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Impact of TNFAIP3 Genetic Polymorphisms on Primary Immune Thrombocytopenia in Egyptian Adults: Case-control Study

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Abstract

BACKGROUND: Immune Thrombocytopenia (ITP) is a common acquired hematological disease. Genetic polymorphisms play an important role in ITP pathogenesis and prognosis. TNF-α-induced protein 3 (TNFAIP3) is a negative regulator of NF-kB in many signaling pathways. Several variants of TNFAIP3 have been associated with various inflammatory autoimmune disorders.

AIM: Our study aimed to study the association of TNFAIP3 single nucleotide polymorphisms (SNPs); rs2230926 & rs5029939 with ITP susceptibility, as well ITP prognosis by follow up the cases for 18 months.

METHODS: One hundred and ten ITP patients as well 110 matched unrelated normal controls were enrolled in our study. The polymorphisms were assessed by real-time polymerase chain reaction (real time PCR).

RESULTS: There were a significant difference between cases and control groups regarding rs2230926 T>G and rs5029939 C>G frequencies with $p < 0.05$. Linkage disequilibrium (LD) analysis of the two variants revealed that there was a significant LD ($p < 0.001$). Non-cutaneous bleeding manifestations were observed mainly in the mutant genotypes of rs2230926 and rs5029939. The ITP patients with mutant genotypes of rs5029939 showed more need to use 2nd line immunosuppressive therapy as well the mutant genotypes of rs2230926 showed more steroid dependence and less complete recovery.

CONCLUSION: Our data concluded the presence of LD between rs5029939 and rs2230926. The mutant genotypes of both variants were associated with increase the susceptibility to ITP and accompanied by worse clinical manifestations and poor response to the treatment in the adult Egyptian patients.

Introduction

Immune thrombocytopenia (ITP) is a prevalent acquired disorder, known by platelet count <100 × 10^9/L. It results from increase immune platelet destruction and/or reduces its production [1]. ITP may be primary with unknown underlying cause or secondary to autoimmune or infectious disease [2], [3]. Primary ITP affects 2–4/100 000 adults per year with a prevalence of 9.5/100,000 adults. Primary ITP accounts for 80% of the diagnosed ITP cases with female predominance [3], [4]. Moreover, in adults, it is often assumes a chronic course that requires persistent monitoring and treatment [5]. The pathogenesis of primary ITP is greatly enhanced, with both genetic and environmental factors are involved its evolution [6]. TNF-α-induced protein 3 (TNFAIP3, also known as A20) exists on the forward strand of six chromosomes (6q23.3), in between the OLIG3 (oligodendrocyte transcription factor 3) gene and PERP (P53 Apoptosis Effector Related To PMP22) gene [7].

TNFAIP3 (A20) is a ubiquitin-editing enzyme, known to be a down regulator of NF-kB in many signaling pathways [8]. A20 is involved in the activation, proliferation, and normal differentiation of specific subsets of B cells. A20 also is a negative regulator of the enhanced immune functions of the dendritic cells (DCs) [9].

A20 expression defect is linked to immune hemostasis disturbance with inflammatory enhancement. Various genetic variants of A20 were found to be related to autoimmune diseases, as multiple sclerosis, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), psoriasis, and Type 1 diabetes. This comes in advance to suggest that TNFAIP3 may have a role in ITP [10].

The coding single nucleotide polymorphism (SNP), *rs2230926 T>G*, leads to change phenylalanine-to-cysteine at 127 residues of the A20 protein with decrease its ability to suppress the TNF enhanced NF-κB activity [11]. This provokes many inflammatory and immunological disorders including RA, SLE [12].

The *rs5029939 C>G* genetic variant in intron 2 of TNFAIP3 gene, considered as functional variant by reducing TNFAIP3 mRNA expression with decrease

A20 protein level [13]. The reduced expression of TNFAIP3 is related to SLE risk and negatively affects its prognosis [14].

Our hypothesis was to explore the role of TNFAIP3 SNPs in ITP risk, presentation features as well therapeutic response. This can identify if *rs2230926 T>G* and *rs5029939 C>G* can be used as biomarkers for ITP risk and targets for therapy.

Materials and Methods

Our case–control study involved 110 adult Egyptian primary ITP patients. They were elected from cases diagnosed at Hematology Outpatient Clinic, Faculty of Medicine, Cairo University during the period from November 2018 to September 2019 with 18 months follow-up. One hundred and ten age, gender, and race-matched healthy controls were enrolled as a control group. Control group was selected among normal people coming for routine check-up with normal examinations and investigations.

