Differential Expression of T-bet and GATA3 in Egyptian Children with Idiopathic Thrombocytopenic Purpura

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Abstract GATA3 and T-box (T-bet) expressed in T-cells are transcriptional factors that play a critical role in development of Th2 and Th1 immunity respectively. GATA3 is expressed during Th2 differentiation and T-bet is expressed exclusively in Th1 cells. Thus, a balance between GATA3 and T-bet is believed to control Th2/Th1 polarization. Therefore, the high expression of T-bet and low expression of GATA3 indicate the existence of Th1 polarization in children with acute immune thrombocytopenic purpura (ITP). This might be related to the regulation of T-bet and GATA3. The objective of this work was to study the expression of transcriptional factors T-bet and GATA3 mRNA in children with idiopathic thrombocytopenic purpura and correlate it with clinical findings, laboratory findings, and outcome of patients. In this study the expression of T-bet and GATA3 genes was analysed in 20 normal healthy individuals and 40 children with ITP (20 acute and 20 persistent) using reverse transcriptase polymerase chain reaction to investigate a possible relation, association or correlation with the type of ITP and prognosis. T-bet was expressed significantly in 60 % of acute ITP children (12/20) (*P* value 0.001) and not expressed in persistent ITP children (0/20), while GATA3 was expressed in 25 % of persistent ITP patients (5/20) (*P* value 0.017) and not expressed in acute ITP patients (0/20). Both

genes were not detected in healthy controls. We concluded that the high expression of T-bet and the low expression of GATA3 indicate the existence of Th1 polarization in children with acute ITP. This might be related to the regulation of T-bet and GATA3. Intensive studies of abnormal cytokine profiles in ITP have led to cytokine therapies that exploit the effects of IFN- γ on Th2 cells, but such therapies are often ineffective to develop safe and effective therapeutic tools. Targeting specific molecules such as T-bet and GATA3 may be a novel therapeutic tool in ITP.

Keywords T-bet · GATA3 · ITP · RT-PCR

Introduction

Immune thrombocytopenic purpura (ITP) is defined as isolated low platelet count (thrombocytopenia) with normal bone marrow in the absence of other causes of thrombocytopenia. It is caused by antibodies against platelets [1].

Most often these antibodies are against platelet membrane glycoproteins IIb–IIIa or Ib–IX and are often immunoglobulin G (IgG) type. The stimulus for auto-antibody production in ITP is probably abnormal T-cell activity [2, 3].

A number of T-cell abnormalities have been demonstrated in patients with ITP and three main mechanisms have been hypothesized:

- T-helper 2 (Th2) bias compared with Th1 particularly in persistent ITP
- The release of cytokines that interfere with megakaryocyte maturation and/or platelet release
- A direct cytotoxic effect of T-cells [4].

GATA-binding protein 3 (GATA3) and T-box (T-bet) expressed in T-cells are transcriptional factors that play a

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critical role in development of Th2 and Th1 immunity respectively [5].

T-bet, the master regulator for Th1 differentiation, is defined not only by its ability to activate the set of genes to promote differentiation of a particular phenotype, but also by that of being able to suppress the development of opposing cell lineages [6, 7].

T-bet is the principal transcription factor, as it significantly enhances the production of interferon gamma (IFN- γ), and plays important role in suppressing the development of Th2 and Th17 [8].

T-bet expression was found to be strongly dependent on signal transducer and activator of transcription 1 (STAT1), rather than IL-12 dependent STAT4 [6].

STAT1 is in turn activated by IFN- γ production by differentiating cells, thereby amplifying T-bet expression and up-regulating the expression of IL-12 receptor β 2. The later cells can then be selected by the abundant IL-12 from the antigen presenting cells (APCs), thus ensuring selective expansion of the differentiating Th1 cells. T-bet suppresses development of Th2 cell by inhibiting the crucial IL-4 gene and impairing the function of the Th2 master regulator GATA3 [9]. And by contrast GATA3 is the master regulator transcription factor [10].

