Article

The relationship between serum TIM-3 and TIM-3 gene expression in a sample of Iraqi patients with multiple sclerosis

Fadel Shaker Hammood*, and Bushra Jasim Mohammed
Institute of Genetic Engineering and Biotechnology for Post Graduate Studies, University of Baghdad, Iraq.
* Correspondence: Email: fadelshaker6@gmail.com, dr.bushrajassim@ige.uobaghdad.edu.iq

Abstract

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) caused by auto-reactive T cells against myelin antigens. T-cell immunoglobulin mucin -3 (TIM-3) is a negative regulator glycoprotein expressed by a range of immune cells. A defect in TIM-3 regulation has been shown in multiple sclerosis patients. This study was planned to investigate the correlation between serum TIM-3 and TIM-3 gene expression in a sample of Multiple Sclerosis Iraqi patients. Three ml of blood samples were collected from fifty Iraqi patients who have Multiple Sclerosis (men and women) with ages ranging between 20-57 years, and 50 healthy volunteers as a control group; 0.25ml of blood was put in Trizol tube for RNA extraction, subsequently to estimate TIM-3 gene expression by one step RT-qPCR, and 2.75 ml of blood placed into gel tube for determination TIM-3 serum level by enzyme-linked immunosorbent assay (ELISA), the Statistical analysis was done by using program of Statistical Analysis System (SAS). There was a significant increase ($P \leq 0.05$) in TIM-3 gene expression for patients (5.30-fold) when compared with control (7.86-fold). Moreover, the result demonstrated a high significant elevated ($P \leq 0.01$) in TIM-3 serum level of patients (0.398 pg/ml) as compared to control (3.17 pg/ml. Furthermore, the findings showed a strong positive association between TIM-3 serum level and TIM-3 mRNA expression with significant differences. The current study concluded that the TIM-3 gene expression and TIM-3 serum level were high in MS patients, and there was a direct positive relationship between TIM-3 gene expression and TIM-3 serum level.

Keywords: MS, TIM-3, RT-qPCR.,ELISA

Introduction

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disorder of the central nervous system (CNS) affecting about 2–3 million people worldwide that is triggered by both environmental and genetic factors 1,2. About 15–30% of MS

patients present the relapsing-remitting clinical course, characterized by acute neurological dysfunctions, such as optic neuritis, sensory disturbances, or motor impairments, usually followed by periods of recovery or remission3. After variable periods, about 50% of relapsing-remitting MS patients progress to a chronic secondary progressive clinical stage that is characterized by steadily worsening disability 4 In about 15% of patients, MS is progressive from the onset and is called primary progressive MS, a clinical course characterized by a gradual and constant decline in neurological functions5 The pathological hallmarks of MS are the breakdown of the blood–brain barrier oligodendrocyte loss, demyelination, astrocytes gliosis, and axonal degeneration 6,7. Inflammation is present at all stages, and pro-inflammatory cytokines and chemokines play a critical role in the pathophysiology of MS by compromising the blood–brain barrier, recruiting immune cells from the periphery and activating resident microglia 8.TIM-3 is a negative regulator of immune responses that is specially expressed on activated Th1 cells, CD8+ T cells and at a lower level on Th17 cells but not on Th2 cells 9-12. TIM-3 expressing regulatory T cells can do a high- level of Th1 and Th17 cell suppression compared to TIM3 negative counterparts 13. Dysregulated expression of TIM-3 on T-cells resulted in enhanced T-cell resulted in enhanced T-cell proliferation and IFN-γ secretion following T-cell stimulation14. TIM-3 interacts with its ligand (Galectin-9) to regulate T-cell responses. Blocking of (Tim-3/Gal-9 interaction with specific monoclonal antibody reduced lymphocyte apoptosis) and augmented production of IFNγ and IL-17 in the relapsing-remitting MS15. Preventing Tim-3 signaling in CD4+ T cells influenced the localization of inflammation in the brain and spinal cord via alteration in the Th17:Th1 ratio 16. Hence, and because of the consequence of MS health problems, the present research was carried out to investigate the correlation between serum TIM-3 and TIM-3 gene expression in a sample of Multiple Sclerosis Iraqi patients

