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The Relationship of the CYP8B1 Gene and Alterations in Blood Parameters with Pediatric Immune Thrombocytopenia Purpura Patients

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Abstract

Immune thrombocytopenia (ITP) in pediatric patients is an autoimmune disorder. The disease is characterized by chronic thrombocytopenia due to immune-mediated platelet loss. CYP8B1 is a critical enzyme in bile acid metabolism, and some studies have shown a relationship between lipid metabolism and hematopoiesis. This study aimed to determine the expression levels of the CYP8B1 gene in pediatric patients with immune thrombocytopenia (ITP) and to detect its association with various basic hematological parameters, including PLT, RBCs, Hb, HCT, and WBCs. One hundred samples (fifty patients with immune thrombocytopenia and fifty healthy individuals) were analyzed. Gene expression of the CYP8B1 gene was evaluated using quantitative real-time polymerase chain reaction (qRT-PCR) and standardized against GAPDH. ROC (receiver operating characteristic) analysis was performed to determine its detection efficiency. The gene expression of CYP8B1 was significantly higher in ITP patients than in healthy controls ($P < 0.001$). In addition, the levels of PLT, RBCs, HB, and HCT were significantly lower in ITP patients ($P < 0.05$), while the white blood cell count was slightly elevated but not statistically significant. ROC analysis showed that the expression of CYP8B1 showed 100% sensitivity and specificity in separating ITP patients from controls (AUC = 1.000, P = 0.001), highlighting its potential as a biomarker for the diagnosis of ITP. The statistics reveal that CYP8B1 has a considerable effect on the development of ITP and could serve as a possible diagnostic marker. Further investigations are needed to establish its mechanistic involvement and therapeutic significance in pediatric ITP.

Keywords: CYP8B1, gene expression, pediatric ITP, qRT-PCR, biomarker

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علاقة جين CYP8B1 والتعديلات في بارامترات الدم مع مرضى نقص الصفيحات المناعي عند الأطفال

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المستخلص

نقص الصفيحات المناعي (ITP) في المرضى الأطفال هو اضطراب مناعي ذاتي. يتميز المرض بنقص الصفيحات المزمن بسبب فقدان الصفائح الدموية بوساطة المناعة. CYP8B1 هو إنزيم حاسم في عملية التمثيل الغذائي للأحماض الصفراوية، وتظهر بعض الدراسات وجود علاقة بين التمثيل الغذائي للدهون وتكوين الدم. تسعى هذه الدراسة إلى تحديد مستويات التعبير عن جين CYP8B1 في المرضى الذين يعانون من نقص الصفيحات المناعي (ITP) عند الأطفال والكشف عن ارتباطه بمعايير دموية أساسية مختلفة، بما في ذلك PLT وRBCs وHB وHCT وWBCs.حيث تم تحليل مائة عينة (خمسون مريضًا بنقص الصفيحات المناعي وخمسون شخصًا سليمًا). تم تقييم التعبير الجيني لجين CYP8B1 باستخدام تفاعل البوليميراز المتسلسل الكمي في الوقت الحقيقي. (qRT-PCR) ومعياريًا ضد GAPDH. تم إجراء تحليل ROC (خاصية تشغيل المستقبل) لتحديد فعالية الكشف عنه. كان التعبير الجيني لجين CYP8B1 أعلى بشكل ملحوظ في مرضى ITP مقارنة بالضوابط الصحية ($P < 0.001$). بالإضافة إلى ذلك، كانت مستويات PLT و RBCsو HBو HCTأقل بشكل ملحوظ في مرضى($P < 0.05$) ITP ، في حين كان عدد خلايا الدم البيضاء مرتفع بشكل طفيف ولكنه لم يكن ذا دلالة إحصائية. أظهر تحليل ROC أن التعبير عن CYP8B1 أظهر حساسية وخصوصية بنسبة 100٪ في فصل مرضى ITP عن الضوابط(AUC = 1.000) ، ($P = 0.001$)، مما يسلط الضوء على إمكاناته كعلامة حيوية لتشخيصITP. تكشف الإحصائيات أن CYP8B1 يلعب تأثيرًا كبيرًا في تطور ITP ويمكن أن

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معلومات البحث
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يكون بمثابة علامة تشخيصية محتملة. هناك حاجة إلى مزيد من التحقيقات لإثبات مشاركتها الآلية وأهميتها العلاجية في ITP عند الأطفال.

