T cell receptor Zeta chain (TCRζ) expression in patients with idiopathic thrombocytopenic purpura

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ABSTRACT

Background: *T cell receptor zeta chain (TCRζ) plays a critical role in signal transduction via TCR. Defective signaling through TCRζ-CD3 components may disrupt peripheral T cell tolerance to autoantigen, giving rise to a variety of autoimmune diseases. The aim of this work was to evaluate TCRζ chain gene expression in patients with ITP, thereby to understand the functioning status of T cells and its possible contribution to disease outcome.* ***Methods****: SYBR Green based Real-time relative quantitative PCR (qRT-PCR) was used to evaluate TCRζ gene expression in 49 ITP patients and 35 age- and sex-matched control subjects.* ***Results:*** *Patients in acute phase of ITP had a significantly lower TCRζ gene expression compared to those in chronic phase and those of the control group; on the other hand TCRζ gene expression was significantly lower in chronic ITP patients than in controls. Patients with active ITP had a significantly lower TCRζ gene expression compared to those in remission and those of control group ($P < 0.001$). TCRζ gene expression was significantly lower in ITP patients in remission state in comparison to the control group. Treated ITP patients had a significantly higher TCRζ gene expression compared to untreated patients, however TCRζ gene expression in the treated group was still significantly lower compared to controls ($P<0.001$). Complete responders had a significantly higher TCRζ gene expression compared to non responders ($P<0.001$), while no significant difference was found between non-responders and partial responders as regards TCRζ gene expression ($P>0.05$). The level of TCRζ gene expression in complete responders was still significantly lower compared to controls ($P<0.001$). Sequential analyses for TCRζ gene expression in 6 patients with stable disease revealed no significant difference in median TCRζ gene expression values in the 3 consequents analyses ($P>0.05$). TCRζ gene expression showed no significant correlation with any of the clinical or hematological data.* ***Conclusion:*** *From these data, it could be suggested that downregulation of TCRζ may represent intrinsic defects of potential pathogenetic significance for ITP. Therapy targeted to normalization of TCRζ expression may represent a new strategy for treatment of ITP patients after being validated in clinical trials.*

Key words: TCRζ, qRT-PCR, ITP.

INTRODUCTION

Idiopathic/immune thrombocytopenic purpura (ITP) is an autoimmune disorder in which anti-platelet antibodies induce platelets destruction because of an imbalanced immune response. Although anti-platelet auto-antibody production is B-cell mediated, however it requires the presence of autoreactive T cells that are normally suppressed by the

regulatory T cells for immune tolerance[1]. In ITP a potentiated autoreactive T cells action secondary to diminished regulatory T cell numbers and/or function, is manifested suggesting loss of peripheral tolerance[2,3].

The T cell receptor zeta chain (TCRζ) is a stable constituent of the TCR, it is present only in T- and natural killer-cells. TCRζ chain, which also known as CD247, plays a critical role in the T-cell differentiation process and the T-cell effector function. Phosphorylation of TCRζ chain is one of the earliest and key events in the T-cell signal transduction through the TCR-CD3 complex[4]. Even anergy or tolerance induction results from an active and selective signaling process involving phospho-ζ chain via the TCR[5,6]. Defective signaling through TCRζ-CD3 components may disrupt peripheral T cell tolerance to autoantigen, giving rise to a variety of autoimmune diseases[7].

التعليق [o1]: Not recent

Previous studies demonstrated a downregulated expression of the TCRζ chain in several autoimmune diseases; systemic lupus erythematosus[8] (SLE), rheumatoid arthritis[9], Type 1 diabetes mellitus[10] and ITP[11].

Thus, detailed characterization of the expression of the TCRζ chain may provide a key to understanding the molecular basis of T cell dysfunction in ITP patients.

Aim of the work:

The aim of this work was to evaluate TCRζ chain gene expression in patients with ITP, thereby to understand the functioning status of T cells and its possible contribution to disease outcome.

