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ORGINAL ARTICLE

The Frequency of Tumor Necrosis Factor Alpha Gene Polymorphism in Patients with Chronic Immune Thrombocytopenia.

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ABSTRACT

Background: The aim of this study was to reveal the frequency of Necrosis Factor _Alpha Gene Polymorphism in evaluation of severity and outcome of chronic immune thrombocytopenia in pediatric department of Zagazig University hospitals.

Methods: We conduct a retrospective case control on 40 children diagnosed with chronic ITP and 40 age and sex healthy matched subjects as a control recruited from outpatient hematology clinic Faculty of Medicine, Zagazig University.

Results: We found a highly significant difference between ITP group and control group as regarding TNF-α alleles where A allele was expressed in 43.7% of cases versus 17.5% 0f control, P value<0.001.Our results showed that, the frequency of TNF-α gene polymorphism genotypes 308G<A (rs1800629) were more expressed in chronic ITP cases versus control group and there was a highly significant difference, P<0.001.We did not find statistically significant differences between patients out come and each of different geno-types of TNF-α, P value =0.128.

Conclusion: The present study, suggests that possible significant association of TNF-α (rs1800629) A allele and 308G<A gene polymorphism in chronic ITP patients might play a role in disease susptability, genetic risk factor, pathogenesis, and development of chronicity.

Key words: Tumor Necrosis Factor-Alpha, immune thrombocytopenia, PCR-ARMS.

INTRODUCTION

Immune thrombocytopenic purpura (ITP) is an autoimmune disease in which antiplatelet antibodies accelerate the destruction of platelets. In addition, platelet production can be impaired because the antiplatelet antibodies can also damage megakaryocytes **[1].**

Immune thrombocytopenic purpura is characterized by transient or persistent decrease of the platelet count to less than 100×10^9/liter. The term 'newly diagnosed ITP' is used to describe all cases at diagnosis. Persistent ITP is defined as ITP lasting between 3 and 12 months from diagnosis while chronic ITP is defined as the presence of ITP for more than 12 months **[2].**

The exact mechanism of this disease is still unknown, and several factors are incriminated in the pathogenesis; although auto reactive B cells producing auto antibodies against self-antigens are considered to play a crucial role **[1].**

Also, there is growing evidence demonstrating that dysfunctional T_lymphocyte mediated immune response is also a key factor in pathogenesis of ITP **[3].**

Moreover, genetic changes can be a potential factor in the development of primary ITP **[4].**

Single nucleotide polymorphisms are among the genetic factors causing a series of changes in the human genome and leading to the development of autoimmune disease e.g immune thrombocytopenic purpura, Some of these polymorphisms may exacerbate the disease or affect therapeutic response while others do not have any effect on the disease process **[4].**

Tumor necrosis factor (TNF)-a (6p21.3) is an inflammatory cytokine that increases the phagocytic activity of macrophages. Several polymorphisms have been reported in the promoter region of this cytokine, which can cause diseases such as ITP **[5].**

Rs1800629 (TNF-a _308G>A) is a polymorphism leading to the increased expression of this cytokine. [6]

On the other hand, the expression of TNF-a has been found to be increased on T-cytotoxic

(TCD8) cells of ITP patients, which leads to the secretion of perforin, granzyme, and activation of apoptotic pathways, all of which cause the destruction of platelets **[3].** Identifying and targeting apoptotic pathways and TNF-a receptors can be an effective therapeutic approach to prevent the destruction of platelets and increase the platelet count in ITP patients.

METHODS

A retrospective case control study was carried out at the outpatient hematology clinic, of Pediatric department and Medical Biochemistry &Molecular Biology department, Zagazig University Hospital during a period from January 2018 until January 2019, Population of the study: Children with chronic ITP who are followed up at outpatient hematology clinic of hematology unit at pediatric department, Zagazig University Hospital.

Written informed consent was obtained from all participants parents; the study was approved by the research ethical committee of faculty of medicine, Zagazig University. The study was done according to the code of ethics of the world medical association (declaration of Helsinki) for studies involving humans.

Inclusion criteria: which approval to share in the study: both sex, Age >I year – 18 year, Chronic ITP (disease duration more than 1 year from the start).

Exclusion Criteria: which refusal to share in the study: Age below 1year, newly diagnosed ITP < (disease duration less than 3 months since diagnosis), Persistent ITP disease duration from 3 to 12 month since diagnosis), Secondary ITP.

Assuming that frequency of TNF-α gene polymorphism in chronic ITP patients and in controlled group is 35%,so,sample size is calculated by OPEN-EPI program to be 80 children (40 in each group) with confidence level 95% and power of test 80%.