Materials and Methods

The current study was conducted in the Institute of Genetic Engineering and Biotechnology for Postgraduate Studies laboratories at the University of Baghdad. A group of 50 MS patients aged 20 to 57 years and 50 age and gender-matched healthy controls were enrolled in the study. Three ml of blood was collected from all individuals; 0.25 ml was put into a Trizol (0.75 ml) preservation tube for RNA extraction using an RNA purification kit (Promega/USA). RNA concentration and purity were estimated according to Mohammed[17] by using Nanodrop (Bioneer/Korea). Subsequently, expression of TIM-3 gene detection was done using One Step RT-qPCR, according to Mohammed[18], using a specific primer supplied by (Macrogen/Korea), depending on this study as illustrated in Table (1). Two

ml of blood was placed into a gel tube to determine TIM-3 serum level by enzyme-linked immunosorbent assay (ELISA) using the Human TIMD3 ELISA kit, as illustrated in Table (6).

Primers name	Sequence 5`- 3`	Annealing Temp. (ºC)	Reference
β-Globin-F	ACACAACTGTGTTCACTAGC	65	This study
β-Globin-R	CAACTTCATCCACGTTCACC		
TIM3_exp-F	ACTCTAGCAGACAGTGGGATC	60	
TIM3_exp-R	GGTGGTAAGCATCCTTGGAAAGG		

Table 1. Primers of TIM3 and β-Globin with their sequences.

Components of PCR mixture reactions of 10µl volume, including qPCR Master Mix (Promega/USA), are shown in Table 2.

Master-mix components	Volume µl
qPCR Master Mix	5
RT mix	0.25
MgCl2	0.25
Forward primer	0.5
Reverse primer	0.5
Nuclease Free Water	2.5
RNA	1
Total	10

Table 2. Reaction component for PCR reactions.

The PCR amplification was done by using RT-qPCR (Molecular System / Australia) according to the program which clarified in Table (3)

Real-Time PCR Program			
Steps	°C	m: s	Cycle
RT. Enzyme Activation	37	15:00	1
Initial Denaturation	95	5:00	40
Denaturation	95	00:20	
Annealing	60, OR 65	00:20	
Extension	72	00:20	

Table 3. Amplification fragments PCR program.

Estimation of TIM-3serum level was done by using the ELISA technique and TIM-3 ELISA kit (Changsheng/China), according to Mohammed [19].
Results analysis was done by using the program Statistical Analysis System-SAS[20] to estimate the effect of different factors in work parameters. T-test was used to compare between means significantly, and the Chi-square test was used to compare percentages significantly.

Results

The comparison of TIM-3 gene expression in patients and control groups revealed a substantial decrease in TIM-3 average folding in patients (5.30 fold) in opposition to the control group (7.86 fold) with significant differences (P ≤ 0.05) as explained in the Table (4) and figures (1and 2).

Group	Mean				
	Beta globin	TIM-3	Delta CT	Delta Delta CT	TIM-3 Folding
Patients	16.16	29.60	13.44	0	5.30
Control	15.19	29.52	14.33	0.89	7.86
P-value					0.044 *
* (P≤0.05).					

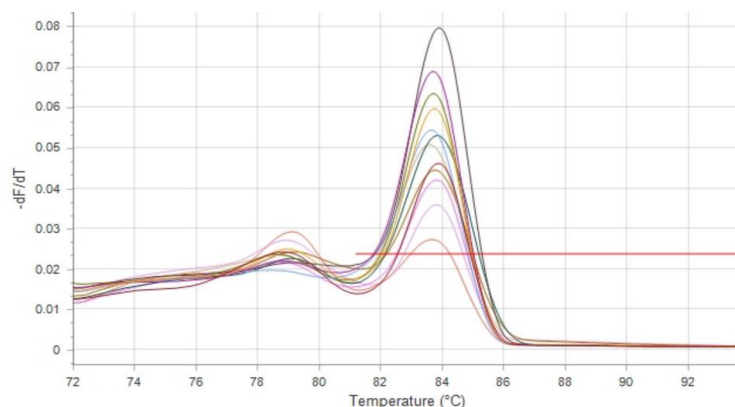**Table 4. Comparison between TIM-3 gene expression in MS patients and control groups.**

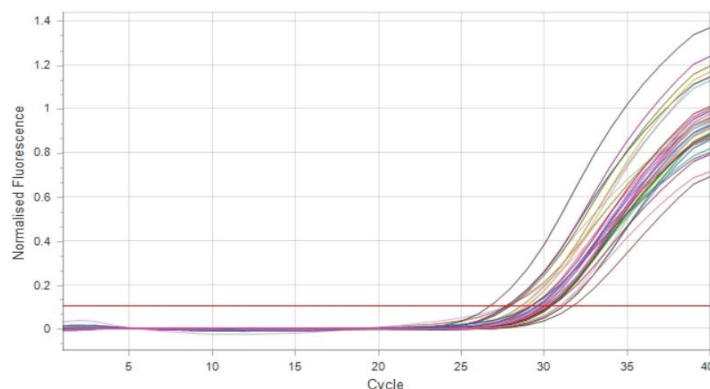**Figure 1. The Tim-3 expression Melting curve.**

Figure 2. The Tim-3 expression cycling curve.