الكلمات المفتاحية : CYP8B1، التعبير الجيني، ITP عند الأطفال، qRT-PCR، العلامة الحيوية

Introduction

Immune thrombocytopenic purpura (ITP) is an autoimmune disorder characterized by persistent thrombocytopenia (peripheral blood platelet count $< 150 \times 10^9$/L), resulting from the binding of autoantibodies to platelet antigens, which accelerates their clearance by the reticuloendothelial system. [1]. ITP is a prominent cause of thrombocytopenia in pediatric populations, with an estimated prevalence of around 1 in 20,000 cases annually [2]. It can manifest as either acute or chronic, with most pediatric cases being acute and self-limiting. However, a significant proportion of children develop chronic ITP, which persists for more than 12 months after diagnosis. [3]. Although studies on childhood ITP outcomes are extensively documented in Western countries, there is limited research available from developing regions.[4-6]. Consequently, examining ITP outcomes in our cohort is essential for comprehending the pathological, clinical, and laboratory determinants that influence disease persistence or progression to chronicity. In order to reduce the possibility of misdiagnosis, the latest Chinese ITP guidelines recommend performing a bone marrow examination together with tests for thyroid function, coagulation parameters, antinuclear antibodies, antiphospholipid antibodies, and antithyroid antibodies.[7]. Furthermore, earlier research has shown that natural killer (NK) cells and B lymphocytes have a role in the development of ITP.[8]. One of the primary pathogenic processes of ITP is the loss of immunological tolerance to platelet autoantigens, which results in abnormal humoral and cellular immune activation. This imbalance leads to increased platelet breakdown and reduced platelet synthesis by megakaryocytes.[9]. Human Cytochrome P450 8B1 (CYP8B1) is a key enzyme in cholesterol metabolism and bile acid production. It particularly hydroxylates the steroid ring at the C12 position, creating cholic acid and so altering the ratio of cholic acid (CA) to chenodeoxycholic acid (CDCA), which in turn regulates the hydrophobicity of the bile acid pool. [10-11]. Although bile acid metabolism has been widely investigated, the role of CYP8B1 in hematopoiesis and its potential connection to platelet generation remains unclear. Considering the major impact of ITP on bleeding risk, quality of life, and long-term management, appropriate diagnostic and treatment procedures are critical for decreasing risks and enhancing patient outcomes.[10]. This study aims to evaluate the expression levels of these genes in clinical peripheral blood samples from ITP patients by real-time quantitative polymerase chain reaction (qRT-PCR) technology and to demonstrate the correlation between the gene expression of these genes and disease severity. This study identifies important genes that may provide insights into the causes of platelet insufficiency and act as potential diagnostic indicators for the illness.

Material and method

Subjects and Design Study

This study involved 50 individuals with pediatric immune thrombocytopenia from Wasit

Governorate Oncology Center and 50 healthy individuals as controls. Samples were tested for genes (CYP8B1) that were normalized by housekeeping genes (GAPDH) and alterations in blood parameters. The samples were collected from September 2024 to January 2025.

Blood sample collections

Blood samples were collected from patients after obtaining their informed consent. A signed authorization from the Faculty of Science was required for sample collection at the hospital. A volume of 250 microliters of the drawn blood was placed into an EDTA tube and subsequently transferred to an Eppendorf tube for further analysis. The samples were then stored in a deep freeze until use.

Hematological Assay

The blood test includes the following tests: RBCs, WBCs, HCT, Hb, and PLT. The examination was conducted using the Huma Count Auto Analyzer after adding 2 ml of blood to the EDTA tube and placing the tube in the designated slot on the device. We then initiate the process by pressing the "Start" button as the device automatically reads the results. Once the results appear, we issue the command "Print" for the device to print them, as per the specifications from Human Company, Germany.

