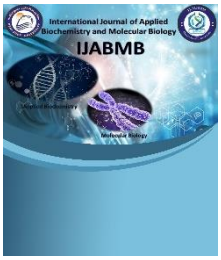
International Journal of Applied Biochemistry and Molecular Biology (IJABMB)



Association of PD-1 and CTLA-4 Gene Variants with Chronic Immune Thrombocytopenia in Pediatric and Adolescent Patients: A Cross-Sectional Study

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Running Title: PD-1 and CTLA-4 Gene Variants in Pediatric ITP

Abstract

Background: Despite the knowledge that complex dysregulation of the immune response is involved in the disease mechanism of chronic immune thrombocytopenia (cITP), pathogenesis remains incompletely understood. The disruption of self-tolerance which leads to the generation of autoreactive T-cells that attack the patient's platelets is one of the proposed underlying mechanisms of cITP. The cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4)/B7 and programmed death 1 (PD-1)/PD-L1 pathways inhibit T cell immunological function at different stages of T-cell activation, influencing immune regulation. Single nucleotide polymorphisms (SNPs) in programmed cell death 1 (PDCD1) and CTLA4 have been associated with susceptibility to specific autoimmune diseases. However, the potential link between them and the risk of chronic immune thrombocytopenia remains a topic of ongoing debate and uncertainty.

Objective: We aimed to explore PDCD1 and CTLA4 gene polymorphisms (PDCD1+ 7209, CTLA4 rs11571315) in cITP to clarify their role in disease pathogenesis and their potential association with progression to chronic disease.

Patients and methods: A retrospective cross-sectional case-control study was conducted, in which 48 cITP patients and 48 matched healthy controls were genotyped for PDCD1+ 7209 and CTLA4 rs11571315 polymorphisms using polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP).

Results: Our study revealed a significant correlation between the heterozygous genotype (C/T), variant T allele of PDCD-1 +7209, and dual variant genotype with reduced platelet counts following therapy (p-value=0.037, 0.049, and 0.018). However, no statistically significant difference was found in genotype distribution for PDCD-1 and CTLA-4 when comparing chronic ITP cases with the control group (p-value=0.627 and 0.251, respectively).

Conclusion: Patients having the variant T allele (C/T genotype of PDCD-1 +7209) and dual genetic variations exhibited lower platelet counts post-therapy when compared to individuals with the C allele (C/C genotype) and those with a single gene mutation, suggesting a suboptimal treatment response. These findings indicate that the T allele variant could potentially increase the incidence of chronic ITP and that focusing on PDCD-1 may pave the way for innovative treatment strategies for patients with chronic ITP. Conversely, the CTLA4 rs11571315 SNP did not appear to influence susceptibility, chronicity, or severity in Egyptian children suffering from chronic ITP.

Keywords

CTLA-4, ITP, PDCD-1, polymorphism.

Introduction:

Primary immune thrombocytopenia is an organ-specific autoimmune disease characterized by a reduced platelet count in peripheral blood (1).

The International Working Group (IWG) for ITP has categorized ITP patients as newly diagnosed ITP (starting from diagnosis to 3 months), persistent ITP patients (with thrombocytopenia lasting from 3 months to 12 months post-diagnosis), and lastly cITP patients (patients with ITP lasting longer than 12 months post-diagnosis) (2).

The exact cause for ITP remains largely unknown; nonetheless, environmental factors such as viral infections, including cytomegalovirus (CMV), Epstein-Barr virus (EBV), Varicella Zoster virus (VZV) (3), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (4), vaccines and some drugs have been suggested as important triggers for this condition. Furthermore, complex deregulation of the immune system plays a role in the causation of this disease (5). Over the past few years, numerous studies have revealed that regulatory T cells (Tregs) play a role in the development of ITP. As previously documented, the numbers of Treg cells in the peripheral blood and bone marrow of ITP patients were notably diminished.

Moreover, the pathophysiology of ITP has been demonstrated to correlate with Treg dysfunction (6, 7). The immune checkpoint pathways serve as essential regulators of the immune system, facilitating the initiation of immune responses while inhibiting the onset of autoimmunity. They represent fundamental mechanisms that govern T-cell-mediated immune responses by encompassing co-stimulation and co-inhibition signal pathways (8).