All ITP patients were picked up according to the American Society of Hematology guidelines of ITP diagnosis [15]. They were subjected to full history taking (especially bleeding, drug intake, family history as well as compliance to therapy), clinical examination (especially for skin, organomegaly, and lymph nodes), and laboratory investigations which involved complete blood count and film, reticulocyte count and erythrocyte sedimentation rate. Patients with secondary ITP due to viral infections, drug-induced, *Helicobacter pylori*, autoimmune diseases as SLE or known to have thyroid disease were excluded from the study.

ITP Treatment was started with platelet count $<30 \times 10^9$/L or in the existence of bleeding manifestations. Prednisolone as 1st line therapy at dose of 1 mg/kg/day was administered for 2–3 weeks followed by gradually tapering. Second-line therapy started in absence response to steroid for 3 months. Different types of 2nd line therapy included splenectomy, immunosuppressive drugs; azathioprine, and thrombopoietin receptor agonists; *romiplostim.*

Response to therapy can be either by complete response as platelet count more than or equal to 100×10^9/L and bleeding absence, response defined by platelet count $\geq 30 \times 10^9$/L and at least 2-fold rise of the baseline count or no response by platelet count $<30 \times 10^9$/L or <2-fold increase of baseline platelet count or bleeding [16].

The present study was approved by the Ethical Committee of Biotechnology and Life Sciences department, Faculty of Postgraduate Studies, Beni-Suef University. It was carried on according to the Declaration of Helsinki involving the ethical principles of medical human research. A written informed consent was obtained from each participant.

Genomic DNA extraction

Two ml of peripheral venous blood was collected on a sterile vacutainer tube with anticoagulant of 5% ethylene diamine tetra-acetic acid. Samples were retained at −20°C till DNA extraction. Genomic DNA extraction was done in consonance with the producer's protocol using DNA Purification Mini Kit (GeneJET; Cat. No. K0781).

Genotyping of TNFAIP3 polymorphisms

TaqMan ready-made SNP assay was utilized (Thermo Fisher; Cat. No. 4351379). The context Sequence [VIC/FAM] of *rs2230926* was GACTTGGTACTGAGGAAGGCGCTGT[G/T]CAGCACGCTCAAGGAAACAGACACA. The Context Sequence [VIC/FAM] of *rs5029939* was GTCACCTAAACTAGTTAGGAGCAGA[C/G]TTAAGCTAGAACCAAGGTCCCCTGG.

Genotyping TaqMan® Master Mix was used for DNA amplification (Thermo Fisher; Cat. No. 4371353). Polymerase chain reaction (PCR) mixture of 20 μL volume was done as 3 μL extracted DNA, 0.5 μL SNP assay, 10 μL Master Mix, and 6.5 μL distilled water.

Real-time PCR equipment (Applied Biosystems 7500) was utilized. Amplification started by holding for 30 s at 95°C followed by 40 cycles as follow: Denaturation at 95°C for 30 s and annealing/extension at 60°C for 1 min.

Statistical analysis

The data were analyzed using statistics software (IBM SPSS; version 18). Independent t-test was done for quantitative data. Chi-square and Fisher's Exact test tests were used for qualitative parameters. Odds ratio (OR) and 95% confidence interval (CI) were also assessed. Linkage disequilibrium (LD) was mathematically calculated [17]. $p \leq 0.05$ was regarded as significant.

Results

Our ITP cases were matched with control group regarding age, sex, and race (Table 1).

Duration of ITP disease at sampling ranged from 1 to 180 months with a median of 8.5 months. ITP cases were selected at different phases of the disease;

Table 1: Demographic, clinical criteria, and treatment modalities

Characteristics	Cases (n. 110)	Controls (n.110)	p-value*
Age (years)			
Mean±SD	33.79±14.41	30.75±11.64	0.086
Range	18–50	20–55	
Gender			
Male n (%)	17 (15.5)	18 (16.4)	0.854
Female n (%)	93 (84.5)	92 (83.6)	
ITP phases at sampling			
Newly diagnosed	35 (31.8)		
Persistant	33 (30)		
Chronic	42 (38.2)		
Clinical Characteristics at diagnosis			
Cutaneous bleeding			
Petechia/purpura	105 (95.5)		
Ecchymosis	44 (40)		
Non-cutaneous Bleeding			
Nasal bleeding	48 (43.6)		
Vaginal bleeding	36 (32.7)		
Gum bleeding	44 (40)		
Treatment modalities at follow up			
Corticosteroids	84 (76.4)		
Immunosuppressive(azathioprine)	32 (29.1)		
Splenectomy	8 (7.3)		
TRO-RA (Romiplostim)	4 (3.6)		
Response at follow up			
Complete response (≥100 × 10^6/ul)	19 (17.3)		
Responsive (≥30 × 10^6/ul)	106 (96.4)		
Non-responsive(<30 × 10^6/ul)	4 (3.6)		
Steroid dependence	88 (80)		
Death	1 (0.9)		

*p value is significant if ≤ 0.05. TRO-RA: Thrombopoitin receptor agonist.

newly diagnosed, persistent, and chronic but all become chronic on follow-up (Table 1).

