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Original article

New aspects in pathogenesis in Patients with Immune Thrombocytopenia

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Abstract

Background: Immune thrombocytopenia is an auto immune disorder characterized by low platelet count due to either peripheral platelet destruction or inappropriate production of platelets by bone marrow. although auto antibodies have been showed as the principle factor in the pathogenesis of ITP, also cellular immune modulation have been identified to have a very important role in the pathophysiology of ITP. the aim of this study was to investigate runt related transcription factor 1 (Runx1) and it`s association to ITP. ***Aim of the Work:*** To determine the expression levels of runt related transcription factor in the peripheral blood of children with ITP and to determine the clinical usefulness of this transcription factor in understanding the pathophysiology of ITP and its relation to disease activity and chronicity. ***Patients and Methods:*** This study was conducted on 49 patients having ITP in beni-suef university hospital and 20 healthy subjects as controls. RUNX1 was analyzed using *ELISA* technique. ***Results:*** The results revealed that RUNX-1 levels were significantly higher in ITP patients compared to controls (1.78 ±0.73 vs. 3.87 ±2.39, p=0.001) in healthy controls and ITP patients respectively. ***Conclusion:*** RUNX1 is involved in the pathogenesis of ITP, it also related to disease activity

1. Introduction:

Immune thrombocytopenic purpura (ITP), is an autoimmune disorder characterized mainly by isolated thrombocytopenia that may present by mild mucocutaneous purpura to life-threatening bleeding. [1]

In ITP there is destruction of platelets by autoantibodies against its glycoprotein surface which will lead to opsonization and destruction of platelets in addition to suppretion of platelets production. Anti-platelet auto antibodies are detected in nearly 60% of patients and they are commonly directed against glycoprotein IIIa and IIb. [2]

ITP can be classified as primary ITP in which there is no evidency of any predisposing cause and secondry as a result of many other associated cases. [1]

Also it could be classified according to the duration into newly diagnosed (less than 3 monthes),persistant (3-12 monthes)and chronic (more than 12monthes. [3]

Recent studies demonstrated that imbalance in the differentiation of Th1 and Th2 cells play an important role in the development and pathogenesis of ITP [4].

Furthermore, in recent years, abnormal differentiation of Th17 cells was reported to be additional mechanism contributing to the development of ITP [5].

Runt related transcription factor 1 is an essential transcription factor that contribute in a variety of cellular processes including, proliferation, differentiation, tissue growth and DNA damage response. [6]

RUNX1 belongs to RUNT domain containing family family of three transcription factors coded by the genes, RUNX1, RUNX2, and RUNX3. [7]

2. Patients and Methods:

The current study was a case-control study conducted on pediatric patients from hematology outpatient clinic in Beni-Suef University hospital, during the period from November 2020 (after the approval of the ethical committee no: FMBSUREC/03112020/HUSSEIN) to November 2021.

Patients:

The children included in this study were selected randomly from beni suef university hospital pediatric hematology outpatient clinic according to the following inclusion and exclusion criteria:

Inclusion criteria:

- Pediatric patients with ITP aged more than 1 month and less than 18 years old
- Healthy controls group will be age and sex matched with our patients.

Exclusion criteria:

- Children with other bleeding disorders
- Children with other autoimmune disorders
- Children suffering from hematological malignancies
- Children infected by h pylori
- Children who are positive for (HBV, HCV, HIV)

Sample size:

The present study included 69 child divided into 2 groups as the following:-

Group (I): 49 patients having ITP, Patients were 19 males (38..8%) and 30(61.2%) females. (subdivided into three groups: active= 20 patients, remission= 11 patients, and chronic= 18 patients), that were confirmed based on the typical clinical picture of ITP, complete blood count and BMA.

Group (II): 20 healthy subjects as controls age and sex-matched individuals, giving no personal or family history of ITP. Control subjects were recruited from patients presenting at the pediatrics outpatient clinic and outpatient laboratory unit and they were selected not to have any autoimmune illness and no interfering medications.