Research has indicated that T-cell activation is meticulously regulated by signals from co-stimulatory and co-inhibitory molecules (9). These molecules have been utilized as targets for immunotherapy, signifying their

crucial involvement in immune modulation and disease resolution (10).

The cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4)/B7 and programmed death 1 (PD-1)/programmed cell death-ligand 1 (PD-L1) are considered the two principal immune checkpoint pathways involved in the negative modulation of T cell immunological function at various stages of T-cell activation (11). Both CTLA4 and PDCD1 were found to impede T-cell activation by obstructing CD28-mediated upregulation (12).

Single nucleotide polymorphisms are genetic variables that induce alterations in the human genome, contributing to the occurrence of autoimmune diseases, including ITP (13).

In this study, we aimed to explore the potential role of PDCD-1 +7209 SNP and CTLA4 rs11571315 SNP in the pathogenesis of chronic ITP.

Patients and methods:

This retrospective cross-sectional case-control study involved forty-eight children who were diagnosed with chronic immune thrombocytopenic purpura (cITP). All patients fulfilled the diagnostic criteria for primary ITP as established by the ITP International Working Group (IWG), with chronic ITP being defined as ITP persisting for over 12 months (2). Forty-eight unrelated, age- and sex-matched healthy children without a history of ITP or other autoimmune disorders were included as a control group.

All patients with ITP underwent a comprehensive history assessment (focusing on bleeding, medication use, familial history, and adherence to therapy), as well as clinical examination and laboratory evaluations, which encompassed complete blood count (CBC) and blood film examination, reticulocyte count, in addition to erythrocyte sedimentation rate (ESR). Excluded patients were those with secondary ITP triggered by viral infections, as well as patients with drug-induced thrombocytopenia, and autoimmune diseases including systemic lupus erythematosus.

Patients were recruited from the pediatric outpatient clinic in the period between January 2023 to August 2023 and the clinical and laboratory data was retrieved from their medical records. All patients fulfilled the diagnostic criteria of cITP where they had isolated thrombocytopenia with platelet count <100 X109 /L, in the absence of other causes or disorders that may be associated with thrombocytopenia that lasted for more than 12 months according to the ITP-IWG (2).

The study was carried out in alignment with the Declaration of Helsinki and received approval from the Ethical Committee (REC code: MS-336). Voluntary written informed consent has been obtained from all legal guardians of the recruited children.

Sampling and DNA extraction:

Two-milliliter whole blood samples were withdrawn in vacutainer blood collection tubes containing ethylenediaminetetraacetic acid (EDTA) under aseptic conditions utilizing clean venipuncture. The genomic DNA was extracted via the spin column method as per the manufacturer's instructions using the Thermo Scientific DNA purification kits (Thermo-Scientific™ Bio-technology, Seongnam-Si, Korea, catalog number K0781). The extracted DNA samples were preserved at -20°C until utilized.

Genotyping

Genotyping of PDCD1 and CTLA4 gene polymorphisms (PDCD1+ 7209, CTLA4 rs11571315) was determined using PCR followed by RFLP. The primers' sequence used for amplification are shown in Table 1 and were supplied by (Invitrogen, Thermo Scientific, USA).

Table (1): Sequence of the primers used for amplification.

Genetic polymorphism	Primer sequence
PDCD1 + 7209	forward: CAGCAACCTCAATCCCTAAAGC reverse: GAAATCCAGCTCCCCATAGTCC
CTLA4 rs11571315.	forward: TTAAAAAGTGAAAAACAAATGTTCCTG reverse: ACTTTAGCCCATGTTATTCTTCTTGT

A PCR reaction mixture volume of 25 uL containing 5uL of purified genomic DNA, 1 uL of each forward and reverse primers, 12.5 uL Master Mix, and 5.5 uL ddH2O. The cycling conditions of the PCR were done according to previously described methods as follows: For PDCD1: denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 30 sec., then annealing at 51°C for 45 sec. and extension at 72°C for 45 sec., followed by a final extension step at 72°C for 7 min, while the cycling conditions for CTLA4 were as follows: a denaturation step at 95°C for 5 min., followed by 35 cycles of 94°C for 30sec., then annealing at 58°C for 30 sec. and extension at 72°C for 1min., followed by a final extension step at 72°C for 10 min.