The results of the comparison between serum TIM-3 levels in MS patients and control groups revealed a significant low in the patient's serum level (0.398pg/ml) as compared to the control group (3.17pg/ml) with highly significant differences (P ≤ 0.01) as shown in table 5 and figure 3.

Group	Mean ofserum TIM-3 (pg/ml)
Patients	0.398
Control	3.17
T-test	0.278**

P-value	0.0001
** (P≤0.01).	

Table 5. Comparison between serum TIM-3 levels in MS patients and control groups.
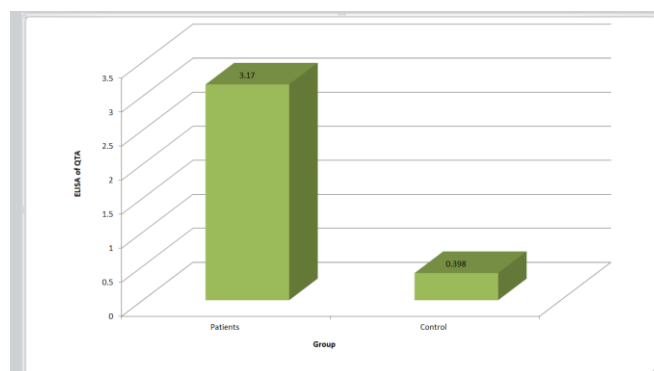
Figure3. Comparison between patients and control groups in TIM-3 serum level.

Correlation between serum TIM-3 and TIM-3 gene expression in MS patients and control groups:

Current findings of the association of serum TIM-3 and TIM-3 gene expression demonstrated that there is a strong positive association between TIM-3 serum level and TIM-3 mRNA expression with significant differences (P≤0.05) and (P≤0.01), as shown in Table 6.

Group	Mean	
	TIM-3 serum (pg/ml)	TIM-3 gene expression (Folding)
Patients	0.398	5.30
Control	3.17	7.86
P-value	0.0093 **	0.044 *
* (P≤0.05), ** (P≤0.01).		

Table 6. Correlation between serum TIM-3 and TIM-3 gene expression in MS patients and control groups.

Discussion

The TIM-3 plays a crucial role in inhibiting adaptive and innate immune responses. Low expression of Tim-3 on effectors T cells indicates T cell motivation, stimulating proliferation and TNF-α and IFN-γ secretion. Upregulation of Tim-3 on macrophages facilitates their polarization and increases IL-6 secretion [21]. Tim-3 appears to have conflicting effects on DCs and NKs as its different ligands. In mice, the interaction of TIM3 with TIM3 ligand appears to regulate both the function of Th1 cells and the ability to induce tolerance [22]. In addition, TIM3-deficient mice are refractory to the induction of high dose tolerance [23]. Galectin-9 has been identified as a ligand of TIM3 and has been shown to suppress IFN-γ secretion [24,25]. Suggested that human TIM3 may similarly play a role in the regulation of Th1 cells and maintenance of tolerance in the context of MS. Specifically, they demonstrate that reduction of TIM3 expression on human CD4+ T cells enhances both proliferation and IFN-γ secretion after T cell stimulation and that CSF clones from MS patients express lower levels of TIM3 than do those from control subjects, yet secrete higher levels of IFN-γ.