Three distinct mechanisms of GATA3 involvement in Th2 differentiation have been postulated, including enhanced Th2 cytokine production, selective proliferation of Th2 cells through recruitment of growth factor independent gene (GFI-1), and inhibition of Th1 differentiation presumably by interacting with T-bet. Moreover, GATA3 was found to suppress Th1 differentiation by down regulating STAT4 in vivo, GATA3 is indispensable for Th2 response [11, 12].

In this study we aimed to study the expression of transcriptional factors T-bet and GATA3 mRNA in children with idiopathic thrombocytopenic purpura and correlate it with clinical findings, laboratory findings and outcome of patients.

Subjects and Methods

The current study was conducted on 40 patients suffering from ITP.

Patients were classified by duration into two groups: The first group (20 patients), termed “Acute (newly diagnosed) ITP” (within 3 months from diagnosis) and the second group, termed “persistent ITP” (between 3 and 12 months from diagnosis, including patients not achieving spontaneous remission or not maintaining complete response after stopping treatment during this period). The different phases and the severity of ITP were defined by the Vicenza Consensus Conference [13, 14].

These patients were diagnosed with primary ITP based on proper history taking, physical examination and laboratory assessment which included (complete blood count revealing platelet count $\leq 100,000/\mu\text{L}$, normal HB concentration and normal WBCs, bone marrow aspiration revealing increased number of megakaryocytes with defective nuclear lobulation, cytoplasmic granulation and platelet budding and antiplatelet antibodies). Exclusion criteria included infants below 6 months of age, recent manifestation of active infection, splenomegaly and secondary causes of ITP.

As regards the 20 acute ITP patients, they were 9 males, 11 females with age ranging from 2 to 17 years (mean 7.4 ± 4.9) years. All acute cases were newly diagnosed and didn't receive any treatment. As regards the 20 persistent patients, they were 12 males, 8 females, with age ranging from 2 to 17 years (mean 9.0 ± 4.1) years. The patients were selected from the pediatric hematology clinic (Beni-Suef university hospital) between January 2012 and June 2012.

The control population consisted of 20 age and sex-matched healthy volunteers. They were 8 males and 12 females with age ranging from 2 to 11 years (mean 6.7 ± 3.7) years. All had complete blood picture with normal platelet count. They had been selected from the children that underwent a health examination in our outpatient department and from the hospitalized patients who had been admitted for elective surgery with no history of recent viral infection, atopy or medication at the time of taking blood samples.

Both the patients and the controls were enrolled into the study after obtaining formal written consent from their parents/guardians, in accordance with Helsinki declaration of Bioethics [15].

Detection of T-bet and GATA3 Genes by RT-PCR

Peripheral blood samples were withdrawn from every patient as well as the healthy volunteers in sterile EDTA vacutainers and centrifuged to obtain mononuclear cells. Total cellular RNA was extracted from mononuclear cells using QIA amp RNA blood minikit (QIAGEN, Catalogue number, 52304) according to the manufacturer's instructions, followed by c-DNA preparation using Revert Aid TM First strand c-DNA synthesis kit (Fermentas, K1621).

A volume of 4 μL cDNA was added to a final PCR reaction mixture of 25 μL polymerase in reaction buffer, MgCl_2 and dNTPs), 1 μL of 25 pmol of each of forward and reverse specific primers of *T-Bet* and *GATA3* gene. For standardization expression of *T-Bet* and *GATA3* gene was correlated with the expression of the internal control primer, β *actin*.

The primers used were as follows:

GATA3:

(F) 5'GAATGCCAATGCGGACCCTG3'

(R) 5'CTAACCCATGGCGGTGACCA3'

T-bet:

(F) 5'CCTCGCACCTGGAGCTGGCTG3'

(R) 5'TTATCAGTTGGGAAAATAGTTA3'

B-actin (House keeping gene):

(F) 5'GTGGGCGCCAGGCACCA3'

(R) 5'CTCCTTAATGTCACGCACGATTT3'

The following PCR cycle was performed for primer amplification:

PCR was run for 33 cycles. Each cycle consisted of 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min. Twenty micro liter aliquots of the amplified product were fractionated on a 3 % agarose gel and visualized by ethidium bromide staining then photographed. The size of the PCR-fragments was estimated using a 100-base-pare ladder (Fermentas™ Germany).

