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The Influence of CYP7A1 and CYP1A2 Genes on the Pathogenesis of Pediatric Immune Thrombocytopenia Purpura

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ABSTRACT

Background: Immune thrombocytopenia (ITP) in pediatric patients is an autoimmune condition Illness characterized by persistent thrombocytopenia resulting from immune-mediated platelet depletion. The actual pathophysiology of ITP remains unclear, with hereditary variables likely impacting illness severity and development.

Objective: The aim of the study is to evaluate the gene expression of the CYP7A1 and CYP1A2 genes in patients with ITP in comparison to healthy controls, and to examine their potential significance as diagnostic biomarkers for the illness.

Methods: One hundred samples (fifty ITP cases and fifty controls) were analyzed from Wasit Oncology Center. Gene expression analysis of CYP7A1 and CYP1A2 was conducted via quantitative real-time polymerase chain reaction (qRT-PCR), normalized against GAPDH. The relative expression levels were evaluated with the Livak technique (2^-ΔΔCT), and a ROC analysis (receiver operating characteristic) was performed to determine its detection efficacy.

Results: The gene expression levels of CYP7A1 and CYP1A2 were significantly higher in ITP patients compared to healthy controls ($P < 0.001$).ROC analysis found that CYP7A1 (AUC = 1.000, cutoff = 9.13) and CYP1A2 (AUC = 1.000, cutoff = 17.72) demonstrated 100% sensitivity and specificity.

Conclusion: This study demonstrated a highly significant association between genes (CYP7A1 and CYP1A2) expression levels in ITP patients. This may indicate the role of genes in regulatory interaction in ITP pathogenesis.

Keywords: *CYP7A1, qRT-PCR, CYP1A2, gene expression, Pediatric ITP.*

1. INTRODUCTION

Immune thrombocytopenic purpura (ITP) is an autoimmune disorder characterized by persistent thrombocytopenia (peripheral blood platelet count < 150 · 10^9/l), caused by autoantibody binding to platelet antigen(s), which accelerates their removal by the reticuloendothelial system." (1). Pediatric ITP is a complicated chronic illness marked by variations in etiology, bleeding pattern, treatment requirement, response, and disease duration (2). Primary ITP in children is considered a diagnosis of exclusion, as there is no conclusive laboratory testing to establish the illness. Global guidelines for diagnosis state that gathering medical and family history, along with a clinical examination, complete blood count assessment, and blood smear analysis, is sufficient to determine the primary type of the disease(3,4). The most recent Chinese ITP guidelines advocate conducting a bone marrow examination in conjunction with testing for antinuclear antibodies, anti-phospholipid antibodies, antithyroid antibodies, thyroid function, and coagulation parameters to mitigate the risk of misdiagnosis(5). Furthermore, Previous investigations have revealed that B lymphocytes and natural killer (NK) cells have a role in the development of ITP(6). The collapse of immunological tolerance to platelet autoantigens, which leads to inappropriate activation of both humoral and cellular immunity, is one of the main pathogenic mechanisms of ITP. This leads to increased platelet breakdown and reduced platelet synthesis by megakaryocytes (7). The clinical importance of ITP lies in its influence on bleeding, quality of life, treatment options, and long-term follow-up, necessitating appropriate diagnostic and therapeutic techniques to reduce risks and improve patients' lives. Cholesterol 7 alpha-hydroxylase (CYP7A1) works as the primary enzyme that controls the rate-limiting stage in the process in the typical bile acid production route. Bile acids returning to the liver via enterohepatic circulation adversely influence this enzyme at the transcriptional level (8) CYP7A1 gene (which encodes the enzyme cholesterol 7α-hydroxylase) (9) (CYP7A1) is the starting enzyme in the traditional pathway that transforms cholesterol into bile acids, which serves as the principal mechanism for cholesterol removal from the body (10), Cytochrome P4501A2 (CYP1A2) has a function in the activation of procarcinogens and the metabolism of many medicinal

medicines. This gene, found in the 15q24.1 chromosomal region, consists of seven exons and six introns, comprising roughly 7.8 kb of DNA(11). This research aims to use real-time quantitative polymerase chain reaction (qRT-PCR) to measure the gene expression of these genes in clinical peripheral blood samples technology and to show how the severity of the disease is correlated with the expression of these genes. This study identifies important genes that may provide insights into the causes of platelet insufficiency and act as potential diagnostic indicators for the illness.