Moreover, T cell clones derived from the cerebrospinal fluid (CSF) of patients with MS that express lower levels of TIM3 than control clones are resistant to tolerance induction. Reduced TIM3 expression may allow uninhibited expansion of IFN-γ--secreting cells in the target organ. Indeed, failure to up-regulate T cell expression of TIM3 in inflammatory sites may represent a novel, intrinsic defect that may contribute to the pathogenesis of MS and other human autoimmune diseases.TIM-3 is a negative regulator of immune responses that is specially expressed on activated Th1 cells, CD8+ T cells and at a lower level on Th17 cells but not on Th2 cells [23,26,27]. TIM-3 expressing regulatory T cells can do a higher Th1 and Th17 cell suppression level than TIM3 negative counterparts [28]. Blocking TIM-3 signaling in CD4+ T cells altered inflammatory localizations in the brain and spinal cord through changes in the Th17:Th1 proportion and aggravated investigational EAE driven through CD8+ T cells. Furthermore, CD4+ T cell clones from MS individuals' CSF released considerably higher IFN-γ than usual but displayed lower amounts of TIM-3 mRNA [29]. In average persons, suppressing TIM-3 throughout T-cell activation stimulated the secretion of IFN-γ but had no impact in reference MS individuals. In current findings, comparing TIM-3 gene expression in patients and control groups reveals a substantial decrease in TIM-3 average folding in patients vs. control group. These findings were consistent with those of Mohammad Zadeh (1), who discovered that CTLA-4, PD-1, and TIM-3 genes were significantly downregulated in MS patients compared to healthy controls. Moreover, Anderson [30] reported that T cells in MS patients' cerebrospinal fluid (CSF) expressed TIM-3 less often and released more IFN-γ than T cells in the CSF of unaffected individuals. Additionally, the inability to establish tolerance in these cells is linked to the lower expression of TIM-3 in CSF T cells from MS patients. Unfortunately, there are no previous researches dealing with this topic of determine serum TIM-3 levels in MS patients, and perhaps the reason for this is due to the novelty of the TIM-3 topic as checkpoint receptor and the focus of researches on the genetic side of the topic regarding the gene expression of mRNA, which is conceders as the basis for the presence of TIM-3 protein in the blood circulation, but it is possible to explain the result of lowered TIM-3 serum level in MS patients logically and scientifically that since the TIM-3 gene expression is low, it is accepted that its presence of TIM-3 protein in the blood circulation is low, and if the TIM-3 gene expression was high and the presence of TIM-3 protein in the blood circulation was low, it can be return the reason to post-translational modifications, but in this case the situation was consistent and the result of the TIM-3 serum level in the is consistent with the low level of gene expression, which gives support to the results of gene expression. Also, previous research has not addressed the correlation between serum TIM-3 and TIM-3 gene expression in MS patients. It is accepted that the decline was simultaneous in both cases, serum TIM-3 and

TIM-3 gene expression, and if the result were opposite, the interpretation of the result would return to post-translation modifications.

Conclusion
The current study concluded that the TIM-3 gene expression and TIM-3 serum level were high in MS patients, and there was a direct positive relationship between TIM-3 gene expression and TIM-3 serum level.