According to the ASH itp guidelines(2019):-

- Remission: Platelet count > 100 x 10^9 /L at 12 Monthes
- Active: patients with a platelet count < 50 x 10^9 /L were defined as active ITP, who had a median platelet count of 20 x 10^9 /L
- Chronic: ITP duration of > 12 month [8]

Sample size calculation method:

Sample size was calculated with G*Power (3.1.9.4) software using a priori analysis with an effect size= 0.25 for F-test: ANCOVA: Fixed effects, main effects and interactions, main effects and interactions using priori a analysis according to the following inputs:

F tests - ANCOVA: Fixed effects, main effects and interactions
Analysis: A priori: Compute required sample size
Input: Effect size f = 0.5
α err prob = 0.05
Power (1-β err prob) = 0.85
Numerator df = 4
Number of groups = 4
Number of covariates = 1
Output: Noncentrality parameter λ =14.7500000
Critical F = 2.5429175
Denominator df = 54
Total sample size = 60
Actual power = 0.8526475

All individuals included in the study were subjected to the following:

- Full history taking.
- Full clinical examination (including liver, spleen, LN examination)
- Complete blood count
- Direct platelet count
- Bone marrow examination
- Pelvi-abdominal ultrasound
- Liver function tests
- Kidney function tests
- H.pylori testing
- Virology testing (HCV,HBV,HIV)
- C reactive protein
- ANA, Anti- ds DNA, C3&C4 and Urine analysis, to exclude systemic auto-immune diseases.
- Runx1 level by ELISA

Treatment:

All patients received treatment with steroids (The dose and mode of administration were determined by platelet count and whether there was active bleeding: in urgent situations, infusions of dexamethasone or methylprednisolone used, while oral prednisone or prednisolone were sufficient in less severe cases). Once the platelet count has improved, the dose of steroid is gradually reduced while the possibility of relapse is monitored.

Methodology:

Measurement of serum Human Runt- related Transcription Factor 1 by ELISA technique(enzyme linked immunosorbant assay) :

RUNT-RELATED TRANSCRIPTION FACTOR 1 : was detected and quantified in serum sample using ELISA technique . Where serum samples and standards added to corresponding wells then conjugate labeled antibody with HRP added after washing completely , TMB substrate solution added , TMB substrate become blue color at HRP enzyme catalyzed , reaction is terminated by addition of stop solution and the color change is measured at a wavelength of 450nm. using a spectrophotometer. In order to measure the concentration of RUNT-RELATED TRANSCRIPTION FACTOR 1 in the serum sample, and including a set of calibration standards. The calibration standards are assayed at the same time as the samples and allow to produce a standard curve of Optical Density versus RUNT-RELATED TRANSCRIPTION FACTOR 1 concentration. The concentration of RUNT-RELATED TRANSCRIPTION FACTOR 1 in the samples is then determined by comparing the O.D. of the samples to the standard curve

Steps:

Add standard 50µl to standard well. Then Add Sample: sample 10µl Then add sample diluent 40µl to testing sample well; Blank well doesn't add anything. Then Add 100µl of HRP-conjugate reagent to each well, cover with an adhesive strip and incubate for 60 minutes at 37°C. then Aspirate each well and wash, repeating the process four times for a total of five washes. Then Add chromogen solution A 50µl and chromogen solution B 50µl to each well. Gently mix and incubate for 15 minutes at 37°C. Protect from light. Then Add 50µl Stop Solution to each well. The color in the wells should change from blue to yellow. Then Read the Optical Density (O.D.) at 450 nm using a microtiter plate reader within 15 minutes**.**

3. Results:

The current study was a case-control study conducted on 49 patients having ITP and 20 healthy subjects as controls. ITP patients' average age was 6.85 ±2.9 years old, and the average age of healthy controls was 5.81 ±3.4 years old with no statistically significant difference between both groups (p=0.114). Female sex was predominant over male sex (30 females Vs. 19 males), parents' consanguinity was detected in 20.4% and other sibling affection was reported in 4.1% of the studied ITP patients.