Quantitative Real-Time PCR (qPCR)

The expression of the CYP8B1 target gene was measured using quantitative real-time PCR (qRT-PCR), normalized against the housekeeping gene GAPDH, in pediatric ITP patients and healthy controls. The procedure was performed using the Real-Time PCR technique, following the method described by[12] and includes the following processes:

Total RNA extraction

As directed by the manufacturer, total RNA was extracted from whole blood samples using the TRIzol® reagent kit. The extraction method was performed as follows:

1. Homogenization: A 250 µL blood sample was mixed with 750 µL of TRIzol® reagent.
2. Phase Separation: A volume of 200 µL of chloroform was added to each tube, followed by vigorous shaking for 15 seconds.
3. The mixture was centrifuged for fifteen minutes at 12,000 rpm and 4°C after being incubated on ice for five minutes.
4. RNA Precipitation: 500 µL of isopropanol was added to a new Eppendorf tube containing the aqueous phase. 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: After removing the supernatant, 1 milliliter of 80% ethanol was added. After a quick vortex, the material was centrifuged for five minutes at 12,000 rpm and 4°C.
6. RNA Drying and Resuspension: The supernatant was disposed of, and the RNA pellet was left to air dry. Lastly, the RNA pellet was dissolved by adding 100 µL of nuclease-free water.
7. Until they were needed later, the RNA isolated from the samples was stored at -20°C.

Estimation of extracted total RNA yield

The extracted total RNA was evaluated and quantified using a Nanodrop spectrophotometer (THERMO, USA). Two quality control

measurements were performed on the extracted RNA: (1) quantification of RNA concentration (ng/µL) and (2) assessment of RNA purity by measuring absorbance at 260 nm and 280 nm using the same Nanodrop instrument. The technique was carried out as follows:

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.

DNase Treatment

Using the DNase I enzyme kit, the RNA that was recovered was processed to remove any leftover genomic DNA. The following approach was applied in accordance with the directions supplied by the manufacturer (Promega, USA) as shown in Table (1).

Table (1): Details of DNase Treatment

Mix	Volume
Total RNA 100ng/ul	10µl
DNase I enzyme	1µl
10X buffer	4µl
DEPC water	5µl
Total	20µl

This was followed by a 30-minute incubation period at 37°C. After incubating for 10 more minutes at 65°C, 1 µL of stop solution was added to the sample to inactivate the DNase enzyme.

qPCR master mix preparation

The qPCR Master Mix kit from GoTaq®, 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 the Target gene (CYP8B1 gene) as shown in Table (2).

Table (2): the qPCR for the Target gene (CYP8B1 gene)

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 the 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

After that, the components of the qPCR master mix were divided into qPCR plate strip tubes and mixed for three minutes in an Exispin vortex centrifuge. After that, the tubes were inserted into the MiniOpticon Real-Time PCR instrument to be amplified.

Thermocycler conditions for qPCR

The qPCR plate was then loaded, and Table (4) shows the subsequent thermocycler process.

Table (4): Details of the qPCR process used in the study

qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	95 °C	5min	1
Denaturation	95 °C	20 sec	40
Annealing\Extention Detection(scan)	60 °C	30 sec	
Melting curve	65-95°C		

2.3 Primers

The NCBI GeneBank database and the Primer3 web tool were used to generate the primers for the target genes and the housekeeping gene (GAPDH) table (5), Scientific Researcher. Co.., Ltd., Iraq, provided these primers:

Table (5): Details of primers used in the study

Primers	Sequence 5'-3'		Product size	Genbank
CYP8B1 genes	**F**	TCTGTGGGCAACTTGGTTTG	111bp	NM 004391.3
	R	TGGAGGCTGATGTTTGCAAG		
GAPDH	**F**	TTGCCATCAATGACCCCTTC	117bp	NM 001256799.3

gene	**R**	TGATGACAAGCTTCCCGTTC		

F: forward primer, R: reverse primer

Result

Blood Parameter Levels in ITP Patients and Healthy Control Participants

The comparison of selected blood parameters between ITP patients and healthy control persons was done, and the findings are shown in Table (6) and Figure (1). White blood cell (WBC) counts were on average 8.35 ± 3.9 in ITP patients and 7.55 ± 1.27 in healthy controls, with the ITP group displaying a slightly higher level; however, the difference was not statistically significant (P = 0.201). In contrast, the mean Red Blood Cell (RBC) count was 4.62 ± 0.73 in ITP patients and 5.09 ± 0.69 in healthy controls, with the ITP group demonstrating a substantially lower count in contrast to the healthy controls (P = 0.002). ITP patients also had mean hemoglobin (Hb) levels that were lower (11.77 ± 1.23) compared to healthy controls (13.96 ± 1.45), and this difference was statistically significant (P = 0.001). Regarding platelet count, ITP patients exhibited significantly lower levels (82.28 ± 12.35) compared to healthy controls (280.58 ± 10.42), having a highly substantial difference (P = 0.001). Furthermore, there was a noteworthy distinction in the hematocrit levels between ITP cases and controls (P > 0.05).