The amplicons were digested using the restriction enzymes supplied by Thermofischer, BstUI (Cat. No.: ER0921) for PDCD1 + 7209 and Tas I for CTLA4 rs11571315 (Cat. No.: ER1351). The digested products were subjected to gel electrophoresis in a 2% agarose gel then visualized via staining with ethidium bromide about a molecular weight marker.

For PDCD-1 (+7209) genetic variant:

The homozygous-wild genotype (C/C) produced 2 bands 340 bp and 114 bp, while the homozygous-variant genotype (T/T) produced a single band of 569 bp long (absence of BstUI cutting site).

For CTLA4 (rs 11571315) genetic variant:

The homozygous-wild genotype (T/T) produced 2 bands 149 bp and 104 bp long, the homozygous-variant genotype (C/C) produced a single band 253bp (absence of Tas I cutting-site) and the heterozygous genotype (T/C) produced 3 bands: 253bp,149 bp, and 104 bp.

Statistical methods:

All data were coded and entered utilizing the Statistical Package for the Social Sciences (SPSS) version 28 (IBM Corp., Armonk, NY, USA). For the quantitative variables we used the mean and standard deviation were used to summarize the results, while in the case of qualitative variables, the counts and percentages were used to express the data. The independent t-test was employed to analyze normally distributed quantitative variables, whereas, for variables that did not adhere to a normal distribution, the nonparametric Mann–Whitney U test was used. The chi-square test was employed to analyze qualitative variables. A statistically significant level was determined by P-values below 0.05.

Results

Forty-eight chronic ITP patients were enrolled in this study as well as 48 age- and sex-matched healthy volunteers as a control group. The patient group comprised 26 females and 22 males, with a mean age of 7.3 years (range from 3 to 14). The laboratory data at the presentation was retrieved from patients' records. The principal demographic and clinical data are displayed in Figure 1 and Hematological data are displayed in Table 2.

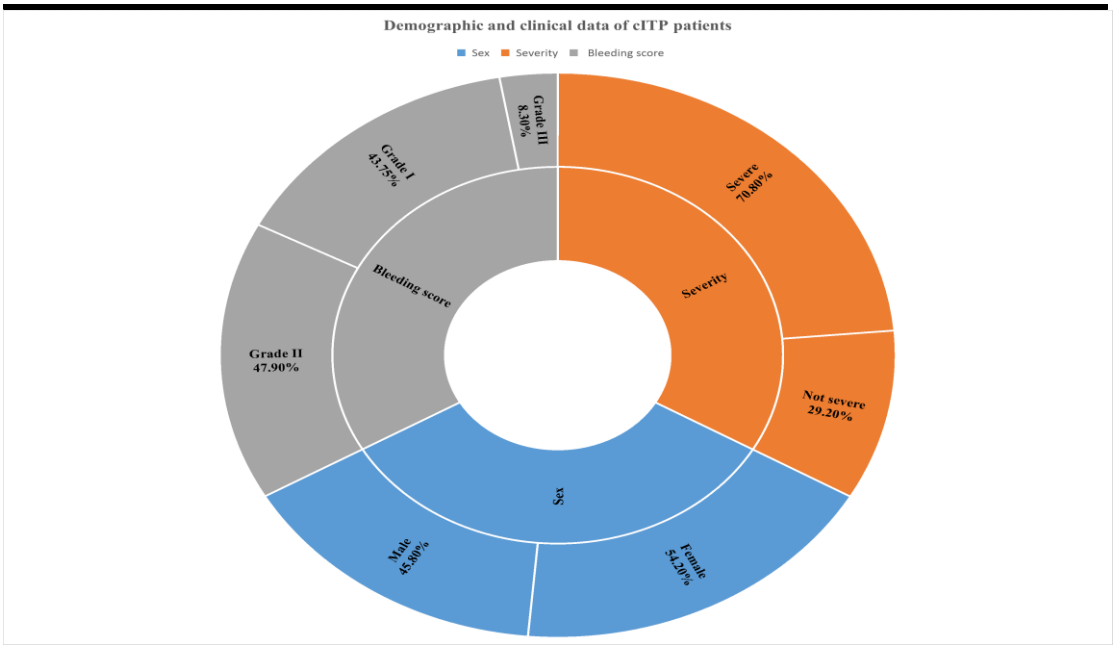

Figure 1. Clinical and demographic data of cITP patients.

