities of suppression of TNF triggered NF-κB activity[34] that triggers various inflammatory and immunological disturbances[35].

The above significant difference between case and control groups regarding rs2230926 can be interpreted as the coding SNP rs2230926 resulting in changing phenylalanine-to-cysteine at residue 127 of the TNFAIP3 protein. The Cys127 TNFAIP3 protein is less efficient in inhibiting NF-κB activity induced by TNF when similar amounts of the two proteins are expressed. This lowered anti-inflammatory activity of TNFAIP3 may increase cellular responses to TNF[36].

As TNFAIP3 is essential for limiting cellular responses stimulated by NOD2, Toll-like receptors, and other pro-inflammatory agents, the hypomorphic TNFAIP3 protein is likely to contribute in multiple aspects of
autoimmunity and excessive inflammation in humans carrying this polymorphism[34].

Regarding bleeding manifestations; the non-cutaneous bleeding manifestations and bleeding gum were evident in the mutant genotypes of both SNPs. data showed that there was a non-significant difference between mutant and wild genotypes of both rs2230926 and rs5029939 regarding cutaneous bleeding. However, it has been found that there is no significant difference between mutant and wild genotypes of rs2230926 also for rs5029939 regarding response to treatment.

Linkage disequilibrium (LD) is a statistical measure of the degree to which particular alleles or SNPs at two loci are related to each other in the population [37]. SNPs and alleles of interest are inherited together if they are physically close to each other producing strong LD[38]. Moreover, LD between marker alleles and traits of interest introduces a fine-scale gene mapping and reproduces the history of natural selection, gene conversion, mutation, and other forces that cause gene-frequency evolution [37].

LD analysis of the two SNPs in this study showed that there is a significant linkage disequilibrium between rs2230926 and rs5029939 among the PITP group indicating that there is a correlation between the two SNPs in producing primary ITP disease. The intronic rs5029939 C>G Transversion Substitution was found to be linked to abnormal immune sensitization[32]. It was studied concerning many TNFAIP3 SNPs which showed a significant LD with rs2230926 T>G in Caucasian ethnic as described in Bates et al., 2009[39]. This agrees with the present data that showed a significant LD between the two SNPs in the Egyptian population. In contrast with our finding Zhou et al. (2015) reported that there was no tight LD in Chinese people. Finally, it is recommended that this study should be repeated in different regions to remove the discrepancy about the relation between the TNFAIP3 and primary ITP patients.

**Conclusion**

The present study showed that TNFAIP3 rs5029939 and rs2230926 polymorphisms are significantly associated with increasing the susceptibility to primary ITP in the Egyptian population. Moreover, patients with rs2230926 wild genotype showed significant improvement than mutant type. Furthermore, there is significant linkage disequilibrium between rs5029939 and rs2230926 genes among the study groups.

**Declarations**

**Funding,**

The authors did not receive support from any organization for the submitted work.

**Conflicts of interest/competing interests,**

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

**Ethics approval**

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Biotechnology and Life Sciences department, Faculty of Postgraduate Studies for advanced sciences Beni-Suef University with approval number (2/019).
Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent to publish

Patients signed informed consent regarding publishing their data.