Clinical manifestations of ITP cases were in the form of cutaneous and noncutaneous bleeding symptoms as shown in Table 1. The median platelet count at the time of diagnosis was 15 × 10^9/L with a range 1.0–29.0 × 10^9/L while the median platelet count at follow-up was 118 with a range of 11.0–400. At sampling, corticosteroid as 1st line therapy was utilized in all our cases. Twelve (10.9%) of patients received second-line therapy (Immunosuppressive; azathioprine) in combination with corticosteroids while splenectomy was done for four patients (3.6%). At follow-up of the patients, the treatment lines in use and patient response to therapy were assessed (Table 1).

Genetic findings among the studied groups

A statistical significance difference was detected in the allelic and genotyping frequencies between cases and controls regarding both variants.

Table 2: Comparison between cases and controls regarding genetic findings

Characteristics	Cases (n = 110)		Controls (n = 110)		OR (95% CI)	p-value*
	n	%	n	%		
rs2230926 T>G					--	*Referance at TT*
Genotypes						0.015*
TT	76	69.1	88	80.0		
GG	7	6.4	0	0.0		
TG	27	24.5	22	20.0		
GG + TG	34	30.9	22	20.0	1.79 (0.97-3.32)	0.063
Alleles					2.06 (1.18-3.60)	0.010*
T	179	81.4	198	90.0		
G	41	18.6	22	10.0		
rs5029939 C>G					--	*Referance at CC*
Genotypes						0.004*
CC	65	59.1	85	77.3		
GG	5	4.5	0	0.0		
CG	40	36.4	25	22.7		
GG + CG	45	40.9	25	22.7	2.35 (1.31-4.23)	0.004*
Alleles					2.29 (1.36-3.87)	0.002*
C	170	77.3	195	88.6		
G	50	22.7	25	11.4		

*p value is significant if < 0.05. CI: Confidence interval; OR: Odds ratio.

The mutant genotypes of both variants carried a risk to ITP disease (Table 2). LD analysis revealed a significant LD between *rs5029939* and *rs2230926* in ITP cases (D' = 0.966 & r^2 = 0.694).

Gender stratification for rs2230926 and rs5029939

By gender-matched stratification, *no significance difference was found between males and females regarding the genotypic and allelic frequencies for both polymorphisms* (Table 3).

Relations of rs2230926 and rs5029939 with Clinical characteristics

The mutant genotypes of both variants were associated with a significant non-cutaneous bleeding manifestations especially bleeding gum. No a significance difference between mutant and wild genotypes of both variants regarding age at presentation, duration and phases of disease (Table 4).

Relations of rs2230926 and rs5029939 with treatment lines and response

On follow-up the patients; the steroid dependence was observed in *rs2230926* mutant

Table 3: Gender stratification for *rs2230926* and *rs5029939*

Characteristics	Cases				p-value*	OR (95% CI)	Controls				p-value*	OR (95% CI)
	Male (n=17)		Female(n=93)				Male (n=18)		Female(n=92)			
	n	%	n	%			n	%	n	%		
rs2230926 T>G												
Genotypes					0.517	--					0.755	--
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		
Alleles					0.552	1.295 (0.551-3.042)					0.715	0.85 (0.36-2.02)
T	26	76.5	133	71.5			28	77.8	148	80.4		
G	8	23.5	53	28.5			8	22.2	36	19.6		
rs5029939 C>G												
Genotypes					0.598	--					1.000	--
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	0	0.0		
Alleles					0.663	1.18 (0.55-2.54)					0.937	1.04 (0.44-2.44)
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 4: Genotypic frequencies of *rs2230926* and *rs5029939* regarding clinical characteristics