SUBJECTS & METHODS

The present study was conducted on 49 patients with ITP as well as 35 age- and sex-matched control volunteers, after informed consent was obtained from each participant. These patients attended Kasr Al Aini Hospital, Cairo University, to be diagnosed in the Clinical Pathology Department between August 2010 and September 2011. ITP was diagnosed in accordance with the guidelines of the American Society of Hematology[12]. Thrombocytopenia was defined as platelet count $<100\times10^9$/L). The mean age of patients under study was 43.4 ± 17.8 years. Among the 49 patients, 34 were females (69%) and 15 were males (31%). The clinical and laboratory data of the patients group are summarized in table 1.

Real-time relative quantitative PCR (qRT-PCR) with SYBR Green I technique was used to examine TCRζ gene expression level in ITP patients and control subjects, beta 2 microglobulin (β_2M) gene was used as an endogenous reference. RNA extraction from whole blood samples was done using QIAamp RNA Blood Mini Kit, (QIAGEN, Germany, catalog number 52304). Conversion of the extracted RNA to complementary DNA (cDNA) was done using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, USA, #K1622) according to the manufacturer's instructions. QRT-PCR was performed for TCRζ and β_2M on Applied Biosystems 7300

التعليق [o2]: Mention the concentrations of the DNA and primers

التعليق [o3]: Be sure of the degree and the time

thermocycler (Qiagen, CA, USA) using SYBR® Green PCR Master Mix, (Applied Biosystems, Warrington, UK, Catalog Number 4309155) as previously described[11]. The 25µl reaction mixture contained 12.5 µl SYBR Green qPCR Mix (2X), 1µl DNA, 1 µl of each primer pair. The primers used for amplification were; β_2M internal control forward: 5′-TACACTGAATTCACCCCCAC-3′, reverse: 5′-CATCCAATCCAAATGCGGCA-3′. TCRζ mRNA forward: 5′-GCCAGAACCAGCTCTATAAC-3′, reverse: 5′-TAGGCCTCCGCCATCTT ATC-3′[11]. After the initial denaturation at 95°C for 2 min, 45 cycles consisting of 95°C for 15 s, 60°C for 1 min and 82°C for 1s were performed. The relative mRNA expression level of TCRζ gene in each sample was calculated using the ΔΔ cycle time (Ct) method, where the target PCR Ct value, that is the cycle number at which emitted fluorescence exceeds the threshold line, is normalized by subtracting the β_2M Ct value from the target PCR Ct.

Statistical analysis:

Data were statistically described in terms of mean ± standard deviation (± SD), median and range, or frequencies (number of cases) and percentages when appropriate. Comparison of numerical variables between the study groups was done using Mann Whitney U test for independent samples when comparing 2 groups and Kruskal Wallis test with posthoc multiple 2-group comparisons when comparing more than 2 groups. For comparing categorical data, Chi square ($\chi 2$) test was performed. Correlation between various variables was done using Spearman rank correlation equation for non-normal variables. P values less than 0.05 was considered statistically significant. All statistical calculations were done using computer programs SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows.

RESULTS

TCRζ gene expression level in ITP patients ranged from 0.0002 to 18.406, with a median of 0.498, while in the control group, it ranged from 4.112 to 15.612, with a median of 6.308. TCRζ gene expression level was significantly lower in ITP patients compared to the control group (P<0.001) (table 1, figure 1).

TCRζ gene expression level ranged from 0.0002 to 18.150, with a median of 0.648 in ITP male patients and from 0.0002 to 18.406, with a median of 0.363 in ITP female patients, with no statistically significant difference (P >0.05) (table 1).

The clinical severity at the time of sampling was assessed by spontaneous bleeding, the bleeder group had a median TCRζ gene expression of 0.368 (range of 0.0001-9.893), while the non-bleeder group had a median TCRζ gene expression of 0.497 (range of 0.0001-18.406). Comparing bleeders and non bleeders as regards TCRζ gene expression revealed no significant difference (P > 0.05) (table 1).