Table (1): Sociodemographic data of the studied population

		ITP Cases (n =49)	Healthy Controls (n=20)	P-value
Age, (years) mean ±SD		6.85 ±2.9	5.81 ±3.4	0.114
Sex; N (%)	**Males**	19 (38.8%)	7 (35.0%)	0.420
	Females	30 (61.2%)	13 (65.0%)	
Consanguinity; N (%)	**-ve**	39 (79.6%)		NA
	+ve	10 (20.4 %)		
Family History; N (%)	**-ve**	47 (95.9 %)		NA
	+ve	2 (4.1 %)		

NA: Not Applicable.

Table (2) demonstrates the clinical presentation symptoms among ITP patients' group. The most frequent presentation among ITP patients was purpuric eruptions in 93.3%, followed by ecchymosis in 73.5%, epistaxis in 38.8%, Bleeding gums in 28.6%, Hematuria in 28.6%, Hematemesis in 18.4% and finally pallor in 14.3%.

Table (2): Clinical presentations of the patients group

	Frequency	*Percent*
Purpuric eruption	46	93.9
Ecchymosis	36	73.5
Epistaxis	19	38.8
Bleeding gums	14	28.6
Hematuria	14	28.6
Hematemesis	9	18.4
Pallor	7	14.3

Table (3) demonstrates response to steroids among ITP patients received treatment, partial response was achieved by half the studied patients (51.7%), while complete response was achieved by 34.5% and only four cases (13.8%) showed no response to steroids.

Table (3): Response to steroid in ITP patients received treatment

		Frequency	Percent
Response	**No response**	4	13.8
	Response	15	51.7
	Complete response	10	34.5
	Total	29	100.0

Table (4) demonstrates a comparison between ITP patients and healthy controls as regard laboratory assessments, Hb concentration was statistically significantly higher among healthy controls as compared with ITP patients, (12.61 ±1.13 vs. 11.97 ±1.01 **g/dL**, p=0.024) in healthy controls and ITP patients respectively, but not clinically significant as non of both groups were anaemic.

Direct platelets count was statistically significantly higher among healthy controls as compared with ITP patients, (320.10 ±86.82 vs. 60.93 ±35.84 **g/dL**, p=0.001) in healthy controls and ITP patients respectively.

RUNX-1 expression levels by ELISA were significantly higher in ITP patients compared to controls (9.1 ±1.5 vs. 19.81 ±4.8, p=0.001) in healthy controls and ITP patients respectively

On the other hand, RUNX-1 levels were significantly higher in ITP patients compared to controls (1.78 ±0.73 vs. 3.87 ±2.39, p=0.001) in healthy controls and ITP patients respectively.

MCV and TLC showed non-statistically significant difference between ITP patients as compared to healthy controls, (p-values >0.05).

Table (4): Comparison between ITP patients and controls as regard laboratory data

	ITP Cases (n =49)	Healthy Controls (n=20)	p-value
HB; g/Dl	11.97 ±1.01	12.61 ±1.13	**0.024***
MCV; fL (femoliter)	76.72 ±10.80	77.55 ±5.29	0.747
TLC; ($\times 10^3$/µl)	9.01 ±2.68	8.51 ±3.21	0.504
Direct platelets ($\times 10^9$/L)	60.93 ±35.84	320.10 ±86.82	**<0.001***
RUNX-1 –ELISA	19.81±4.8	9.1±1.5	**<0.001***

Table (5) demonstrates a correlation analysis between RUNX1 expression levels and other studied variables, there was a significant linear negative correlation between direct platelets count and RUNX1 expression levels (r= -0.436, p=0.002).

Other studied variables showed non-statistically significant correlation with RUNX1 expression levels among ITP patients, (p-values >0.05).