Characteristics	*rs2230926 T>G*			*rs5029939 C>G*		
	Mutant (n = 34)	Wild (n = 76)	p-value*	Mutant (n= 45)	Wild (n= 65)	p-value*
	n (%)	n (%)		n (%)	n (%)	
Age at presentation (years)						
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
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
Phase of ITP at sampling						
Newly diagnosed	8 (23.5)	27 (35.5)	0.356	12 (26.7)	23 (35.4)	0.509
Persistent	6 (17.6)	15 (19.7)		8 (17.8)	13 (20)	
Chronic	20 (58.8)	34 (44.7)		25 (55.6)	29 (44.6)	
Clinical characteristics at diagnosis						
Cutaneous bleeding						
Peticheaa/purpura	33 (97.1)	72 (94.7)	1.000	43 (95.6)	62 (95.4)	1.000
Ecchymosis	13 (38.2)	31 (40.8)	0.801	17 (37.8)	27 (41.5)	0.692
Non-cutaneous bleeding	30 (88.2)	54 (71.1)	0.050*	40 (88.9)	44 (67.7)	0.010*
Nasal bleeding	18 (52.9)	30 (39.5)	0.188	25 (55.6)	23 (35.4)	0.036
Vaginal bleeding	12 (35.3)	24 (31.6)	0.701	19 (42.2)	17 (26.2)	0.077
Gum Bleeding	19 (55.9)	25 (32.9)	0.023*	25 (55.6)	19 (29.2)	0.006*

*p value is significant if ≤ 0.05. CI: Confidence interval; OR: Odds ratio.

genotypes, while, the administration of immunosuppressive drugs was noticed in the mutant *rs5029939* genotypes. Complete response was evident in the wild genotype of *rs2230926* with p < 0.05 (Table 5).

Discussion

ITP is greatly associated with genetic variants that affect the immune hemostasis [18]. TNFAIP3 is a zinc-finger cytoplasmic protein, dumping the inflammatory reactions induced by NF-κB activation throughout various ways as nod-like receptor, toll-like receptor, interleukin-1, and TNF ligands [19]. TNFAIP3 genetic defects have been associated with various human disorders that mainly of immune inflammatory nature [20].

Our study confirmed the association of rs2230926 and rs5029939 SNVs of TNFAIP3 with ITP risk and prognosis in Egyptian population. Regarding rs2230926; our results revealed that TT, GG, TG genotypic frequencies showed a statistically significant difference between the cases and controls with p = 0.015. The G allele was found to have a 2.06-fold raised risk of ITP (p = 0.010, OR = 2.06 and 95% CI = 1.18–3.60). For rs5029939; the mutant genotypes (GG + CG) as well the G allele were found to have a higher risk to ITP incidence ([OR = 2.35, 95% CI = 1.31–4.23, p = 0.004] and [OR = 2.29, 95% CI = 1.36–3.87, p = 0.002]), respectively.

In concomitant to our results, Zhou *et al*., 2015 studied 222 ITP Chinese patients and 153 healthy controls for TNFAIP3 SNPs (*rs2230926* and *rs5029939*). They detected the polymorphisms by PCR–restriction fragment length polymorphism with subsequent confirmation of more than 10% of results by direct sequencing [10].

Regarding rs2230926, Zhou *et al*., 2015 reported that the frequencies of TT, TG, GG genotypes were 76.7%, 23.3% and 0%, respectively, in the case group versus 90.2%, 9.8% and 0% independently in controls (OR = 2.79, 95% CI = 1.51–5.18 and p < 0.05). As well G allele in ITP cases was of higher frequency 11.6% than in controls 4.9% (OR = 2.56, 95% CI = 1.41–4.64 and p < 0.05). Regarding rs5029939, they stated that the frequencies of CC, CG and GG genotypes in cases were 69.9%, 30.1% and 0, respectively, while were 92.8%, 7.2% and 0% in controls (OR = 5.57, 95% CI = 2.83–10.97 and p < 0.05). Furthermore, they found that G allele was of a higher risk to ITP (OR = 4.76, 95% CI = 2.47–9.17 and p < 0.05) [10].

TNFAIP3 is an essential negative regulator of inflammatory response induced by NF-κB [10]. The immune reactions induced *in-vivo* in mice in presence of A20 disturbance lead to profound activation of NF-κB with subsequent inflammatory response of multiple organs. As well, Kool *et al*. reported the association of A20 to checkpoints proteins that control DCs activation

Table 5: *rs2230926* and *rs5029939* genotypic frequencies and treatment lines and therapy response