References

1. Izak M, Bussel JB (2014) Management of thrombocytopenia. F1000Prime Rep 6:
2. Lamb MP, Gernsheimer TB (2017) Clinical updates in adult immune thrombocytopenia. Blood 129:2829–2835
3. Khodadi E, Asnafi AA, Shahrabi S, et al (2016) Bone marrow niche in immune thrombocytopenia: a focus on megakaryopoiesis. Ann Hematol 95:1765–1776
4. Zufferey A, Kapur R, Semple JW (2017) Pathogenesis and therapeutic mechanisms in immune thrombocytopenia (ITP). J Clin Med 6:16
5. Xuan M, Li H, Fu R, et al (2014) Association of ABCB1 gene polymorphisms and haplotypes with therapeutic efficacy of glucocorticoids in Chinese patients with immune thrombocytopenia. Hum Immunol 75:317–321
6. Argoff CE (2014) An Introduction to Pharmacogenetics in Pain Management: Knowledge of How Pharmacogenomics May Affect Clinical Care. In: Practical Management of Pain. Elsevier, pp 132–138
7. Rasi S, Rossi D, Gaidano G (2010) TNFAIP3 (tumor necrosis factor, alpha-induced protein 3). Atlas Genet Cytogenet Oncol Haematol
8. Zhao H, Wang L, Luo H, et al (2017) TNFAIP3 downregulation mediated by histone modification contributes to T-cell dysfunction in systemic lupus erythematosus. Rheumatology 56:835–843
9. Matsuzawa Y, Oshima S, Takahara M, et al (2015) TNFAIP3 promotes survival of CD4 T cells by restricting MTOR and promoting autophagy. Autophagy 11:1052–1062
10. Zhou H, Yang J, Liu L, et al (2016) The polymorphisms of tumor necrosis factor-induced protein 3 gene may contribute to the susceptibility of chronic primary immune thrombocytopenia in Chinese population. Platelets 27:26–31
11. Neunert C, Lim W, Crowther M, et al (2011) The American Society of Hematology 2011 evidence-based practice guideline for immune thrombocytopenia. Blood 117:4190–4207. https://doi.org/10.1182/blood-2010-08-302984
12. Dacie J V, Lewis SM (1991) Practical haematology, ELBS with Churchill Livingston
13. Houte AJ van, Bartels PCM, Schoorl M, Mulder C (1994) Methodology-dependent variations in reticulocyte counts using a manual and two different flow cytometric procedures
14. Sinton JR (1948) Clinical Value of Erythrocyte Sedimentation Rate. Br Med J 1:391
15. Biggs R, Macfarlane RG (1962) Christmas disease. Postgrad Med J 38:3
16. Wanger C, Dati F (1998) Prothrombin time (PT) test. Clinical Lab diagnostics Frankfurt TH-books Verlagsges 599–601
17. Kaplan LA (1984) Glucose. Clin Chem 30:1032–1036
18. Schumann G, Klaue R (2003) New IFCC reference procedures for the determination of catalytic activity concentrations of five enzymes in serum: preliminary upper reference limits obtained in hospitalized subjects. Clin Chim Acta 327:69–79
19. Murray R, Kaplan A (1984) ClinChem The CV Mosby Co. St Louis. Toronto
20. Emlen W, O’Neill L (1997) Clinical significance of antinuclear antibodies. Comparison of detection with immunofluorescence and enzyme-linked immunosorbent assays. Arthritis Rheum Off J Am Coll Rheumatol 40:1612–1618
21. Theis SR, Hashmi MF (2019) Coombs Test
22. Wolters G, Kuijpers LPC, Kaçaki J, Schuurs AHWM (1977) Enzyme-linked immunosorbent assay for hepatitis B surface antigen. J Infect Dis 136:S311–S317
23. Choo QL, Weiner AJ, Overby LR, et al (1990) Hepatitis C virus: the major causative agent of viral non-A, non-B hepatitis. Br Med Bull 46:423–441
24. Rodeghiero F, Stasi R, Gemsehimer T, et al (2009) Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group. Blood 113:2386–2393
25. Michel M (2013) Immune thrombocytopenia nomenclature, consensus reports, and guidelines: what are the consequences for daily practice and clinical research? In: Seminars in hematology. Elsevier, pp S50–S54
26. Khan MA Introduction to different measures of linkage disequilibrium (LD) and their calculation
27. Goubran H, Hart C, Othman I, Seghatchian J (2018) Flow cytometry and immune thrombocytopenic purpura. Transfus Apher Sci 57:800–803
28. Samson M, Fraser W, Lebowitz D (2019) Treatments for Primary Immune Thrombocytopenia: A Review. Cureus 11:
29. McKenzie CGJ, Guo L, Freedman J, Semple JW (2013) Cellular immune dysfunction in immune thrombocytopenia (ITP). Br J Haematol 163:10–23
30. El Ghannam D, Fawzy IM, Azmy E, et al (2015) Relation of interleukin-10 promoter polymorphisms to adult chronic immune thrombocytopenic purpura in a cohort of Egyptian population. Immunol Invest 44:616–626
31. Swinkels M, Rijkers M, Voorberg J, et al (2018) Emerging concepts in immune thrombocytopenia. Front Immunol 9:880
32. Vereecke L, Beyaert R, van Loo G (2011) Genetic relationships between A20/TNFAIP3, chronic inflammation and autoimmune disease. Biochem Soc Trans 39:1086–1091. https://doi.org/10.1042/BST0391086
33. Ma A, Malynn BA (2012) A20: linking a complex regulator of ubiquitylation to immunity and human disease. Nat Rev Immunol 12:774–785
34. Musone SL, Taylor KE, Lu TT, et al (2008) Multiple polymorphisms in the TNFAIP3 region are independently associated with systemic lupus erythematosus. Nat Genet 40:1062
35. Adrianto I, Wen F, Templeton A, et al (2011) Association of a functional variant downstream of TNFAIP3 with systemic lupus erythematosus. Nat Genet 43:253–258
36. Broen JCA, Coenen MJH, Radstake TRDJ (2012) Genetics of systemic sclerosis: an update. Curr Rheumatol Rep 14:11–21
37. Slatkin M (2008) Linkage disequilibrium—understanding the evolutionary past and mapping the medical future. Nat Rev Genet 9:477–485. https://doi.org/10.1038/nrg2361
38. Kager L, Evans WE (2018) Pharmacogenomics and hematologic diseases. In: Hematology. Elsevier, pp 79–91