According to the guidelines of the British Society for Hematology[13], 30 (61%) of our patients were classified

as acute ITP patients (disease duration < 6 months), and 19 (39%) were classified as chronic ITP patients, this classification was done at the time of sampling. Median TCRζ gene expression was 0.200 (range of 0.0002-18.406) and 0.742 (range of 0.0002-7.378) in the acute and chronic ITP groups respectively. Patients in acute phase of ITP had a significantly lower TCRζ gene expression compared to those in chronic phase and those of the control group, on the other hand TCRζ gene expression was significantly lower in chronic ITP patients than in controls (P<0.001) (table 1, figure 1).

Defining the disease state at the time of sampling according to ZHOU et al. (2009)[11], 39 out of 49 patients (79.5%) were in activity (platelet count $<100\times10^9$/L), and 10 out of 49 patients (20.5%) were in remission (platelet count $\geq100\times10^9$/L). Patients with active ITP had a significantly lower TCRζ gene expression (median 0.188, range 0.0001-18.405) compared to those in remission (median 0.916, range 0.130-7.378) (P < 0.001), and those of control group (P < 0.001) (table1, figure 1). On the other hand, TCRζ gene expression was significantly lower in ITP patients in remission state in comparison to the control group (P<0.001) (table 1, figure 1).

At the time of sampling, 37 of the included ITP patients (75.5%) were under steroid therapy, among those steroid-treated patients 9 (18%) were receiving combination of steroids and Immuran. Treated ITP patients had a significantly higher TCRζ gene expression (median 0.569, range 0.0002-9.894) compared to untreated patients (median 0.165, range 0.0001-18.405) (P < 0.001), however TCRζ gene expression in the treated group was still significantly lower compared to controls (P < 0.001) (table 1, figure 1).

Clinical and laboratory response were evaluated in 39 of the included patients (79.5%) who were available for a short period of follow up (3-6 months). They were further classified based on their platelet counts according to Francesco et al. (2009)[14] into complete responders (platelet count ≥100 X 10^9/L), partial responders (platelet count between 30 and 100 X 10^9/L, and at least doubling of the baseline count) and non responders (platelet count lower than 30 X 10^9/L or less than doubling of the baseline count). The definition of response required concurrent resolution of bleeding symptoms. Corticosteroid- or other treatment-dependent patients were considered non-responders. Complete responders, partial responders and non responders, had a range TCRζ gene expression of: 0.001-13.792, median of 0.823, 0.001-18.406, median of 0.138 and 0.001-1.469, median of 0.009 respectively. Complete responders had a significantly higher TCRζ gene expression compared to non responders and to partial responders (P< 0.001), while no significant difference was found between non responders and partial responders as regards TCRζ gene expression (P>0.05). The level of TCRζ gene expression in complete responders was still significantly lower compared to controls (P<0.001) (table 1, figure 1).

In order to examine for variability in the expression level of TCRζ gene, sequential analyses for TCRζ gene expression at two time points (2 months interval) after the base line expression at first sampling, were done for 6 chronic ITP patients selected according to the stability in the clinical course and the platelets count (<10% variation from sampling time count). No significant differences were found between the median expression levels of TCRζ gene in the 1st (0.523, range of 0.0002-6.325), 2nd (0.498, range of 0.001-5.807) and 3rd (0.509, range of 0.008-4.956) analyses respectively (P>0.05) (table 1, figure 1).

TCRζ gene expression showed no significant correlation with any of the clinical (patient age and disease duration) or hematological data (hemoglobin concentration, total leucocytes count and platelets count at sampling time and at follow up as well).

Table 1: Descriptive data of ITP patients, and a comparison of TCRζ expression level in different groups of the studied ITP patients.