Table (6): Mean Values of Blood Parameters in Healthy Control Subjects and ITP Patients

Parameters	**Comparison of cases and controls**		***P value***
	ITP Patients ***n* = 50**	**Healthy control** ***n* = 50**	
White Blood Cell count.			
Mean± SD	**8.35 ± 3.9**	**7.55 ± 1.27**	**0.201** † **NS**
Range	1.59 – 20.99	4.18- 10.69	
Red Blood Cell count (million/ µl)			
Mean± SD	**4.62 ± 0.73**	**5.09 ± 0.69**	**0.002** † **S**
Range	3.17 – 6.73	3.93- 6.44	
Hemoglobin (Hb) (g/dl)			
Mean± SD	**11.77 ± 1.23**	**13.96 ± 1.451**	**0.001** † **S**
Range	8.50 -13.80	11.59-16.91	
Hematocrit (HCT)			
Mean± SD	**34.53 ± 3.39**	**44.31 ± 5.03**	**0.001** †
Range	26.40 -40.0	36.02-51.87	

			S
Platelet Count (thousand/ µl)			
Mean± SE	**82.28 ± 12.35**	**280.58 ± 10.42**	**0.001**
Range	3.00 -318.00	153.25-392.57	**†** **S**

SD: standard deviation; SE: standard error; n: number of instances; †: independent samples t-test; HS: P < 0.001 indicates high significance. NS: not significant if P is greater than 0.05;"

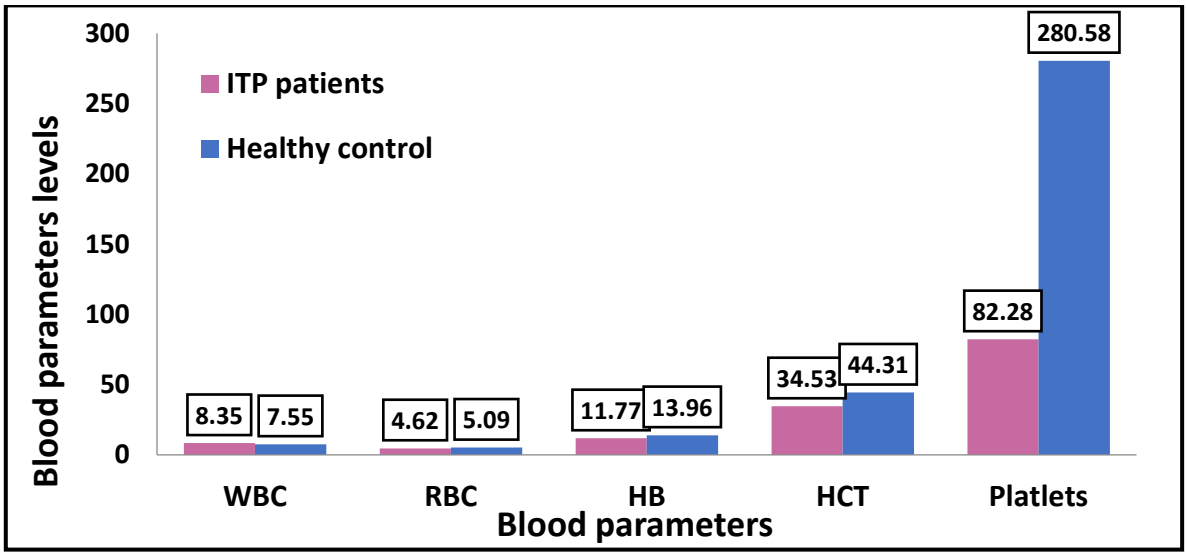

Figure (1) Mean Values of a Few Blood Parameters in Healthy Control Subjects and ITP Patients

Correlation between some blood parameters (WBC, RBC, HGB, HCT, and Platelets) in ITP patients

The connections between key blood parameters (WBC, RBC, HGB, HCT, and Platelets) in ITP patients are illustrated in Tables (7). The present results suggest there was a strong positive association between WBC count and RBC count (r=0.364 and p=0.001), RBC count and HGB level (r=0.522 and p=0.001), RBC count and HCT level (r=0.618 and p=0.001), and HGB level and HCT level (r=0.866 and p=0.001). However, the present data demonstrate a non-significant link between all other parameters in ITP patients.