Table (2): Hematological data of cITP patients

	Mean ±SD	**Median (range)**
HB (initial)	11.19 ±1.34	11.00 (6.50-13.80)
WBCs (initial)	7.63 ±2.67	7.00 (3.80-14.00)
Platelets (initial)	17.90 ±15.54	12.00 (0.00-64.00)
HB after therapy	11.66 ±1.09	12.00 (8.80-14.50)
WBCs after therapy	7.94 ±3.30	7.00 (4.00-16.00)
Platelets after therapy	99.88 ±29.82	100.00 (37.00-145.00)

Genotyping:

In this study, we examined PDCD-1 and CTLA-4 polymorphisms. None of the patients nor the control group showed a homozygous TT genotype of PDCD-1. The genotyping and allele distribution of PDCD-1 gene polymorphism did not show any statistically significant difference between the cITP cases and the healthy controls (p-value =0.627 and 0.650 respectively), also the genotyping and allele distribution of CTLA4 gene polymorphism did not show any statistically significant difference when comparing the cITP cases with the healthy controls (p-value = 0.251 and 0.341 respectively), Results of genotyping and allele frequency distribution are summarized in Table 3.

Table 3. Comparison of genotype and allele frequency distributions of the PDCD-1 and CTLA-4 polymorphisms between cITP patient group and control group

Frequency		**Controls**		**Cases**		**P value**
		Count	**%**	**Count**	**%**	
PDCD1 Genotypes	**Wild (C/C)**	36	75.0%	38	79.2%	0.627
	Heterozygous (C/T)	12	25.0%	10	20.8%	
PDCD1 alleles	**Wild allele (C)**	84	87.5%	86	89.6%	0.650
	Mutant allele (T)	12	12.5%	10	10.4%	
CTLA4 Genotypes	**Wild (T/T)**	3	63%	8	16.7%	0.251
	Heterozygous (T/C)	19	39.6%	15	31.3%	
	Homozygous (C/C)	26	54.2%	25	52.1%	
CTLA4 alleles	**Wild allele (T)**	25	26%	31	32.3%	0.341

Correlation studies of platelet count post-therapy and genotyping and allele distribution of PDCD-1 gene polymorphism revealed a statistically significant difference in the platelet count after therapy between the homozygous-wild (C/C) and heterozygous (C/T) chronic ITP cases (p-value =0.037 and 0.049 respectively), (Figure 2), while we couldn't find any correlation between post-therapy platelet count and CTLA-4 genetic variants, (Figure 3).

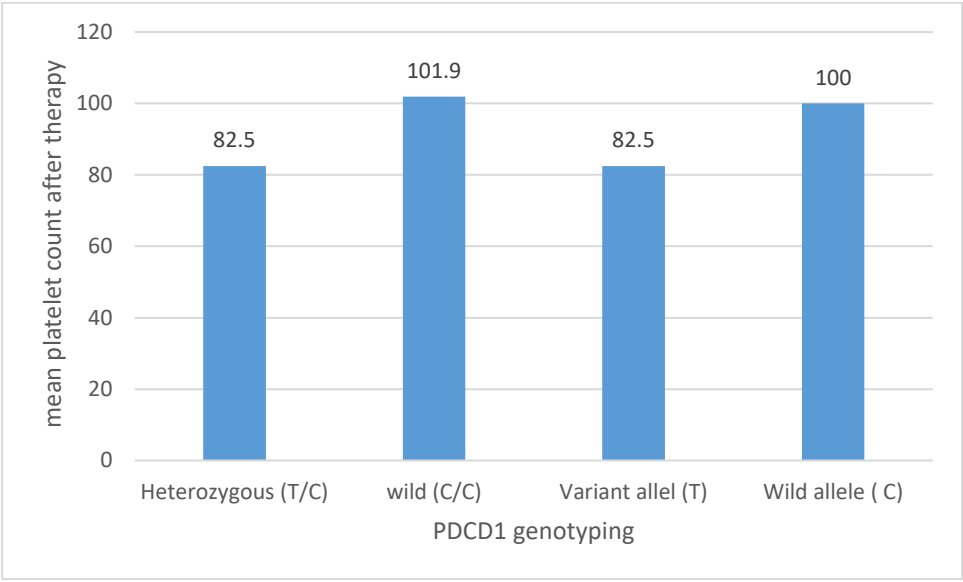

Figure 2: Comparison between PDCD1 genotyping and allele frequency regarding platelet count after therapy