Variable	**Value** (Number,%)/(Mean±SD)	**TCRζ expression** Median, range	**P-value**
• ITP patients	49	0.498 (0.0002- 8.406)	<0.001
• Controls	35	6.308 (4.112 to 15.612)	
• Male	15 (31)	0.648 (0.0002-18.150)	> 0.05
• Female	34 (69)	0.363 (0.0002 - 18.406)	
Age in years	43.4 ± 17.8	------	------
Hemoglobin at sampling, g/dl	11.0 ± 2.2	------	------
TLC at sampling, $x10^3/\mu l$	7.5 ± 4.4	------	------
Platelet at sampling, X 10^9/L	13 ± 60.3	------	------
Platelet at follow up, X 10^9/L	145 ± 113	------	------
• Acute	30 (61)	0.200 (0.0002-18.406)	< 0.001
• Chronic	19 (39)	0.742 (0.0002-7.378)	
• Activity	39 (79.5)	0.188 (0.0001-18.405)	< 0.001
• Remission	10 (20.5)	0.916 (0.130-7.378)	
• Bleeder	14 (29)	0.368 (0.0001-9.893)	> 0.05
• Non bleeder	35 (71)	0.497 (0.0001-18.406)	
No treatment	12(24.5)	0.165 (0.0001-18.405)	< 0.001
Treatment	37 (75.5)	0.569 (0.0002-9.894)	
• Steroids alone	28 (57.5)	-----	
• Steroids & Immuran	9 (18)	-----	
Total patients in follow up	39 (79.5)	------	
• Complete response	24 (49)	0.823 (0.001-13.792)	< 0.001[a,b]
• Partial response	8 (16.3)	0.138 (0.001-18.406)	> 0.05[a]
• No response	7 (14.2)	0.009 (0.001-1.469)	------
Sequential analysis:			
• 1st analysis		0.523(0.0002-6.325)	
• 2nd analysis	--------	0.498 (0.001-5.807)	> 0.05
• 3rd analysis		0.509 (0.008-4.956)	

a: compared to no response b: compared to partial response

التعليق [o4]: What about the control sex??

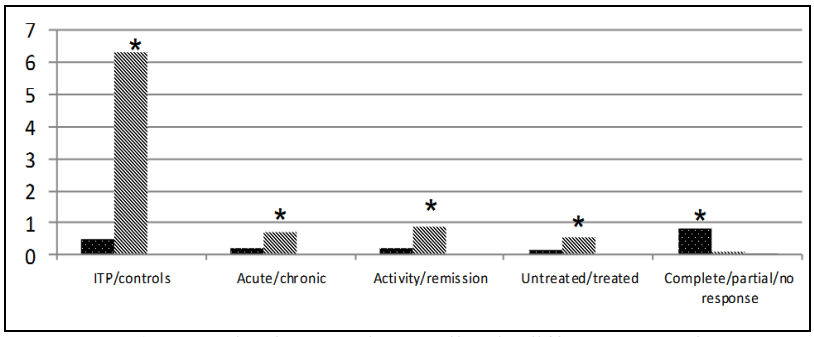

Figure1: TCRζ expression in controls as well as in different ITP patients groups. * = P<0.05 (significant difference).

DISCUSSION

T lymphocytes use a cell surface multisubunit structure, the TCR/CD3 complex, as an antigen-specific recognition site. The TCR αβ (or γδ) chains are responsible for specific antigen binding, but they are unable to transmit any intracellular signals due to the very short cytoplasmic stretches they possess. Signal transduction is carried out by the T lineage-specific CD3 molecule, which is associated with the Ag-binding TCR chains, as well as with the TCRζ chain dimer[15,16].

One aspect of T cell physiology which is influenced significantly by the absence of the ζ chain is the positive and negative selection of the immature thymus-dependent cells. Absence of the ζ chain decreases both positive and negative selection. The decreased negative selection may enhance an autoimmune repertoire, and the decreased positive selection may be the basis for the decreased regulatory immune responses against autoreactivity[17]. Further support for the former hypothesis comes from a study reporting an enhanced autoimmune potential for the T cells produced by ζ-knockout mice[18].

This study demonstrated that TCRζ gene expression level was significantly lower in ITP patients compared to the control group. Reviewing literature, our study finding was in agreement with the only study that was found to analyze TCRζ gene expression in ITP (18 chronic patients) [11]. In confirmation to the current study results, a significant decrease in TCRζ gene expression was reported in other autoimmune diseases, including SLE[19], RA[20,21] and Type 1 diabetes[10]. On the other hand, **Pang et al.** [22] reported that flow cytometric analysis demonstrated a significant decrease in the expression of TCRζ in SLE, but in the other rheumatic diseases (RA, systemic sclerosis and primary Sjögren's syndrome), they noted a trend toward a decreased expression of TCRζ but with no statistical significance.