39. Bates JS, Lessard CJ, Leon JM, et al (2009) Meta-analysis and imputation identifies a 109 kb risk haplotype spanning TNFAIP3 associated with lupus nephritis and hematologic manifestations. Genes Immun 10:470–477

Tables

Table 1: Demographic and clinical characteristics regarding genotypes among PITP group.
|                      | rs2230926 Mutant (n= 34) | Wild (n= 76) | P-value | rs5029939 Mutant (n= 45) | Wild (n= 65) | P-value |
|----------------------|--------------------------|--------------|---------|--------------------------|--------------|---------|
|                      | No. (%)                  | No. (%)      |         | No. (%)                  | No. (%)      |         |
| 1.Age at presentation|                          |              |         |                          |              |         |
| Median (Range)       | 24.9 (10.0-59.0)         | 28.5 (6.0-66.4) | 0.142   | 24.9 (19.0-32.0)         | 29.0 (20.0-42.0) | 0.102   |
| 2.Duration of disease(months) |                          |              |         |                          |              |         |
| Median (Range)       | 16.0 (1.0-120.0)         | 6.5 (1.0-180) | 0.074   | 12.0 (1.0-120.0)         | 7.0 (1.0-180.0) | 0.174   |
| 3.Family history     | 0 (0)                    | 4 (5.3)      | 0.309   | 0 (0)                    | 4 (6.2)      | 0.143   |
| 4.Phase at baseline  |                          |              |         |                          |              |         |
| 4.1.Newly diagnosed  | 8 (23.5%)                | 27 (35.5)    |         | 12 (26.7%)               | 23 (35.4%)   |         |
| 4.2.Persistent       | 6 (17.6%)                | 15 (19.7)    |         | 8 (17.8)                 | 13 (20%)     |         |
| 4.3.Chronic         | 20 (58.8%)               | 34 (44.7)    |         | 25 (55.6%)               | 29 (44.6%)   |         |
| 5.Clinical characteristics |                          |              |         |                          |              |         |
| 5.1.PLT at basal     |                          |              |         |                          |              |         |
| 30       <       | 34(100%)                  | 76(100%)     |         | 45(100%)                 | 65(100%)     |         |
| 30 >30     | 0 (0.0 %)                 | 0 (0.0%)     |         | 0 (0.0 %)                | 0 (0.0 %)    |         |
| 5.2.PLT in response  |                          |              |         |                          |              |         |
| 30       <       | 3 (8.8%)                  | 4 (5.3%)     |         | 3(6.7%)                  | 4(6.2%)      |         |
| 30 >30     | 31(91.2%)                 | 72(94.7%)    |         | 42(93.3%)                | 61(93.8%)    |         |
| 6.Cutaneous bleeding |                          |              |         |                          |              |         |
| 6.1.Petichae/purpura | 33(97.1)                 | 72(94.7)     | 1.000   | 43(95.6)                 | 62(95.4)     | 1.000   |
| 6.1.Ecchymosis      | 13(38.2)                 | 31(40.8)     | 0.801   | 17(37.8)                 | 27(41.5)     | 0.692   |
| 6.2.Non-cutaneous bleeding |                  |              |         |                          |              |         |
| 6.2.1.Nasal bleeding | 18(52.9)                 | 30(39.5)     | 0.188   | 25(55.6)                 | 23(35.4)     | 0.036   |
| 6.2.2.Vaginal bleeding | 12(35.3)                | 24(31.6)     | 0.701   | 19(42.2)                 | 17(26.2)     | 0.077   |
| 6.2.3.Gum Bleeding  | 19(55.9)                 | 25(32.9)     | 0.023*  | 25(55.6)                 | 19(29.2)     | 0.006*  |
PLT at basal: means the platelet count of patients at presentation and before receiving treatment.