2. METHODS

Subjects and Design Study:

This study involved 50 individuals with pediatric immune thrombocytopenia from HEMATO - Oncology Center-Wasit. The samples were obtained from the patients and 50 healthy individuals as control. samples were tested with 2 genes (CYP7A1 and CYP1A2) associated with ITP that normalized by housekeeping gene (GAPDH). The samples collected were from September 2024 to January 2025.

Method of selecting ITP patients:

The following criteria are used to select ITP patients: sex (male and female) and age (1 to 15 years old.

Quantitative Real-Time PCR (qPCR)

The gene expression of CYP7A1 and CYP1A2 target genes was calculated using real-time quantitative polymerase chain reaction (qRT-PCR) normalized against the housekeeping gene GAPDH, in pediatric immune thrombocytopenia cases and controls. This method was performed following the protocol outlined (12).

Total RNA extraction

The TRIzol® reagent kit was used to extract total RNA from whole blood samples by the manufacturer's instructions. The following is how the extraction technique was carried out:

1. Homogenization: A 250 µL blood sample was mixed with 750 µL of TRIzol® reagent.

2. Phase Separation: A volume of 200 µL chloroform was added to each tube, followed by vigorous shaking for 15 seconds.

3. After five minutes of incubation on ice, the mixture was centrifuged for fifteen minutes at 12,000 rpm and 4°C.

4. RNA Precipitation: When the aqueous phase was in a fresh Eppendorf tube, 500 µL of isopropanol was added. The combination underwent four to five gentle inversions, ten minutes of incubation at 4°C, and ten minutes of centrifugation at 12,000 rpm at 4°C.

5. RNA Washing: One milliliter of 80% ethanol was added after the supernatant was removed. Following a brief vortex, the material was centrifuged at 12,000 rpm and 4°C for five minutes.

6. RNA Drying and Resuspension: The RNA pellet was allowed to air dry while the supernatant was disposed of. Finally, 100 µL of nuclease-free water was added to dissolve the RNA pellet.

7. The RNA extracted from the samples was kept at -20 °C until it was needed.

Estimation of extracted total RNA yield

A Nanodrop spectrophotometer was used to assess and measure the extracted total RNA (THERMO, USA). The extracted RNA was subjected to two quality control measurements: (1) RNA concentration (ng/µL) quantification and (2) RNA purity evaluation by measuring absorbance at 260 and 280 nm using the same Nanodrop device. The following is how the technique was used:

1. After launching the Nanodrop program, the appropriate application (Nucleic Acid To RNA) was chosen.

2. A dry swab was used to clean the measuring pedestals. Then, 2 µL of nuclease-free water was gently pipetted onto the lower measuring pedestal to obtain a blank measurement.

3. The pedestals were cleaned again, and 1 µL of the total RNA sample was pipetted onto the measuring pedestal for analysis.

Primers

In Table (1), the NCBI GeneBank database and the Primer 3 online design tool were used to create real-time primers for the target genes and the housekeeping gene (GAPDH). These primers were supplied by Scientific Researcher Co. Ltd., Iraq.

Table (1): Details of primers used in the study.

Primer	Sequence 5'-3'		Product size	GenBank
CYP7A1 genes	F	TCGCAAGCAAACACCATTCC	74bp	NM 000780.4
	R	GCTTTCATTGCTTCTGGGTTCC		
CYP1A2 gene	F	AACGTCATTGGTGCCATGTG	96bp	NM 000761.5
	R	AGTCTCCACGAACTCATGAGTG		
GAPDH gene	F	TTGCCATCAATGACCCCTTC	117bp	NM 001256799.3
	R	TGATGACAAGCTTCCCGTTC		

qPCR master mix preparation

The GoTaq® qPCR Master Mix kit, which uses GAPDH amplification in a Real-Time PCR device and SYBR Green dye for target gene detection, was used to make the qPCR master mix. The following elements were part of the master mix:

1-qPCR for Target genes (CYP7A1 and CYP1A2 genes) as shown in table (2)

Table (2): The qPCR for Target genes (CYP7A1 and CYP1A2 genes).

qPCR master mix	volume
cDNA template (100ng)	5µL
Forward primer(10pmol)	1 µL
Reverse primer (10pmol)	1 µL
qPCR Master Mix	10µL
Nuclease Free water	3 µL
Total	20 µL

2- qPCR for Target gene (GAPDH gene) as shown in table (3).

Table (3): The qPCR for the Target gene(GAPDH gene).

qPCR master mix	volume
cDNA template (100ng)	5µL
GAPDH gene Forward primer(10pmol)	1 µL
GAPDH gene Reverse primer (10pmol)	1 µL
qPCR Master Mix	10µL
Nuclease Free water	3 µL
Total	20 µL

The qPCR master mix's ingredients were then divided into qPCR plate strip tubes and combined in an Exispin vortex centrifuge for three minutes. The tubes were then placed inside the MiniOpticon Real-Time PCR apparatus to undergo amplification.

3. RESULTS

Real-time PCR Quantification of *CYP7A1 Expression*

The study's findings contrasting the expression of the CYP7A1 gene in ITP case and control subjects are shown in Table 4. The mean CYP7A1 gene expression was 75.71 ± 6.73 in the ITP case and 1.00 ± 0.42 in the controls, as shown in Figure 1. The mean expression levels were substantially greater in the ITP case compared to controls, with a significant difference (P < 0.001).

Table (4): Comparison of mean of *CYP7A1 gene expression* between patients and healthy controls

Groups	Mean	SD	SE	p-value
ITP patients	75.71	6.79	3.78	0.001**
Control	1.00	0.42	0.24	

****SD: standard deviation; SE: standard error; †: one-way ANOVA; : significant at *P* < 0.05**

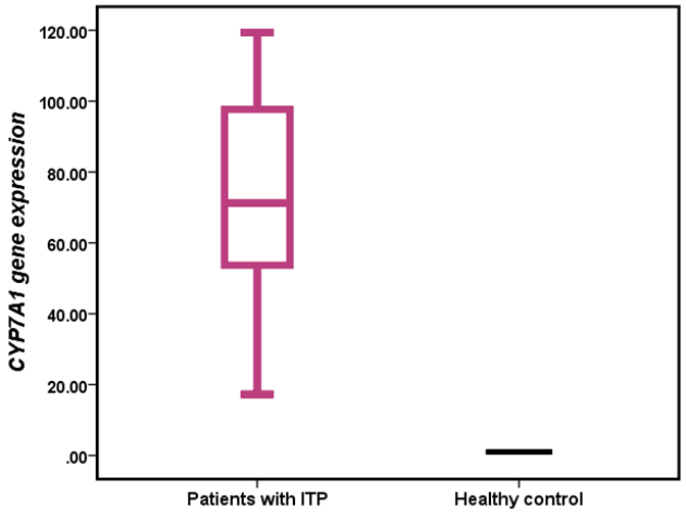


Figure (1): The means *CYP7A1 gene expression* in patients and control groups

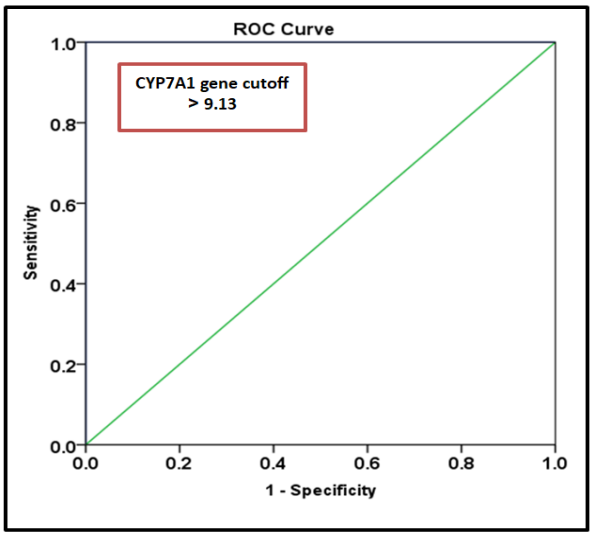
Diagnostic accuracy of *CYP7A1 gene expression*