A clear sharp band at 343 bp indicated GATA3 gene.

A clear sharp band at 334 bp indicated T-bet gene.

A clear sharp band at 520 bp indicated β -actin gene.

Treatment of ITP Patients

All persistent ITP patients received Corticosteroids; prednisone in a dose 2 mg/kg/day, maximum dose 60 mg/day.

Statistical Analysis

Data was analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL). Numerical data of scores were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi square test (Fisher's exact test) was used to examine the relation between qualitative variables. For not normally distributed quantitative data, comparison between two groups was done using Mann–Whitney test (non-parametric *t* test). Comparison between three groups was done using Kruskal–Wallis test (non-parametric ANOVA) then post-Hoc “Scheffe test” on rank of variables was used for pair-wise comparison. Spearman-rho method was used to test correlation between numerical variables. Survival analysis was done using Kaplan–Meier method and comparison between two survival curves was done using log-rank test. Odds ratio (OR) with it 95 % confidence interval (CI) were used for risk estimation. The receiver operating characteristic (ROC)

curve was used for prediction of cut off values. Kappa test was used to evaluate agreement between two diagnostic methods. A *P* value <0.05 was considered significant.

Results

The current study was carried out on 40 diagnosed ITP pediatric patients among which 20 were acute ITP and 20 were persistent. Twenty age and sex matched normal individuals were studied as controls. All our cases and controls were subjected to detailed history taking, complete clinical examination, and laboratory investigations including complete blood count, bone marrow aspiration for patients, and T-bet and GATA3 gene expression by reverse transcriptase polymerase chain reaction (RT-PCR). Clinical and laboratory data of the studied groups are presented in Table 1.

Among the 20 acute ITP patients 12 were found to be positive for the T-bet gene expression with a percentage 60 % while 8 patients were negative with a percentage of 40 % (Figs. 1, 2, 3). It was found that GATA3 gene was not expressed in any of the acute ITP cases with a percentage of 0 %.

Among the 20 persistent ITP patients 5 were found to be positive for the GATA3 gene expression with a percentage 25 % while 15 patients were negative with a percentage of 75 %. It was found that T-bet gene was not expressed in any of the persistent ITP cases with a percentage of 0 %.

Both T-bet and GATA3 genes were not expressed in any of the control group with a percentage of 0 %.

Comparing Acute, Persistent and Control Groups as Regards Their Clinical Data (Table 1)

There was no statistically significant difference found between acute, persistent and control groups as regards age (*P* value 0.342) and sex (*P* value 0.504).

Comparing Acute, Persistent ITP and Control Groups as Regards Their Laboratory Data (Table 1)

There was no statistically significant difference between acute, persistent and control groups as regards total leucocytic count (*P* value 0.147), red cell count (*P* value 0.220) and hemoglobin (*P* value 0.119) while there was high statistically significant difference as regards platelets (*P* value 0.001).

Comparing Acute to Persistent ITP Groups as Regards Their Laboratory Data

There was no statistically significant difference between acute and persistent groups as regards total leucocytic

Table 1 Clinical and laboratory data of the studied groups

	Acute ITP N = 20	Persistent ITP N = 20	Control N = 20	<i>P</i> value
Age (years)				
Mean $\pm$ SD	7.4 $\pm$ 4.9	9.0 $\pm$ 4.1	6.7 $\pm$ 3.7	0.342
Range	0.6–17	2.0–17	2–11	
Sex				
Male (No %)	9 (45 %)	12 (60 %)	8 (40 %)	0.504
Female (No %)	11 (55 %)	8 (40 %)	12 (60 %)	
WBCs $\times 10^3$				
Mean $\pm$ SD	10.7 $\pm$ 4.8	10.3 $\pm$ 6.8	6.8 $\pm$ 1.4	0.147
Range	4.4–21	3.6–30.4	4.9–9.5	
RBCs $\times 10^3$				
Mean $\pm$ SD	4.3 $\pm$ 0.8	4.4 $\pm$ 0.5	4.7 $\pm$ 0.4	0.220
Range	3.2–6.7	3.1–5.2	3.9–5.2	
PLTs $\times 10^3$				
Mean $\pm$ SD	24.4 $\pm$ 23.4	105.1 $\pm$ 32.8	263.5 $\pm$ 72.7	0.001*
Range	5–106	64.6–145	180–410	
Hb (g/dl)				
Mean $\pm$ SD	10.8 $\pm$ 2.6	11.4 $\pm$ 1.2	12.3 $\pm$ 0.7	0.119
Range	1.9–15	8.9–13.4	11.2–13.2	