Table (5): Correlations between RUNX1 levels and study variables

	R	P
Age	-0.026	0.861
Age at onset	0.072	0.621
Duration of illness	-0.220	0.129
HB	0.007	0.960
MCV	-0.066	0.660
TLC	-0.138	0.344
Platelets	**-0.436**	**0.002***

4. Discussion:

Immune thrombocytopenia is a heterogeneous autoimmunity disease, characterized by immune cells-mediated platelet destruction and/or platelet production defect [16], leading to a lower platelet count, putting patients on a higher risk of bleeding [17]. More and more evidence demonstrated that imbalance of the differentiation of Th1 and Th2 cells play an important role in the development and pathogenesis of ITP [4]. However, in recent years, abnormal Th17 cells differentiation was reported to be another factor contributing to the development of ITP [5].

Runt-related transcription factor (RUNX) proteins are a family of heterodimeric transcription factors which have been shown to have crucial roles in the regulation of the differentiation and function of T lymphocytes [18]. The RUNX family is composed of three members, RUNX1, RUNX2 and RUNX3, each of which forms a functional complex with a core binding factor b (Cbfβ) partner protein [19].

A potential role of the RUNX family in the development of chronic autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and psoriasis, has been reported by several authors [23]. RUNX1 and RUNX3 have primarily been found to regulate genes expressed in the myeloid and lymphoid lineages [19].

A variety of abnormalities within the T cell population in ITP have been described [26], including increased autoreactive T cell reactivity and decreased regulatory T cell function [27]. The current study was designed with an aim to study the expression level of (Runx1) in immune thrombocytopenic patients as compared with healthy controls.

In the present study, female sex was predominant over male sex (30 females Vs. 19 males), however; gender distribution between active, remission and chronic ITP patients showed non-statistically significant difference, similar to this observation the reported equal gender distribution among patients with acute and chronic ITP by ***Bahoush et al.*** [28]. Also, ***Kohli & Chaturvedi*** [2] demonstrated that chronic ITP occurred with equal frequency in males and females, on the opposite side, ***Schmidt et al.*** [29], found significantly more chronic cases among females of 6–14 years of age as compared

to males in the same age category. ***Wong et al.*** [30], reported a predominance of females with chronic ITP.

In the present study, we demonstrated that RUNX-1 levels were significantly higher in ITP patients compared to controls. These results are in line with the reported in a similar study conducted on (30) active ITP patients, (20) ITP in remission and (20) age and gender matched healthy controls to evaluate the expression profile of RUNX1 in patients with active ITP as well as ITP patients with remission, they reported a significantly higher expression of RUNX1 in active ITP patients compared to controls, which was restored into normal value in ITP patients in remission, suggesting association of abnormal expression of RUNX1 with the development of ITP [31].

In the current study, by dividing the ITP patients' group into three subgroups according to disease activity, active ITP patients showed significantly higher levels of expression compared to remission ITP and control groups with no significant differences between the remission subgroup and healthy controls. At the same time, there was non-statistically significant difference between active and chronic ITP groups, while RUNX1 expressions was significantly higher among chronic ITP patients as compared to remission and controls group. Those findings were comparable with the reported in a similar study conducted by ***Bal et al.*** [32], demonstrated a statistically significant downregulation of RUNX1 expression in the chronic ITP patients.

Furthermore, a link between a heterozygous variant in the RUNX1 gene and chronic thrombocytopenia has been found in an adult female aged 29 years who had a life –long tendency for brusing easily and chronic thrombocytopenia.

Another recent study reported similar findings as the current study, they found significant increase of RUNX1gene in active ITP patients, which was restored to normal levels in both ITP patients in remission and controls ($P<0.001$) [34].

5. Conclusion:

In the present study, we evaluate the level of RUNX1 in patients with active ITP as well as normal healthy individuals, and we demonstrated aberrant expression of RUNX1 was observed in ITP patients as compared to healthy controls,

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