A ROC analysis (receiver operating characteristic) was performed to determine its detection efficacy. Was conducted to assess the diagnostic accuracy of using the CYP7A1 gene to distinguish ITP patients from healthy controls. An AUC value of 1.000 (95% CI: 1.000-1.000, P=0.001), sensitivity, specificity, PPV, and NPV of 100.0% was obtained with an optimum CYP7A1 gene cut-off value greater than 9.13. According to the current findings, the CYP7A1 gene is regarded as a superior diagnostic marker for distinguishing ITP patients from healthy controls, as shown in Table (5) and Figure (2).

Table (5): ROC curve of *CYP7A1 gene expression*

Characteristic	*ITP patients/control*
Cutoff value	> 9.13
P value	0.001
Sensitivity %	100.0 %
Specificity %	100.0 %
PPV %	100.0 %
NPV %	100.0 %
AUC (95% CI)	1.000 (1.000- 1.000)

"CI: Confidence Interval; AUC: Area Under the Curve."



"Figure (2): Receiver Operating Characteristic (ROC) curve for the CYP7A1 gene to differentiate ITP patients from healthy control subjects."

Real-time PCR Quantification of *CYP1A2 Expression*

The study findings contrasting the expression of the CYP1A2 gene in ITP case and control subjects are shown in Table 6. The Mean of CYP1A2 gene expression was 146.86 ± 35.04 and 1.00 ± 0.41 in ITP patients and healthy control, respectively, as shown in Figure (3), respectively; the mean values were greater in ITP patients compared to healthy control, and the difference was very significant (P < 0.001).

Table (6): Comparison of mean of *CYP1A2 gene expression* between patients and healthy controls

Groups	Mean	SD	SE	p-value
ITP patients	146.86	35.04	10.61	0.001**
Control	1.00	0.41	0.12	

"SD: standard deviation; SE: standard error; †: one-way ANOVA : significant at P > 0.05