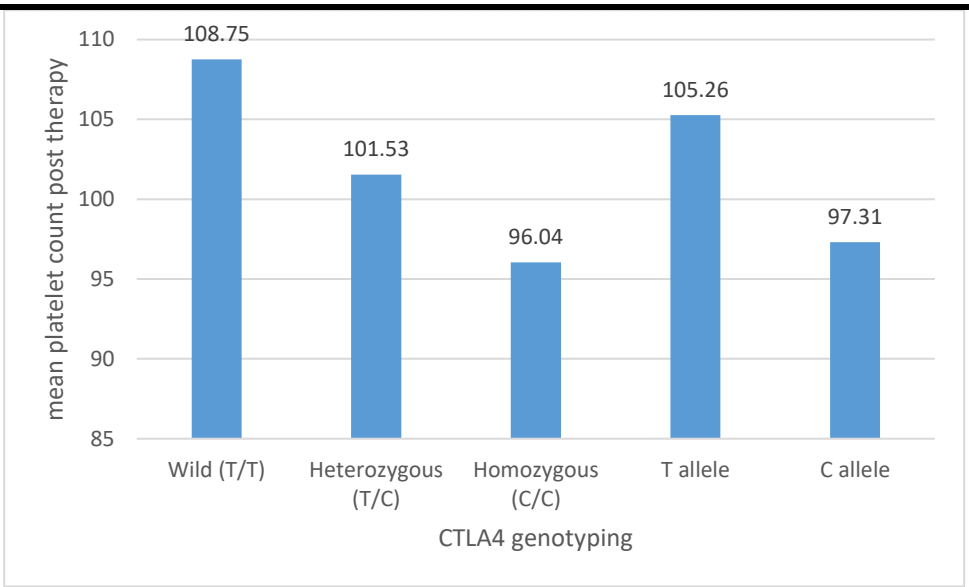

Figure 3: Comparison between CTLA4 genotyping and allele frequency regarding platelet count after therapy

Also, when comparing the dual variant genotypes and single variation regarding post-therapy platelet count, patients with dual variant genotypes were found to have a significantly lower platelet count when compared to those with a single variant genotype (Figure 4). Regarding the results of the genotype frequency of dual variation (PDCD-1 and CTLA4) polymorphism, our results showed that among the ITP patients: 6 cases (12.5%) were of the dual variant type and 38 cases (79.2%) were of the single variation of one gene. In comparison, the control group showed 11 cases (22.9%) who had a dual variant type and 35 cases (72.9%) who were of the single variation of one gene. Correlation studies showed no significant association between either of the studied polymorphisms (PDCD-1 and CTLA4) and platelet count (before treatment), bleeding score, or disease duration in cITP patients. Also, when comparing the dual variant genotypes and single variation regarding clinical variables, no significant differences could be found regarding the platelet count before treatment, disease duration, or bleeding score (Table 4).

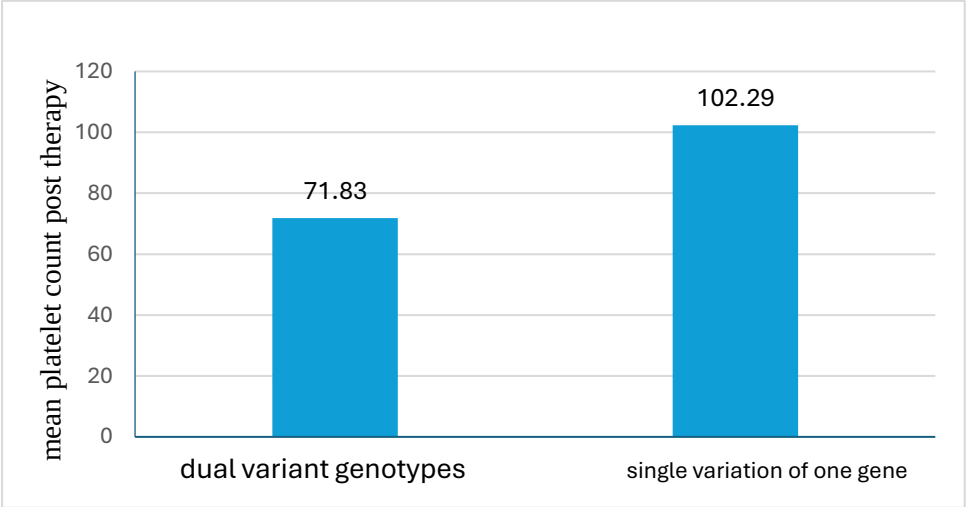

Figure 4: Comparison between dual variant genotype and single variation of one gene regarding platelet count after therapy