Group (1): Included 40 patients with chronic ITP (24 males and 16 females).

Group (2): Included 40 healthy children (16males and 24 females) who served as a control group (age and sex-matched).

All children were subjected to a detailed history with emphasis on disease duration, history of bleeding (skin, mucus membrane, frank bleeding).

While laboratory investigation including: complete blood count with manual platelet count, Laboratory investigations to exclude secondary causes (C3, ANA, antiDNase), bone marrow examination. But there are specific investigations such as:

TNF-α_308G<A (rs1800629) gene polymorphism by amplification-refractory mutation system-polymerase chain reaction (ARMS-PCR) method.

Criteria of response to therapy were defined as follow: complete response (CR) was defined as any platelet counts of 100 × 109/L or more; partial response (PR) was defined as any platelet count between 30 and 100 ×109/L or doubling of the baseline count; total response was referred to both CR and PR; and no response (NR) was defined as any platelet count lower than 30 ×109 /L or less than doubling of the baseline count.

Blood sampling: 2 ml of peripheral venous blood, were taken from each participant under complete aseptic condition and were collected in K ethylenediamine tetra acetic acid (EDTA K2) and stored at -20 °C for genomic DNA extraction.

For all participants, genomic DNA from a peripheral blood was extracted and purified using the kit provided by Quick-gDNATM MiniPrepkit (Catalog No.D3025). Typing of the TNF-α −308 GNA (rs1800629) SNP was performed using the polymerase chain reaction with amplification refractory mutation system (PCR-ARMS) as described by (Pravica et al., 2000). The PCR amplification was carried out using recombinant Taq polymerase master mix (Dream taq tm green pe,code number k1081,LOT:00643300) (thermo fisher scientific Ballics UAB ,V A Cracino 8 ,LT-002241 Vilnius,Lithuania) in a 20 µl total volume containing about 100 ng of genomic DNA template, 2 pM of each primer, 2 mM of each dNTP, 1.5 mM of MgCl2, 1× PCR buffer and 0.6 U of Taq DNA polymerase. TNF-α Primer sequences were as follows: TNF-α G forward primer: 5′-ATA GGT TTT GAG GGG CAT GG3′; TNF-α A forward primer: 5′-AATA GGT TTT GAG GGG CAT GA-3′; and reverse primer, 5′-TCT CGG TTT CTT CTC CAT CG-3′. Reaction conditions were carried out in a thermocycler at 95 °C for 1 min followed by 10 cycles of 95 °C for 15 s, 65 °C for 50 s, and 72 °C for 40 s and then 20 cycles of 95 °C for 20 s, 56 °C for 50 s, and 72 °C for 50 s. The amplified PCR products were analyzed by 2% agarose gel and ethidium bromide staining followed by ultraviolet visualization. The PCR product for TNF-α −308 was detected at 184 bp.

Statistical analysis

All data were collected, tabulated and statistically analyzed using SPSS version 19.Continuous Quantitative variables was expressed as the mean SD& median (range), and categorical qualitative variables were expressed as absolute frequencies (number) & relative frequencies (percentage). Mann-Whitney test was used to compare between two groups of not-normally distributed data. Independent student's t test was used to compare between two groups of normally distributed data.

Categorical data were compared using chi square and single Chi-square goodness of fit tests. Odds ratio was used for risk quantification. The tests were two sided with p-value < 0.05 was considered statistically significant (S), p-value < 0.001 was considered highly statistically significant (HS), and p-value ≥ 0.05 was considered statistically insignificant (NS).The statistical analysis was based on the intention-to-treat population. According to age and sex, that there was significant difference between the studied groups as regarding age and consanguinity, however, the difference between them was non-significant as regarding sex.

RESULTS

We found a highly significant difference between ITP group and control group as regarding TNF-α alleles where A allele was expressed in 43.7% of cases versus 17.5% 0f control, P value<0.001, (table1). Our results showed that, the frequency of TNF-α gene polymorphism genotypes 308G<A (rs1800629) were more expressed in chronic ITP cases versus control group and there was a highly significant difference, P<0.001, (table2).We did not find statistically significant differences between patients out come and each of different geno-types of TNF-α ,P value =0.128, (table3).