PLT in response: means the platelet count of patients after receiving treatment.

*P value is significant if < 0.05.

Table 2: Allelic and genotypic frequencies of rs2230926 & rs5029939 SNPs regarding sex.

| Case          | Cases | P-value | OR (95% CI) | Control | P-value | OR (95% CI) |
|---------------|-------|---------|-------------|---------|---------|-------------|
|               |       |         |             |         |         |             |
|               | Male  | Female  |             | Male    | Female  |             |
|               | No.   | %       | No.         | %       | No.     | %           |
| rs2230926     |       |         |             |         |         |             |
| genotypes     |       |         |             |         |         |             |
| TT            | 12    | 70.6    | 64          | 68.8    | 14      | 77.8        |
|               |       |         |             |         | 74      | 80.4        |
| GG            | 3     | 17.6    | 24          | 25.8    | 4       | 22.2        |
|               |       |         |             |         | 18      | 19.6        |
| TG            | 2     | 11.8    | 5           | 5.4     | 0       | 0.0         |
|               |       |         |             |         | 0       | 0.0         |
| rs2230926     |       |         |             |         |         |             |
| alleles       |       |         |             |         |         |             |
| T             | 26    | 76.5    | 53          | 28.5    | 28      | 77.8        |
|               |       |         |             |         | 148     | 80.4        |
| G             | 8     | 23.5    | 133         | 71.5    | 8       | 22.2        |
|               |       |         |             |         | 36      | 19.6        |
| rs5029939     |       |         |             |         |         |             |
| genotypes     |       |         |             |         |         |             |
| CC            | 11    | 64.7    | 54          | 58.1    | 14      | 77.8        |
|               |       |         |             |         | 71      | 77.2        |
| GG            | 6     | 35.3    | 34          | 36.6    | 4       | 22.2        |
|               |       |         |             |         | 21      | 22.8        |
| CG            | 0     | 0.0     | 5           | 5.4     | 0       | 0.0         |
| rs5029939     |       |         |             |         |         |             |
| alleles       |       |         |             |         |         |             |
| C             | 22    | 64.7    | 113         | 60.8    | 28      | 77.8        |
|               |       |         |             |         | 142     | 77.2        |
| G             | 12    | 35.3    | 73          | 39.2    | 8       | 22.2        |
|               |       |         |             |         | 42      | 22.8        |

*P value is significant if < 0.05. CI, confidence interval; OR, odds ratio.