No statistically significant difference was found in this study between males and females as regards TCRζ gene expression levels. This

was in agreement to **Zhang et al.** [11] who reported no significant correlation between TCRζ gene expression level and gender of ITP patients.

The current study reported that patients in acute phase of ITP had a significantly lower TCRζ gene expression compared to those in chronic phase and those of the control group. On the other hand, TCRζ gene expression was significantly lower in chronic ITP patients than in controls. Confirming to our findings, **Zhang et al.** [11] reported that chronic ITP patients had a significantly lower TCRζ gene expression compared to healthy controls, it's worth noting that in their study none of the included ITP patients was in acute phase of the disease.

This study demonstrated that active ITP patient group had a significantly lower TCRζ gene expression compared to those in remission and those of control group. On the other hand, TCRζ gene expression was significantly lower in ITP patients in remission state in comparison to the control group. No similar analysis was found in literature for ITP patients. As regards other autoimmune diseases, **Pang et al.** (2002)[22] reported that one-third of the SLE patients exhibited a significant change in TCR ζ expression at different levels of disease activity, while two-thirds of the patients showed a relatively stable low expression. No significant difference in the expression level of TCRζ between the active and inactive patients was reported by **Liossis et al.** [17] in SLE patients. This discrepancy needs to be further studied with consideration to different disease pathogenesis.

In the present study a significant up-regulation for TCRζ was demonstrated in the steroid treated ITP patients versus untreated patients. In SLE, although **Pang et al.** [22] reported that three SLE patients in whom treatment could normalize the level of TCR ζ expression, however their final statistical conclusion was that deficient TCRζ chain expression is treatment unrelated. **Liossis et al.** [17] demonstrated that the decrease in TCR ζ did not correlate with the dose of prednisolone.

In the current study among patients who were available for follow up, complete responders had a significantly higher TCRζ gene expression compared to non responders and partial responders while no significant difference was found between the latter groups as regards TCRζ gene expression.

Sequential analyses for TCRζ gene expression revealed no significant differences between the median expression levels of TCRζ gene in the 1st , 2nd and 3rd analyses. This was in agreement to **Pang et al.** [22] as they reported that two-thirds of the included SLE patients showed a relatively stable low expression of TCRζ gene at two time points.

In this study, TCRζ gene expression showed no significant correlation with any of the clinical or hematological data at sampling time and at follow up as well. In confirmation **Zhang et al.**[11] demonstrated no correlation between TCRζ gene expression level with either age or gender of ITP patients.

Many theories have been postulated as how TCR ζ defects lead to autoimmunity. In TCR ζ chain knock-out mice, a shift from conventional thymic differentiated T cells to intestinal intraepithelial lymphocytes (iIEL), which differentiate from the extrathymic environment, has been reported[18]. As it has been suggested that autoreactive T cell clones are more numerous in iIEL[23], this could account for the autoimmune features in ITP with ζ defects, as in ζ knock-out mice.

The alternative hypothesis arises from demonstrations that autoreactive clones are not deleted by negative selection, but selected positively in thymic differentiation, and expanded in the periphery in TCR ζ chain knock-out mice[18,24,25], suggesting a role of the TCRζ chain in negative selection in the thymus.

In addition, it has been proposed that the T cell anergy and altered receptor signaling in regulatory T cells (secondary to defective TCRζ expression) may lead to autoimmunity by breaking the peripheral tolerance[26].

Understanding the underlying defects within T lymphocytes is of critical importance not only for understanding disease pathophysiology, but also for identifying predictive biomarkers and better therapeutic targets.