Table (7): Correlation of some blood parameters (WBC, RBC, HGB, HCT, and Platelets) in ITP patients

Parameters	**blood parameters**									
	WBC		**RBC**		**HGB**		**HCT**		**Platelets**	
	r	***P***	***r***	***P***	***r***	***P***	***r***	***p***	***r***	***p***
WBC	1									
RBC	0.364	0.001*	1							
HGB	-0.078	0.591	0.522	0.001*	1					

HCT	0.033	0.820	0.618	0.001*	0.866	0.001*	1		
Platelets	-0.082	0.570	0.037	0.801	0.035	0.810	-0.089	0.541	1

r: Pearson correlation.

Real-time PCR Quantification of CYP8B1 *Expression*

The comparison of CYP8B1 gene expression between ITP patients and healthy control participants was undertaken, and the results are provided in Table (8). The mean CYP8B1 gene expression was 20.39 ± 6.1 in ITP patients and 1.00 ± 0.24 in healthy controls as shown in Figure (2). The expression levels were substantially greater in ITP patients compared to healthy controls, with a very significant difference (P < 0.001).

Table (8): Comparison of the mean of *CYP8B1 gene expression* between patients and healthy controls

Groups	Mean	SD	SE	p-value
ITP patients	20.39	6.1	2.27	0.001**
Control	1.00	0.24	0.035	

"*SD: Standard Deviation; SE: Standard Error; †: One-way ANOVA;* **:** *Significant at P* < 0.05"

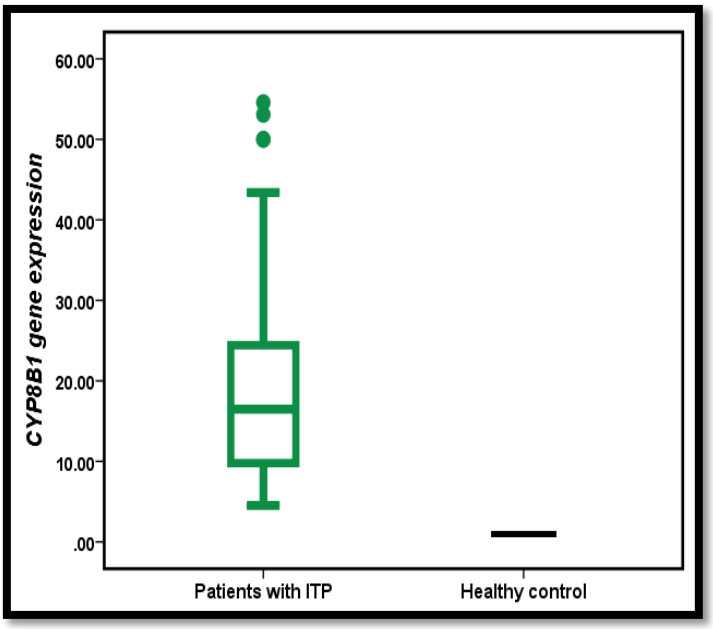

Figure (2): Mean CYP8B1 Gene Expression in ITP Patients and Healthy Control Groups

Diagnostic accuracy of *CYP8B1 gene expression*

A ROC analysis (receiver operating characteristic) was undertaken to assess the accuracy of diagnostics using the CYP8B1 gene to identify ITP patients from healthy control people. An ideal cut-off value of 4.75 for the CYP8B1 gene resulted in an area under the curve (AUC) of 1.000 (95% confidence interval [CI100.0% sensitivity, 100.0% specificity, 100.0% positive predictive value (PPV), and 100.0% negative predictive value (NPV) were found in the 1.000-1.000 range (P = 0.001). These results demonstrate that the

CYP8B1 gene is a strong diagnostic marker for identifying ITP patients from healthy controls as shown in Table (9) and Figure (3).