Table 4. Comparison between dual variant genotype and single variation of one gene regarding platelet count before and after therapy, disease duration, and bleeding score

	dual variant genotypes	**Single variant**	**P value**
	Median (range)	**Median (range)**	
Platelets (initial)	16.5 (4-45)	11.5 (0-64)	0.652
Disease duration (yrs)	3 (2-8)	2 (1.5-4)	0.091
bleeding score	1.5 (1-2)	2 (1-3)	0.803

In an attempt to detect the association between PDCD-1 +7209 SNP and CTLA4 rs11571315 SNP and ITP severity, the chronic ITP patients were divided into severe (platelet count <20.000/L) and non-severe groups (platelet count >20.000/L), and genotypes were compared between both

groups but no statistically significant difference between genotype distribution or allele frequency between the different studied groups regarding both polymorphisms could be demonstrated (Table 5).

Table 5. Association between PDCD1 and CTLA4 allele frequency and severity of the disease

	PDCD1 alleles				**P value**
	Wild allele (C)		**Variant allele (T)**		
	Count	**%**	**Count**	**%**	
Severe Platelet count <20000	60	69.8%	8	80%	0.718
Not severe Platelet count>20000	26	30.2%	2	20%	
	CTLA4 alleles				**P value**
	Wild allele (T)		**Variant allele (C)**		
	Count	**%**	**Count**	**%**	
Severe Platelet count <20000	**22**	**71%**	**46**	**70.8%**	0.984
Not severe Platelet count>20000	**9**	**29%**	**19**	**29.2%**	

Discussion

ITP involves complex immune mechanisms that interact to influence therapeutic responses. Although these mechanisms are not yet fully understood, T cell dysregulation plays a crucial role in its pathogenesis. Immune checkpoint pathways serve as essential regulators of the immune

system, facilitating the activation of immune responses while inhibiting the development of an autoimmune response. PDCD-1 and CTLA-4 are integral components of the immune checkpoint pathways, both acts as key inhibitory receptors that critically affect peripheral T-cell tolerance and function and are implicated in the pathogenesis of many autoimmune disorders (14, 15).

Previous studies indicate that genetic factors significantly influence the production of PD-1 and CTLA-4. Single-nucleotide polymorphisms (SNPs) in the genes encoding these proteins (PDCD1 and CTLA4) were found to be associated with the pathogenesis of various immunological diseases (16, 17). The current case-control study aimed to investigate the role of PDCD1+ 7209 and CTLA4 rs11571315 SNPs in susceptibility and treatment response in chronic ITP. Despite being widely studied in other immune disorders, to the extent of our knowledge only a few studies have considered the role of PDCD1+ 7209 and CTLA4 rs11571315 SNPs in chronic ITP. In the current study, we found that patients who had the heterozygous (C/T) genotype showed a statistically significant lower platelet count after therapy when compared to those who had the homozygous-wild (C/C) genotype (p-value =0.037). Also, the patients carrying the variant allele (T) showed a lower platelet count after therapy when compared to those carrying the wild allele (C) with a statistically significant difference. However, we couldn't find any statistically significant difference in the genotyping results or allele frequency of the PDCD1 +7209 SNP between the patient group and the control group. This association between the variant T allele in the heterozygous group as well as the T allele frequency and the poorer response of platelets to therapy may be an indication of susceptibility to ITP chronicity. A study conducted by Kasamatsu et al. in 2018 examined the association between the PDCD-1+7209 SNP and the susceptibility to cITP. Patients with cITP exhibited a significantly higher frequency of the PDCD1 +7209 TT genotype when

compared to healthy controls. Thus, the T/T genotype was suggested to be associated with an increased susceptibility to chronic ITP in the Japanese population (15).