Table (1): Comparison of demographic data among the studied groups:

Variable	ITP group (n=40)		Control group (n=40)		Test	P value
Age: (years) Mean ± SD Median Range	7.98 ± 4.69 6 3 – 18		5.08 ± 3.28 3.75 2 – 12		**-3.299#**	**0.001 (S)**
	No	**%**	**No**	**%**	χ^2	**P**
Sex: Female Male	16 24	40 60	24 16	60 40	3.200	0.074 (NS)
Consanguinity: Negative: Positive:	31 9	77.5 22.5	38 2	95 5	**5.165**	**0.02 (S)**

Table (2): Comparison of laboratory data among the studied groups:

Variable	ITP group (n=40)		Control group (n=40)		Test	P value
Initial platelets ×10³/ul : Mean ± SD Median Range	21.98 ± 15.7 17.5 5 – 80		331.4 ± 94.6 316.5 199 - 501		**-7.704#**	**<0.001 (HS)**
Hemoglobin (gm/dl): Mean ± SD Range	10.6 ± 2.07 4.3 – 16		10.3 ± 1.05 9 – 12.2		0.733	0.466 (NS)
	No:	**%**	**No:**	**%**		
Bone marrow examination: Hyper cellular: Norm cellular:	12 28	30 70	---	---	---	---

Table (3): Markers of autoimmunity among the ITP studied group:

Variable	Studied group (n=40)		Test	p-value
	No	%		
C3: Normal: Abnormal:	 40 0	 100 0	0.00	1.00 (NS)
ANA: Positive: Negative:	 2 38	 5 95	**32.4**	**<0.001** **(HS)**
Anti-DS: Positive: Negative:	 2 38	 5 95	**32.4**	**<0.001** **(HS)**
Anti-platelet antibody: Positive: Negative:	 3 37	 7.5 92.5	**28.9**	**<0.001** **(HS)**
IgA: Normal: Increased: Decreased:	 18 19 3	 45 47.5 7.5	**12.05**	**0.02** **(S)**

Table (4): Outcome of the ITP studied group:

Variable	Studied group (n=40)	
	No	%
Outcome: Complete response (platelets >100,000 mcl) Partial response ((30 - 100,000/mcl) No response (<30.000 mcl)	 0 32 8	 0 80 20

Table (5): Frequency distribution of genotype polymorphism among the studied groups:

Variable	ITP group (n=40)		Control group (n=40)		OR (95%CI)	P value
	No	%	No	%		
Allele frequency: A allele: G allele:	 35 45	 43.7 56.3	 14 66	 17.5 82.5	 **3.66 (1.77-7.58)** **0.27 (0.131-0.563)**	 **<0.001** **(HS)**
TNF-α: AA: AG: GG:	 1 33 6	 2.5 82.5 15	 2 10 28	 5 25 70	 2.33 (0.108-30.1) **15.4 (4.97-47.6)** **Reference**	 0.556 **<0.001**