P value >0.05 non Significant*P* value $\leq$ 0.05 significant* *P* value $\leq$ 0.01 highly significant

count (*P* value 0.816), red cell count (*P* value 0.495) and hemoglobin (*P* value 0.296), while there was high statistically significant difference as regards platelets (*P* value 0.001).

Comparing Acute ITP Patients to Control Group as Regards Their Laboratory Data

There was no statistically significant difference between acute ITP group and control group as regards total leucocytic count (*P* value 0.063), red cell count (*P* value 0.084) and hemoglobin (*P* value 0.041) while there was high statistically significant difference between persistent ITP group and control group as regards platelet count (*P* value 0.001).

Comparing Persistent ITP Patients to Control Group as Regards Their Laboratory Data

There was no statistically significant difference between persistent group and control group as regards total leucocytic count (*P* value 0.234) and hemoglobin (*P* value 0.223), while there was high statistically significant difference between persistent ITP group and control group as regards platelet count (*P* value 0.001).

Fig. 1 Comparison between acute, persistent ITP and control group as regards age and laboratory data

Comparing All Groups as Regards GATA3 Gene Expression (Table 2)

There was statistically significant difference between acute, persistent, and control groups as regards GATA3 gene expression (*P* value 0.017).

Fig. 2 T-BET genes expression by RT-PCR. T-BET gene expression by RT-PCR showing a 334 bp PCR product. B-actin specific primers (520 bp) served as an internal control. *Lanes 1, 2, 3, 4, 5, 7* show positive T-BET cases. *Lanes 6, 8, 10, 12, 13* show negative T-BET cases. *Lane C* control cases negative T-BET gene expression. *Lane L* 100–1000 bp ladder size marker

Fig. 3 GATA3 genes expression by RT-PCR. GATA 3 gene expression by RT-PCR showing a 343 bp PCR product. B-actin specific primers (520 bp) served as an internal control. *Lanes 1, 2, 3, 5, 6, 8* show positive GATA3 cases. *Lanes 4, 7* show negative GATA3 cases. *Lane 9* show weak positive case. *Lane C* control case negative GATA3 gene expression. *Lane L* 100–1000 bp ladder size marker

Table 2 GATA3 gene expression in the studied groups

GATA3	Acute ITP no (%)	Persistent ITP no (%)	Control no (%)	<i>P</i> value
Positive	0 (0 %)	5 (25 %)	0 (0 %)	0.017*
Negative	20 (100 %)	15 (75 %)	20 (100 %)	

Comparing All Groups as Regards T-bet Gene Expression (Table 3)

There was high statistically significant difference between acute, persistent and control groups as regards T-bet gene expression (*P* value 0.001).

Discussion

Idiopathic thrombocytopenic purpura (ITP) is an autoimmune disorder characterized by thrombocytopenia (peripheral blood platelet count $<150 \times 10^9/L$) due to autoantibody binding to platelet antigen(s) causing their premature destruction by the reticuloendothelial system, particularly in the spleen [16].

ITP diagnosis is based on low platelet number in the absence of other hematologic abnormalities or other causes of thrombocytopenia [17]. The annual incidence of pediatric ITP is about 4–6 cases per 100,000. About 50 % of childhood ITP cases show an acute onset following a viral or bacterial infection that commonly resolves within weeks to months without treatment.

Nevertheless, about one-fourth of these patients go on to develop a persistent disease, defined by a platelet count less than $150 \times 10^9/L$ at 6 months after diagnosis [18, 19].