Table (9): ROC curve of CYP8B1 *gene expression*

Characteristic	*ITP patients / control*
Cutoff value	> 4.75
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"

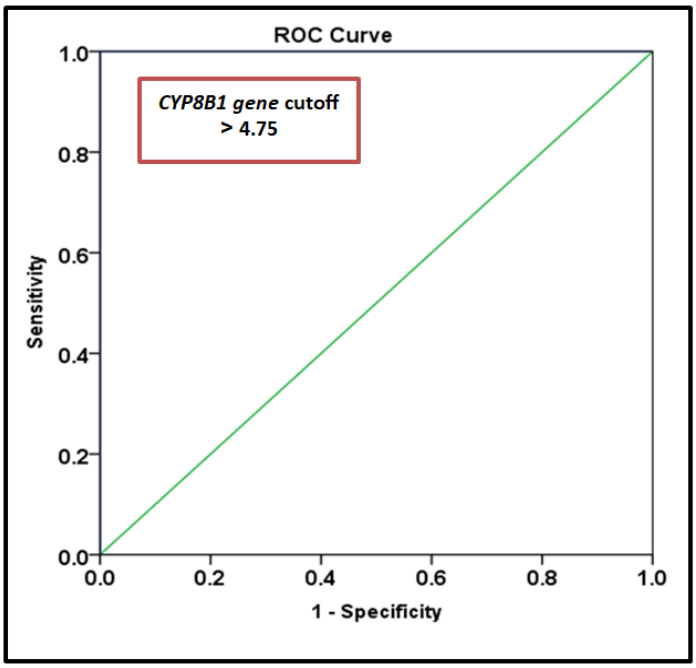

Figure (3): Receiver Operating Characteristic (ROC) Curve for CYP8B1 Gene to Differentiate ITP Patients from Healthy Control Subjects

Discussion

Previous metabolomics studies have found that ITP patients and healthy individuals have different metabolic characteristics [13], providing new information about the mechanisms that cause thrombocytopenia. These facts can help diagnose to some extent, even if the profiles of these genes are still unknown. A gene related to metabolism and bile acid metabolism was studied during this work, and this gene was studied in relation to how it affects the immune response of ITP patients. In this research, the CYP8B1 gene was identified as a diagnostic marker for ITP and compared with healthy controls. In the context of this research, the

results of blood parameters showed a decrease in the levels of RBC, HCT, HGB, and PLT, which are shown in Table (6) compared to controls, which is statistically significant (P = less than 0.05), which is consistent with the studies of [14–17]which showed that thrombocytopenic patients suffer from a sharp decrease in these variables or parameters as a result of platelet destruction by the immune system, where the immune system attacks and destroys healthy platelets in the spleen. Thrombocytopenia can also affect the bone marrow's ability to produce blood cells, such as red blood cells and white blood cells, and also leads to a decrease in hematocrit. In addition, medications and treatments used, such as cortisone and steroids, affect the production of blood cells. The results of blood parameters also showed a slight increase in white blood cells in patients with thrombocytopenia compared to healthy controls, as it was not statistically significant, which contradicts the study [18], which showed a slight increase in white blood cells. In patients with thrombocytopenia, compared to the control group. In another study by [19], it was shown that both white blood cells and red blood cells were not affected and remained within normal limits. This may be due to the possibility of early diagnosis, a recent injury, or other reasons. The relationship between these blood variables was also tested in patients with thrombocytopenia in Table (7). The results showed a positive statistical relationship between both white blood cells and red blood cells, as well as red blood cells with hemoglobin.

CYP8B1 encodes a sterol 12α-hydroxylase, which is essential for the synthesis of cholic acid [20]. In this study, the expression of CYP8B1 in ITP patients was higher than that in healthy individuals. Consistently, the expression of CYP8B1 was increased in peripheral blood samples of ITP patients. Therefore, it was speculated that CYP8B1 mediates the pathogenesis of ITP through the MAPK pathway by influencing the growth and proliferation of natural killer cells. This is consistent with the [21] study that confirmed an increased expression of the cyp8b1 gene, explained or justified by the same reason above. In this study, ROC, a diagnostic test that plays a major role in modern medicine, was used to determine the diagnostic ability of the CYP8B1 gene [22]. The results indicated that the area under the curve (AUC) approached 1000, demonstrating remarkable diagnostic accuracy, as it could be identified between patients and healthy individuals with 100% accuracy. The threshold value (cut-off value) was set at 4.75, meaning that patients with gene expression above this threshold could be identified as affected, while those with expression below it were classified as healthy. This threshold had a sensitivity and specificity of 100%, meaning that all patients were accurately identified. This was used in the study of [23], which used the same procedure on ITP but with different genes.

Conclusions

The statistics reveal that CYP8B1 has a considerable effect on the development of ITP and could serve as a possible diagnostic marker. Further investigations are needed to establish its mechanistic involvement and therapeutic significance in pediatric ITP.

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