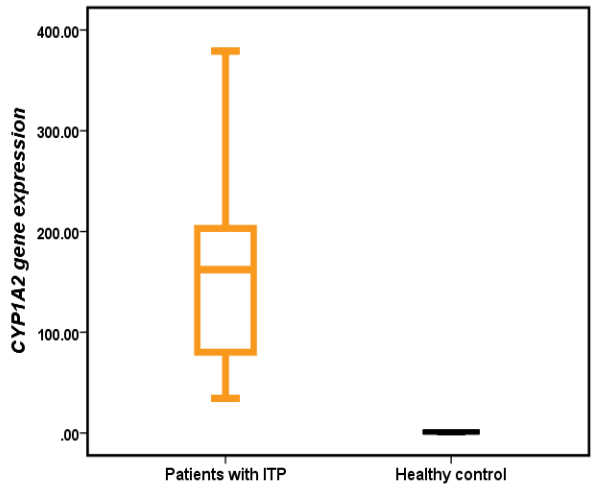


Figure (3): The means *CYP1A2 gene expression* in patients and control groups

Diagnostic accuracy of *CYP1A2 gene expression*

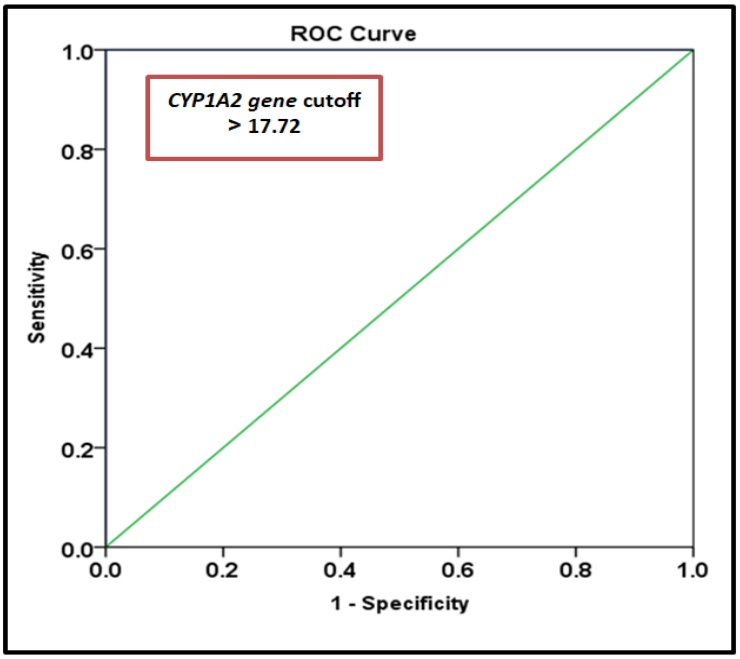
A ROC analysis (receiver operating characteristic) was undertaken to establish the diagnostic accuracy of utilizing the CYP1A2 gene to identify ITP patients from healthy control subjects. The findings demonstrated that an ideal CYP1A2 gene

cut-off value larger than 17.72 was associated with an AUC value of 1.000 (95% CI: 1.000-1.000, P=0.001), sensitivity, specificity, PPV, and NPV of 100.0%. According to the current findings, the CYP1A2 gene is thought to be a reliable diagnostic marker for distinguishing ITP patients from healthy controls, as shown in Table (7) and Figure (4).

Table (7): ROC curve of CYP*1A2 gene expression*

Characteristic	*ITP patients/control*
Cutoff value	> 17.72
P value	0.001
Sensitivity %	100.0 %
Specificity %	100.0 %
PPV %	100.0 %
NPV %	100.0 %
AUC (95% CI)	1.000 (1.000- 1.000)

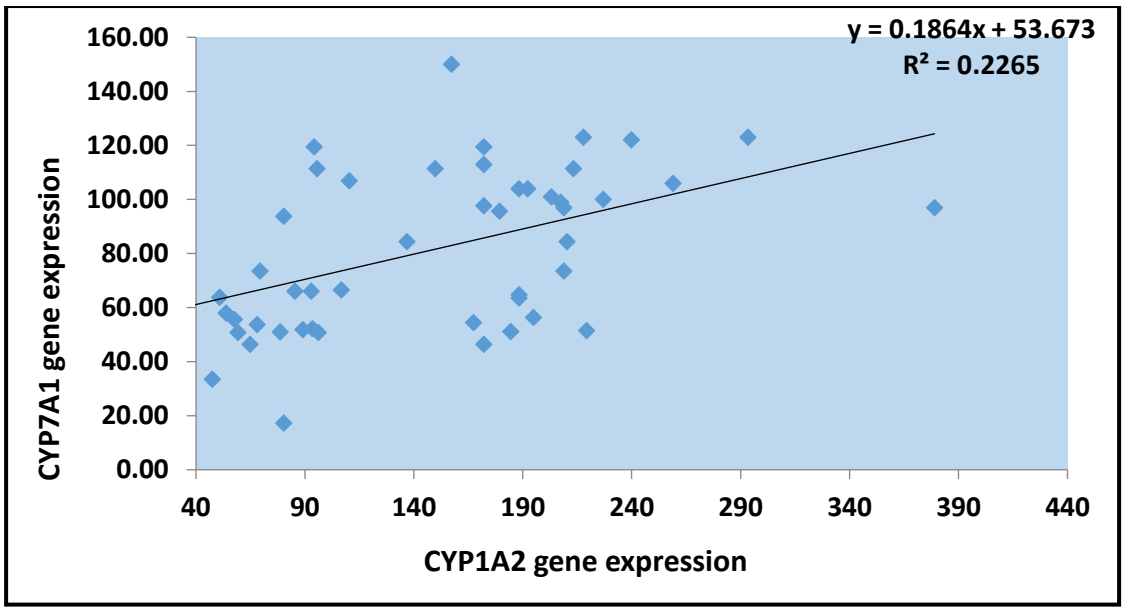
"CI: Confidence Interval; AUC: Area Under the Curve."