Although the immunopathogenesis of ITP is autoantibody mediated, the exact mechanism of immune dysfunction is not known. However, there is substantial evidence to suggest that T-cells and their cytokines play a pivotal role in the control of antiplatelet autoantibodies [17, 18]. A number of T-cell abnormalities have been demonstrated in patients with ITP and three main mechanisms have been hypothesized: (1) a T-helper (Th)1 bias compared with Th2, particularly in persistent ITP; (2) the release of cytokines that interfere with megakaryocyte maturation and/or platelet release; and (3) a direct cytotoxic effect of T-cells [4].

Recently many intracellular molecules have been reported to regulate cytokine expression and T-cell differentiation [5].

GATA3 and T-box expressed in T-cells (T-bet) are transcriptional factors that play a critical role in development of Th2 and Th1 immunity respectively [5].

Induction of the transcription factors GATA-3 and T-bet are critical for differentiation of naïve T-cells into Th2 and Th1 cells respectively [20].

GATA-3 belongs to the GATA family of transcription factors. Six members (GATA-1 to GATA-6) of the family have been identified in avians, with homologues in mammals and avians. Based on their expression profile and structure, the GATA proteins may be classified as haematopoietic (GATA-1 to GATA-3) and non haematopoietic (GATA-4 to GATA-6). Naïve CD4⁺ T-cells express low levels of GATA-3 m-RNA [21]. The expression of GATA-3 is however, markedly upregulated in cells differentiating along the Th2 lineage, and is downregulated in cells differentiating along the Th1 pathway [22].

GATA-3 is expressed during Th2 differentiation via pathways that probably involve the IL-4 dependent activation of the signal transducer and activator of transcription (STAT)-6 and the expression of GATA-3 is critical for Th2 development [23, 24]. In addition, GATA-3 transactivates the expression of genes for IL5 and IL13 directly in Th2 cells [25].

By contrast, T-bet is expressed exclusively in Th1 cells and its expression is mediated mainly by a STAT4 dependent pathway [26]. T-bet, a member of the T-box family of transcription factors, is a master determinant of Th1 lineage [27]. T-bet is induced by STAT-1 mediated signals, which strongly promote IFN- γ and IL-12R β 2 expression for differentiation into Th1 cells. Th1 cytokines are known to inhibit allergic responses [22]. Thus, a balance between GATA-3 and T-bet is believed to control Th2/Th1 polarization. Therefore the high expression of T-bet and low expression of GATA3 indicate the existence of Th1 polarization in children with acute ITP. This might be related to the regulation of T-bet and GATA3 [28].

In the present work, the expression of transcriptional factors T-bet and GATA3 m RNA in children with idiopathic thrombocytopenic purpura (ITP) was studied using

Table 3 T-bet gene expression in the studied groups

T-bet	Acute ITP no (%)	Persistent ITP no (%)	Control no (%)	<i>P</i> value
Positive	12 (60 %)	0 (0 %)	0 (0 %)	0.001*
Negative	8 (40 %)	20 (100 %)	20 (100 %)	

RT-PCR and the results was correlated with clinical findings, laboratory findings and outcome of patients.

Fourty diagnosed ITP patients admitted to Beni Suef university hospital; pediatric hematology unit (20 acute and 20 persistent) as well as 20 normal age and sex volunteers that served as a control group controls were included in this study, the clinical and laboratory data of which are presented in Table 1.

Among the acute ITP patients 12/20 were found to be positive for the T-bet gene expression with a percentage 60 % while 8/20 patients were negative with a percentage of 40 % while none of the acute ITP patients (0/20) expressed GATA3 gene with a percentage of 0 %.

Among the persistent ITP patients 5/20 were found to be positive for the GATA3 gene expression with a percentage 25 % while 15/20 patients were negative with a percentage of 75 % while none of the persistent ITP patients (0/20) expressed T-bet gene with a percentage of 0 %.

Both T-bet and GATA3 genes were not expressed in any of the control groups.