Table 3: Comparison according to rs2230926 and rs5029939 regarding follow up among PITP group
| Treatment line                  | rs2230926 Mutant (n= 34) | rs2230926 Wild (n= 76) | P-value | rs5029939 Mutant (n= 45) | rs5029939 Wild (n= 65) | P-value |
|--------------------------------|--------------------------|------------------------|---------|--------------------------|------------------------|---------|
|                                 | No. (%)                  | No. (%)                |         | No. (%)                  | No. (%)                |         |
| 1.1. Corticosteroids            | 29 (85.3)                | 55 (72.4)              | 0.140   | 35 (77.8)                | 49 (75.4)              | 0.771   |
| Steroid dependence             | 31 (91.2)                | 57 (75)                | 0.050*  | 38 (84.4)                | 50 (76.9)              | 0.332   |
| 1.2. Immunosuppressive (azathioprine) | 13 (38.2)            | 19 (25)                | 0.158   | 16 (35.6)                | 16 (24.6)              | 0.214   |
| Immunosuppressive change:      |                          |                        |         |                          |                        |         |
| Added                          | 8 (23.5)                 | 17 (22.4)              |         | 9 (20.0)                 | 16 (24.6)              |         |
| Removed                        | 1 (2.9)                  | 4 (5.3)                | 0.107   | 2 (4.4)                  | 3 (4.6)                | 0.013*  |
| Continue                       | 5 (14.7)                 | 2 (2.6)                |         | 7 (15.6)                 | 0 (0)                  |         |
| Persist absent                 | 20 (58.8)                | 53 (69.7)              |         | 27 (60.0)                | 46 (70.8)              |         |
| 1.3. TRO-RA (Romiplostim)      | 2 (5.9)                  | 2 (2.6)                | 0.586   | 2 (4.4)                  | 2 (3.1)                | 1.000   |
| 1.4. Splenectomy               | 1 (3)                    | 6 (7.8)                | 0.672   | 2 (4.5)                  | 5 (7.6)                | 0.700   |
| 2. Response                    |                          |                        |         |                          |                        |         |
| 2.1. Responsive                | 32 (94.1)                | 74 (97.4)              |         | 42 (93.3)                | 64 (98.5)              |         |
| 2.2. Non responsive            | 2 (5.9)                  | 2 (2.6)                | 0.586   | 3 (6.7)                  | 1 (1.5)                | 0.303   |
| 3. Complete recovery           | 1 (2.9)                  | 18 (23.7)              | 0.008*  | 5 (11.1)                 | 14 (21.5)              | 0.155   |
| 4. Death                       | 0 (0)                    | 1 (1.3)                | 1.000   | 1 (2.2)                  | 0 (0)                  | 0.409   |

*P value is significant if < 0.05. CI, confidence interval; OR, odds ratio

Table 4: Comparison between PITP and control regarding genetic findings
### Table 5. Linkage disequilibrium between rs5029939 and rs2230926 genes among study groups

| Genotypes                  | No. (%) | OR (95% CI) | P-value |
|----------------------------|---------|-------------|---------|
| **Cases (n= 110)**         |         |             |         |
| **No.** | **%**  | **No.** | **%**  |         |
| 1.rs2230926 genotypes      |         |             |         |
| TT                         | 76      | 69.1        | 88      | 80.0    | 0.015* |
| GG                         | 7       | 6.4         | 0       | 0.0     | -      |
| TG                         | 27      | 24.5        | 22      | 20.0    |        |
| rs2230926 genotypes        |         |             |         |
| GG + TG                    | 34      | 30.9        | 22      | 20.0    | (0.97-3.32) | 0.063 |
| rs2230926 alleles          |         |             |         |
| T                          | 179     | 81.4        | 198     | 90.0    | 2.06   | 0.010* |
| G                          | 41      | 18.6        | 22      | 10.0    | (1.18-3.60) |      |
| 2.rs5029939 genotypes      |         |             |         |
| CC                         | 65      | 59.1        | 85      | 77.3    |        |
| GG                         | 5       | 4.5         | 0       | 0.0     | -      | 0.004* |
| CG                         | 40      | 36.4        | 25      | 22.7    |        |
| rs5029939 genotypes        |         |             |         |
| GG + CG                    | 45      | 40.9        | 25      | 22.7    | (1.31-4.23) | 0.004* |
| rs5029939 alleles          |         |             |         |
| C                          | 170     | 77.3        | 195     | 88.6    | 2.29   | 0.002* |
| G                          | 50      | 22.7        | 25      | 11.4    | (1.36-3.87) |      |

*P value is significant if < 0.05. CI, confidence interval; OR, odds ratio
| rs5029939 | p | Degree of the theoretical maximum |
|-----------|---|----------------------------------|
|           |   | C       | G       |
| Case      |   |         |         |
| rs2230926 | T | 172 (78.2%) | 11 (5.0%) |
|           | G | 1 (0.5%)   | 36 (16.4%) |
| Control   |   |         |         |
| rs2230926 | T | 191 (86.8%) | 4 (1.8%)  |
|           | G | 0 (0.0%)   | 25 (11.4%) |

* P value is significant if ≤ 0.05. #Chi square test. &Fisher's Exact test.

**Figures**

**Figure 1**

Comparison between PITP and control regarding rs2230926 gene.
Figure 2

Comparison between PITP and control groups regarding rs5029939.