Treatment of SLE patients with rapamycin, (also known as sirolimus), led to normalization of TCRζ expression and calcium flux in T cells[27]. Lupus-prone mice treated with sirolimus showed reduced proteinuria, nephritis, and anti-dsDNA antibodies[28]. In a preliminary report, seven of nine SLE patients refractory to conventional immunosuppression showed an improvement in disease activity scores after treatment with sirolimus[29]. Phase II trials of sirolimus in SLE and lupus nephritis are in progress.

In conclusion, from these data, it could be suggested that downregulation of TCRζ may represent intrinsic defects of potential pathogenetic significance for ITP. Further experiments, for example showing the normalization of TCR-CD3 expression with genetic manipulation of TCRζ to normal level, and concerning the detailed characterization of the downstream signaling molecules are required to understanding the molecular basis of TCR-CD3 dysfunction in ITP patients. Therapy targeted to normalization of TCRζ expression may represent a new strategy for treatment of ITP patients after being validated in clinical trials.

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التعبير عن سلسلة زيتا فى مستقبلات الخلايا الليمفاوية تي في مرضى نقص الصفائح الفرفرى غير المسبب

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سلسلة زيتا من مستقبل الخلايا الليمفاوية تي (TCRζ) تلعب دورا حاسما في نقل الإشارات عبر مستقبل الخلايا تى. تعطيل نقل الأشارات عبر CD3-TCRζ قد يؤدى الى فقد التواؤم الذاتى مما يؤدى إلى مجموعة متنوعة من أمراض المناعة الذاتية. يهدف هذا العمل الى تقييم التعبيرعن TCRζ كمحاولة لفهم الحالة الوظيفية للخلايا T في مرضى فرفرية نقص الصفائح مجهول السبب ودراسة إمكانية وجود علاقة بين مستوى التعبير عن TCRζ ومسار المرض. تم تقييم التعبير عن TCRζ باستخدام سلسلة البوليميرازالكمي في الوقت الفعلي بتحليل رد الفعل (PCR-qRT)، في ٤٩ من المرضى الذين يعانون من آي تي بي، فضلا عن خمسة وثلاثون من الأصحاء كمجموعة ضابطة مطابقة فى العمر والجنس. اسفرت الدراسة عن وجود انخفاض مستوى التعبيرعن TCRζ فى مرضى ITP مقارنة بمجموعة الأصحاء. لم يظهر فرق ذا دلالة احصائية بين النساء والرجال فى التعبيرعن TCRζ. كان مستوى التعبيرعن TCRζ أقل في مرضى ITP النشطة مقارنة مع الموجودة في هدأة والمجموعة الضابطة بينما كان مستوى التعبيرعن TCRζ أقل في مجموعة ITP الهدأة عن المجموعة الضابطة. كان مستوى التعبيرعن TCRζ أقل في مجموعةITP الحاد بالمقارنة مع المرضى الذين يعانون منITP المزمن والمجموعة الضابطة . برغم الارتفاع ذا الدلالة الاحصائية فى مستوى التعبيرعنTCRζ بعد العلاج عن ما قبله الا انه لم يصل الى مستوى التعبيرفى المجموعة الضابطة . فى مرضى المتابعة كان مستوى التعبيرعن TCRζ أعلى مرضى ITP المستجيبين مقارنة مع الغير مستجيبين ومرضى الإستجابة الجزئية بينما لم يختلف مستواه فى المجموعتين الأخيرتين. التحليل التتابعى لمستوى التعبيرعن TCRζ فى ٦ من المرضى مستقرى الحالة لم يسفر عن اختلاف احصائى. لم يثبت وجود علاقة بين مستوى التعبيرعنTCRζ وأى من البيانات الاكلينكية او المعملية للمرضى. .الدراسة الحالية تؤكد التقارير السابقة التى تشير إلى انخفاض التعبير عن TCRζ في المرضى الذين يعانون من مرض ITP النشط. وكانت الاستجابة للعلاج والهدأة فى المرض مصاحبه للزيادة في التعبير عن TCRζ معبرة عن اتصاله بشدة المرض.المعالجة التي تستهدف الحث على التعبيرعن TCRζ تحمل أملا جديدا لمرضى فرفرية نقص الصفائح مجهولة السبب.