In accordance to our study, Ren [28] studied the expression of the transcriptional factors T-bet and GATA3 mRNA and the levels of IFN- γ and IL-4 in blood of children with acute ITP to investigate the tendency of polarization of Th1/Th2 in children with ITP using RT-PCR for T-bet and GATA3 and ELISA (Enzyme linked immune sorbent assay) for IF- γ and IL-4. They found that blood T-bet mRNA expression and IFN- γ levels in children with ITP in acute stage were markedly higher than those in the remission stage and controls ($P < 0.01$), in contrast, blood GATA3 mRNA expression and IL-4 levels in children with ITP in acute stage were significantly lower than those in the remission stage and controls ($P < 0.01$). They concluded that high expression of T-bet and IFN- γ , and low expression of GATA3 and IL4 indicates the existence of Th1 polarization in children of acute ITP and this might be related to the regulation of T-bet and GATA3.

A different study Arakawo [5] identified intracellular molecules that are responsible for the pathogenesis and the imbalance of cytokines in atopic dermatitis. Semiquantitative RT-PCR revealed that in peripheral blood mononuclear cells without any stimulation the levels of mRNA for GATA-3 and SOCS-3 were elevated, the levels of mRNA for Txk were depressed and the levels of mRNA for T-bet and SOCS-5 were comparable in patients with atopic dermatitis as compared with healthy controls. In addition, successful therapy normalized levels of mRNA for GATA-3 and Txk, although those for the others including IL-4, IL-5, IL-10, IL-13 and IFN- γ did not change. Levels of Txk mRNA correlated with those of IFN- γ , while the mRNA levels of the other regulators did not correlate with those of any of the cytokines. These results suggest GATA-3 and

Txk might be involved in skin lesions, while SOCS-3 might be associated with an imbalance of cytokines that is difficult to normalize in atopic dermatitis.

In their study, Kawashima and Miossec [29] studied interferon γ (IFN γ) production and the expression of T-bet and GATA-3, the transcription factors associated with Th1 and Th2, in peripheral blood mononuclear cells (PBMC) from patients with rheumatoid arthritis before and during infliximab treatment, so as to distinguish between a disease specific and a disease activity dependent defect.

They obtained rheumatoid PBMC at weeks 0 and 6 of infliximab treatment and cultured for 7 days with or without interleukin IL12 or the combination of IL12 and IL18. IFN γ concentrations in supernatants were determined by ELISA. mRNA expression of IFN γ , IL4, T-bet, and GATA-3 was determined by real time RT-PCR in whole blood at weeks 0 and 22. They determined a reduction in spontaneous IFN γ production and in the response to Th1 inducing cytokines in rheumatoid PBMC. Reduction of systemic inflammation with infliximab treatment increased IFN γ production in response to IL12 or IL12 + IL18. The IFN γ /IL4 expression ratio of rheumatoid blood before treatment was lower than in healthy controls but was increased by infliximab treatment. T-bet expression or T-bet/GATA-3 ratio of rheumatoid blood was less than in controls. The T-bet/GATA-3 ratio was not influenced by infliximab treatment.

They concluded that Regulation of T-bet and GATA-3 or IFN γ and IL4 expression appeared different. The IFN γ /IL4 ratio might express the blood Th1/Th2 balance better than the T-bet/GATA-3 ratio. Reduced IFN γ production by rheumatoid PBMC and levels of IFN γ and IL4 mRNA expression in blood were linked to disease improvement, indicating an association between this systemic Th1 feature and disease activity.

Finally we conclude that the high expression of T-bet and the low expression of GATA3 indicate the existence of Th1 polarization in children with acute ITP. This might be related to the regulation of T-bet and GATA3. Intensive studies of abnormal cytokine profiles in ITP have led to cytokine therapies that exploit the effects of IFN- γ on Th2 cells, but such therapies are often ineffective. To develop safe and effective therapeutic tools, we must target specific molecules, such as, T-bet and GATA-3. Further studies to support these findings are highly recommended. Studies involving adult ITP patients and also studies measuring IFN- γ and correlating its levels to GATA-3 and T-bet are thought to add to our findings.

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Compliance with Ethical Standards

Conflict of interest No conflicts of interest to be declared.

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