"Figure (4): Receiver Operating Characteristic (ROC) curve for the CYP1A2 gene to differentiate ITP patients from healthy control subjects."

Logistic regression correlations between gene expression parameters in ITP patients.

The Logistic regression model shows that the correlation gene expression parameters, such as the CYP7A1 gene, have directly correlated with CYP8B1 genes among ITP patients as in Figure (5). This result might indicate that the ITP condition enhances the production of the CYP7A1 gene in relation to the expression of the CYP1A2 gene.



"Figure (5): The Logistic scatter CYP7A1 and CYP1A2 expression among patients."

4. DISCUSSION

Previous metabolomics investigations identified changes in metabolic parameters between healthy individuals and patients with thrombocytopenia (13) and gave fresh insights into the processes producing thrombocytopenia. These data can help the diagnosis somewhat, although the profiles of these genes are still unknown. In this investigation, two genes associated with metabolism and drug metabolism were found. These data reveal an increase in the gene expression of the gene (cyp7a1) compared with healthy subjects (p: 0.001 statistically significant). This may be attributed to a change in the metabolism, which is the major enzyme for synthesizing bile acids and initiates the conventional pathway for synthesizing bile acids (14,15). This is in agreement with what was indicated in the work of Xu et al., 2024, where an increase in the gene expression of this gene was discovered (12) as well as the study of (16). These results also demonstrated an increase in the gene expression of the gene (cyp1a2) compared to healthy subjects (p: 0.001). This may be due to its involvement with drug and toxin metabolism and its link with autoimmunity. This was revealed in the study, which explained this rationale (17). Furthermore, one of the studies conducted in Canada showed that the gene cyp1a2 plays a role in the production of reactive oxygen species (18), and since reactive oxygen species are a product of oxidative metabolism (19), overall the expression of this gene was high, which is consistent with the study by Xu et al., 2024, which linked this gene to thrombocytopenia through what it does to enhance the production of reactive oxygen species (12). In this investigation, ROC was utilized, which is a diagnostic test that plays a key role in modern medicine, and it was used to determine the diagnostic ability of the gene CYP7A1 (20). The results indicated that the area under the curve (AUC) approached 1,000, which signifies remarkable diagnostic accuracy since it may be recognized through it. Between patients and healthy individuals by 100%, the value of the specific threshold (cutoff value) was also set at >9.13, which implies that patients who have a gene expression higher than this limit can be defined as infected, while those who have less are classified as healthy. This threshold has a sensitivity and specificity of 100%, which implies that all patients were accurately identified, and this is what was used in the study (21) that employed the same procedure. The findings of the ROC analysis of the CYP1A2 gene likewise indicated an area under the curve value of 1.000, and the diagnostic threshold value was calculated at >17.72, which suggests that persons with greater gene expression are deemed unwell, and those with less than this are healthy. In addition, this threshold also revealed a sensitivity and specificity of 100%, which may be applied to accurately identify them. When comparing these results with the (21) study that utilized the same technique on patients with thrombocytopenia but with different gene analyses, we can claim that this analysis provides a powerful aid in diagnosis despite the difference in genes between the two studies. This emphasizes the hypothesis that gene expression can be an essential biological indicator in identifying thrombocytopenia. The findings of the logistic regression analysis indicated a relationship between the gene expression of CYP7A1 and CYP1A2 in patients with thrombocytopenia. The strength of the connection was $R^2 = 0.2265$, meaning that 22.65% of the expression of the CYP7A1 gene can be explained by the expression of the CYP1A2 gene, whereas 77.35% is related to other factors. In sum, the genes (CYP7A1, CYP1A2) can be considered a good diagnostic tool, This is in line with a study (22) that used the same analysis on patients with immune thrombocytopenia. The considerable overexpression of CYP7A1 and CYP1A2 genes in ITP patients indicates their putative significance in the disease. Therefore, we can use this expression as an early accurate diagnostic marker and for the progression development of ITP.

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