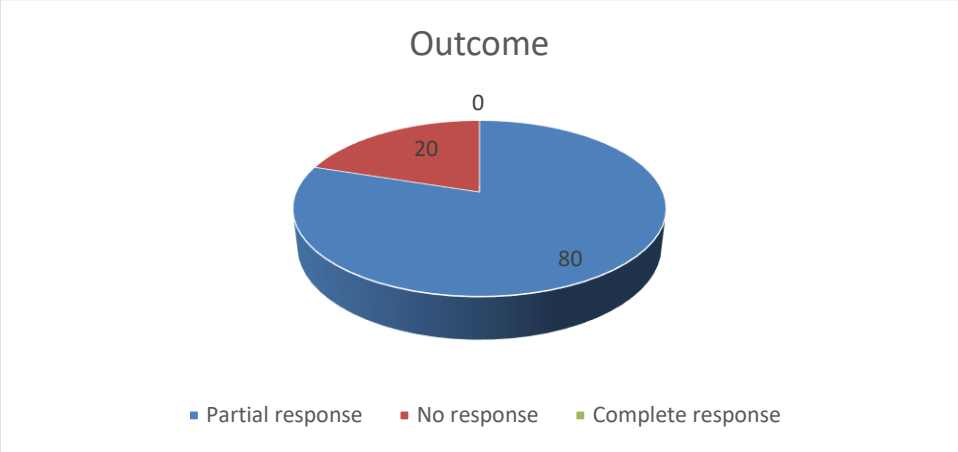

Figure (1): Pie diagram showing the outcome among the studied group

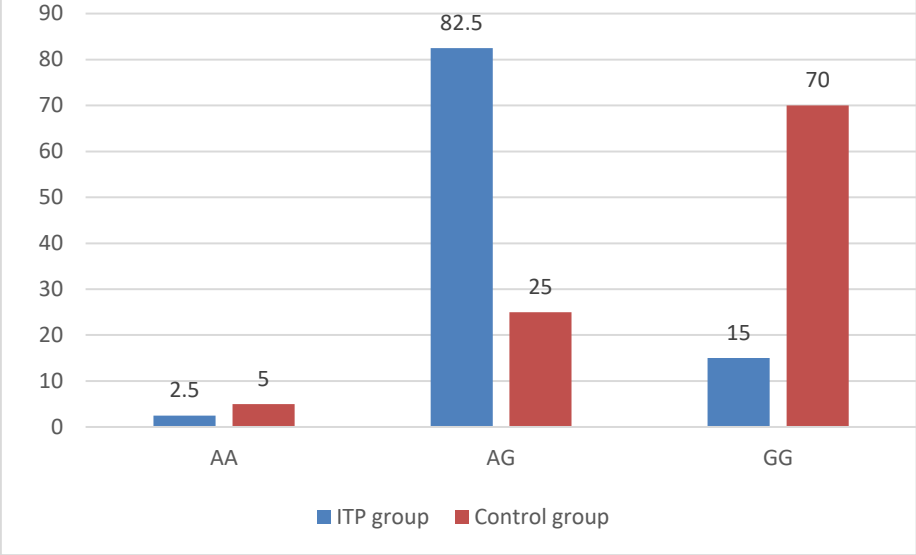

Figure (2): Frequency distribution of TNF-α genotype among the studied groups

DISCUSSION

In this study, our results showed that there was highly significant difference between ITP group and control group as regarding TNF-α alleles. In the contrary; Akar and Hasypek reported that there was not any difference between the patients and controls in the frequency of the TNF-α allele, in Turkish Pediatric patients **[10].**Our results showed that the frequency of TNF-α gene polymorphism genotypes 308G<A (rs1800629) were studied in chronic ITP versus control group and there was a highly significant difference between ITP cases and control (P<0.001). Where, 33 patients (82.5%) express heterozygous A/G genotype,6 patients (15%) express homozygous GG genotype and 1 patient (2.5%) express homozygous AA genotype. while in control group 10 patients (25%) express heterozygous A/G genotype, 28 patients (70%) express homozygous GG genotype and 2 patients(5%) express AA genotype. Similarly, Mokhtar et al. showed that the all patients with ITP were higher frequency of TNF-α GA heterotype, in Egyptian children **[6].** Similar to us, Pehlivan et al, reported it their studies that the high expression of TNF-alpha (308) AG genotype was demonstrated to increase 3.1-fold in ITP cases with no significant different, in Turkish children **[8].** In the contrary, Elsissy et al. noticed that TNF-α AA homotype was significantly higher in ITP cases than in controls, in Egyptian children **[7].** Okulu et al. revealed that there was highly significant difference between ITP patients and control group as regarding TNF-α genotypes, P value<0.001 in Turkish children **[12].** In the contrary, Yadav et al., revealed no significant difference in distribution of TNF-a 308G>A heterozygous genotype (GA) among patients and controls with, P-value 0.638. In TNF-a 308G>A, homozygous variant genotype (AA) was absent both in patients and controls in Indian population **[5]**.So, In this study, we could find association of TNF-α gene polymorphism with susceptibility in developing ITP, P value<0.001. In contrast, several studies have reported that polymorphism in TNF-α 308G>A not contributes to

susceptibility to ITP. This discrepancy might be due to the differences in racial and geographical characteristic of study subjects. In our study we revealed that the AG genotype was highly significant among the ITP patients compared to controls and conferred increased ITP risk (OR=15.4, 95% CI=4.97-47.6).In the contrary, Okulu et al., investigated the role of TNF-α (–308) G/A polymorphisms in the development and clinical progression of childhood ITP and found that the frequency of TNF-α (–308) G/A polymorphism was 22.9% in the chronic ITP patients, P > 0.05 and the risk of developing ITP and clinical progression were not associated with TNF-α (–308) G/A polymorphisms **[12]**. In our study we found that there were highly statically significant difference between ITP patients and controls among (AG) genotypes. Also , Foster et al., observed GA and AA genotypes of TNF-α (–308) polymorphisms in 11% of chronic ITP compared with 29% of controls, suggesting that allele G is over presented in chronic ITP and that genotypes of TNF associated with decreased cytokine expression could protect against chronic ITP.

The study limitations are relatively small sample size due high cost concerning PCR-ARMS technique. It was a single-center study (Zagazig University). Moreover, chronic ITP take more time to be detected and take of our consideration acute ITP.

CONCLUSION

The present study, suggests that possible significant association of TNF-α (rs1800629) A allele and 308G<A gene polymorphism in chronic ITP patients might play a role in disease susptability, genetic risk factor, pathogenesis, and development of chronicity.

- Conflict of Interest: None.
- Financial Disclosures: None.
- Supplementary materials: The following are available online at Table 6, Table 7and Table 8

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