Characteristics	*rs2230926 T>G*			*rs5029939 C>G*		
	Mutant (n = 34)	Wild (n = 76)	p-value*	Mutant (n = 45)	Wild (n = 65)	p-value*
	n (%)	n (%)		n (%)	n (%)	
1st line therapy at follow-up						
Corticosteroids	29 (85.3)	55 (72.4)	0.140	35 (77.8)	49 (75.4)	0.771
2nd line therapy at follow-up						
Immunosuppressive added at follow-up	8 (23.5)	17(22.4)	0.107	9 (20.0)	16 (24.6)	0.013*
TRO-RA (Romiplostim)	2 (5.9)	2 (2.6)	0.586	2 (4.4)	2 (3.1)	1.000
Splenectomy	1 (3)	6 (7.8)	0.672	2 (4.5)	5 (7.6)	0.700
Response to therapy on follow-up						
Complete response (≥100 × 10^6/ul)	1 (2.9)	18 (23.7)	0.008 *	5 (11.1)	14 (21.5)	0.155
Responsive (≥30 × 10^6/ul)	32 (94.1)	74 (97.4)	0.586	42(93.3)	64(98.5)	
Non responsive(<30 × 10^6/ul)	2 (5.9)	2 (2.6)		3 (6.7)	1 (1.5)	0.303
Steroid dependence	31 (91.2)	57 (75)	0.050*	38 (84.4)	50 (76.9)	0.332
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.

and apoptosis. TNFAIP3 disruption of DCs regulation makes them highly sensitive to pro-survival signals of RANKL and CD40L with the overstimulation of anti-apoptotic proteins; Bcl-x and Bcl-2. They stated that mice with A20 disruption DCs suffered from ectopic hematopoiesis and SLE [21].

The rs2230926 T>G is located in exon 3, with transversion substitution leads to amino acid change at residue 127. This substitution dampens the anti-inflammatory activities of A20, increasing the susceptibility and activity of various immunological disorders, for example, SLE, RA, and Sjögren's syndrome [18]. The rs5029939 C>G is intronic transversion substitution that has been related to immune disturbance and systemic sclerosis susceptibility and prognosis [22].

Our findings demonstrated a high LD between the both variants (D' = 0.966 and r^2 = 0.694). This is matching with Bates *et al*., 2009 meta-analysis for rs2230926 and rs5029939 in Caucasian population [13]. They demonstrated a strong LD between both variants (r^2 = 0.99). As well Zhou *et al*., 2015 found a moderate LD between both variants in Chinese ethnic (D' = 0.359 and r2 = 0.108) [10].

In the gender-matched analysis, we observed no significant difference between males and females for both polymorphisms < 0.001). No a statistical significance difference between males and females for rs5029939 genotypic and allelic frequencies was detected. In opposite to our findings, Zhou *et al*., 2015 showed a statistical significance difference for rs5029939 distribution between males and females that did not found regarding rs2230926 distribution. This difference can be related to ethnic variability regarding genetic distribution [10].

The present study documented the association of the non-cutaneous bleeding symptoms mainly of bleeding gum character in the mutant genotypes of *rs2230926* with p = 0.05 and 0.023, respectively, as well in the mutant genotypes of *rs5029939* with p=0.01 and 0.006 independently. Our results demonstrated no a significance difference for genotypic frequencies of both variants regarding the age at onset of disease as well for the duration and phases of ITP. Also, no difference for both variants was found regarding the cutaneous bleeding symptoms.

Our results demonstrated the linkage of the mutant genotypes of *rs2230926* with steroid dependence and lack of complete response with p = 0.05 and 0.008, respectively. As well the cases with mutant genotypes of *rs5029939* were in need to 2nd line therapy in form of immunosuppressive drugs (azathioprine) to control the disease (p = 0.013). We are the first to demonstrate these relations in ITP regarding *rs2230926*.

In contrast to our finding, El-hady *et al*. 2021 examined *rs5029939* in 40 pediatric ITP patients in comparison to 50 normal controls. They revealed no association of *rs5029939* genotypic and allelic frequencies to risk of ITP as well to demographic data, clinical findings, treatment modalities, and therapeutic response with p > 0.05. Their findings were not matching to our results may be referred to the difference in sample size and the type of their studied subjects [23].

Our study provides a proper insight to the effect of TNFAIP3 SNPs in ITP risk and prognosis. Further studies on a large scale are recommended to establish them as predictors and therapeutic targets in ITP.

Defect financing was a limiting factor to our study to confirm our results by another technique. Presence of confirmation in other research of different ethnic was our base to establish our findings.

Conclusion

Our study concludes the pivotal role of TNFAIP3 rs5029939 and rs2230926 polymorphisms in ITP risk with their high LD in the Egyptian population. The patients of the mutant genotypes of both variants are more liable to non-cutaneous bleeding manifestations and poor therapeutic response.

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