The genotype of CTLA4 (rs11571315) revealed no statistically significant difference in the distribution of the genotypes and allele frequency between chronic ITP cases and the control groups (p-value 0.251 and 0.341 respectively). These results came in agreement with the results of a previous study conducted by Yao et al. in 2019, where they examined the association between CTLA4 (rs11571315) and the susceptibility to chronic ITP and revealed no significant differences in SNP genotypes and allele frequencies between ITP patients and the control group. However, a decreased expression of CTLA4 was observed in ITP patients compared to the control group (18). Their findings indicated that ITP may primarily be influenced by environmental factors such as viral infections, rather than genetic ones.

Contrary to our findings, a study by Chen et al. (2023) in Taiwan investigated the relationship between the CTLA4 SNP (rs11571315) and the risk of chronic ITP.

Their research revealed a notable difference in rs11571315 of CTLA4 SNP when comparing ITP cases to healthy controls. The control group demonstrated a significantly higher prevalence of heterozygous genotypes in comparison to ITP cases. The researchers determined that rs11571315 is a susceptibility SNP for primary ITP within the Taiwanese population (10).

The discordance between these results and the findings of our study might be attributed to the diverse ethnic populations examined and the restricted sample size.

In a recent Egyptian study conducted by El Demerdash et al. 2024, where they focused on the role of CTLA polymorphisms in the risk of immune thrombocytopenic purpura (ITP). Although they have studied a different SNP, their findings indicated that the

CTLA-4 CT 60 A/G polymorphism may influence susceptibility to ITP (19).

When chronic ITP patients were divided into severe (platelet count <20.000/L) and non-severe groups (platelet count >20.000/L), we couldn't find any association with the polymorphisms studied and the disease severity. Referring to the findings of Kasamatsu et al., who studied 4 different SNPs of PDCD-1 and CTLA4 in 119 cITP patients, they have shown similar findings to our results regarding the PDCD-1+7209 SNP, which didn't correlate with the disease severity, however, they have found a correlation between CTLA4 CT60 GG genotype and severe clinical presentation and lower platelet count at diagnosis, which contradicted with our findings, such contradiction might be owed to studying different SNPs of CTLA4 (CTLA4 −1577 GG and CTLA4 CT60 GG) (15).

Previous studies investigated the role of immune checkpoint in response to treatment in ITP patients, Zhu et al. 2015, assessed the CTLA-4 levels in plasma in 37 patients with ITP after 4 consecutive days of dexamethasone therapy. They observed a significant elevation of CTLA-4 levels in plasma samples of both patients with acute ITP as well as responders, indicating a potential association between CTLA-4 and treatment efficacy (20). Also, in another study by Guo et al. 2016, they observed that there was a dynamic change in the expression of both CTLA-4 and CD28 following high-dose dexamethasone therapy in 28 patients with ITP, indicating that an altered balance of CD28/CTLA-4 may play a role in the immunopathogenesis of ITP (21). Thus, we studied the association between the platelet count after treatment and PDCD-1 and CTLA4 SNPs. Patients with dual variant genotypes showed statistically significant lower platelet counts after initial treatment when compared to patients with a single variation of one of the genes (p-value =0.018). These results aligned with the findings of El Demerdash et al. 2024, who studied the same ethnic group but different SNP,

and found that a single variation of CTLA-4 + 49 A/G and CT60 A/G polymorphisms did not affect the response to various treatment regimens in ITP patients (19).These results were contradicting with Wang et al. who found an association between the allelic or genotypic frequencies of CTLA4 (rs231779) and corticosteroid sensitivity in ITP (22).

This study has some limitations that should be acknowledged. First, our findings were derived from a relatively small cohort of patients; thus, we recommend further studies that aim to replicate these findings in larger, more diverse patient populations to strengthen their validity.

Additionally, the genetic variants identified in this study were not correlated with the serum levels of CTLA-4 and PDCD-1, which could provide further insights into the functional impact of these variants. We recommend conducting studies that integrate genetic findings with plasma protein level measurements to enhance our understanding of the underlying biological mechanisms.

In conclusion, our study revealed that the C/T genotype of PDCD-1+7209, Patients who had T allele and patients with dual genetic variation are associated with lower platelet count after therapy when compared with those who had C/C genotype, patients who had the C allele and patient with only one single gene mutation respectively, which may implicate the presence of the variant T allele as a risk factor for ITP chronicity. CTLA4 rs11571315 SNPS showed no impact on the susceptibility, severity, or response to treatment in Egyptian children with chronic ITP.

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