References

1. Thompson, A.J.; Banwell, B.L.; Barkhof, F.; Carroll, W.M.; Coetzee, T.; Comi, G.; Correale, J.; Fazekas, F.; Filippi, M.; Freedman, M.S.; et al.Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. Lancet Neurol., 2018; 17, 162–173.
2. Dendrou, C.A.; Fugger, L.; Friese, M.AImmunopathology of multiple sclerosis. Nat. Rev. Immunol.,2015;15, 545–558.
3. Brownlee, W.J.; Hardy, T.A.; Fazekas, F.; Miller, D.H. Diagnosis of multiple sclerosis: Progress and challenges. Lancet,2017; 389, 1336–1346.
4. Lorscheider, J.; Buzzard, K.; Jokubaitis, V.; Spelman, T.; Havrdova, E.; Horakova, D.; Trojano, M.; Izquierdo, G.; Girard, M.; Duquette, P.; et al. We are defining secondary progressive multiple sclerosis. Brain,2016; 139, 2395–2405.
5. Lassmann, H.; van Horssen, J.; Mahad, D. Progressive multiple sclerosis: Pathology and pathogenesis. Nat. Rev. Neurol.,2012; 8, 647–656.
6. Trapp, B.D.; Nave, K. A multiple sclerosis: An immune or neurodegenerative disorder? Annu. Rev. Neurosci.,2008; 31, 247–269.
7. Baecher-Allan, C.; Kaskow, B.J.; Weiner, H.LMultiple sclerosis: Mechanisms and immunotherapy. Neuron,2018; 97, 742–768.
8. Thompson, A.J.; Baranzini, S.E.; Geurts, J.; Hemmer, B.; Ciccarelli, O. Multiple sclerosis. Lancet, 2018;391, 1622–1636.
9. Kuchroo VK, Meyers JH, Umetsu DT, DeKruyff RH. TIM family of genes in immunity and tolerance. AdvImmunol,2006; 91:227-249.
10. Sabatos CA, Chakravarti, S.; Cha E, et al. Interaction of Tim-3 and Tim-3 ligand regulates T helper type 1 responses and induction of peripheral tolerance. Nature Immunol, 2003;4(11):1102-1110.
11. Hastings,WD.; Anderson, DE.; Kassam, .; Koguchi ,K.;,Greenfield,. EA.; et al. TIM- 3 is expressed on activated human CD4+T cells and regulates Th1 and Th17 cytokines. *Eur J Immunol*, **2009**;*39(9)*:2492-2501.
12. Gorman, JV.; Colgan, JD. Regulation of T cell responses by the receptor molecule Tim-3. Immunol Res, 2014;59(1-3):56-65.
13. Gautron ,AS.; Dominguez-Villar, M,.;de Marcken ,M.; Hafler, DA .Enhanced suppressor function of TIM-3 FoxP3 regulatory T cells.*Eur J Immunol*, **2014**;*44(9)*:2703-2711
14. Koguchi, K,.; Anderson, DE.; Yang,L.; O'Connor, KC.; Kuchroo,VK,.; Hafler ,DA . Dysregulated T cell expression of TIM3 in multiple sclerosis. *J Exp Med*, **2006**;*203(6)*:1413- 1418.
15. Saresella ,M.; Piancone ,F.; Marventano ,I.; et al. .A role for the TIM-3/GAL-9/BAT3 pathway in determining the clinical phenotype of multiple sclerosis. *FASEB J*, **2014**;*28(11)*:5000- 5009.
16. Lee, SY.; Goverman, JM. The influence of T cell Ig mucin-3 signaling on central nervous system autoimmune disease is determined by the effector function of the pathogenic T cells. *J Immunol*, **2013**;*190(10)*:4991-4999.

17. Mohammed, B. Investigation on the effect of different concentrations of chlorine drinking water on mice livers. *Biochemical and Cellular Archives*, *18*(Suppl. 1),**2018**; 1069-1073.

18. Mohammed, B.. Effect of gasoline inhalation on tumor necrosis factor-alpha (TNF-alpha) expression in liver of rats. *Bioscience Research*, 2017;*14*(3), 566-573.

19. Mohammed,B.. Association between TNF-α level and TNF-α gene polymorphisms in liver cirrhosis of Iraqi patients, Bioscience research,2018; 15(2): 1342-1349

20. SAS. Statistical Analysis System, User's Guide. Statistical. Version 9.6^{th} ed. SAS.Inst.Inc. Cary. NC. USA,2018.

21. Yan,W,.;Liu ,X.; Ma ,H.; Zhang ,H.; Song, X.; Gao, L.; Liang ,X,.;Ma, C. Tim-3 fosters HCC development by enhancing TGF-beta-mediated alternative activation of macrophages. Gut.2015; 64(10):1593–604.

22. Sánchez-Fueyo, A.; Tian, J.; Picarella, D.; Domenig, C.; Zheng, X. X.; Sabatos, C. A.; ... and Strom, T. B. Tim-3 inhibits T helper type 1–mediated auto-and alloimmune responses and promotes immunological tolerance. Nature immunology, 2003;4(11), 1093-1101.

23. Sabatos, C.; Chakravarti,S,.;Cha, E.; et al. Interaction of Tim-3 and Tim-3 ligand regulates T helper type 1 responses and induction of peripheral tolerance. Nature Immunol, 2003;4(11):1102-1110

24. Zhu, C.;Anderson, A. C.; Schubart, A.; Xiong, H.; Imitola, J.; Khoury, S. J.; and Kuchroo, V. K. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. Nature immunology,2005; 6(12), 1245-1252.

25. Koguchi, K., Anderson, D. E., Yang, L., O'Connor, K. C., Kuchroo, V. K., and Hafler, D. A. Dysregulated T cell expression of TIM3 in multiple sclerosis.*The Journal of Experimental Medicine*,**2006**; *203(6)*, 1413-1418.

26. Hastings, WD,.;Anderson ,DE.; Kassam ,N .;Koguchi ,K.; Greenfield, EA.; et al. TIM cells and regulates Th1 and Th17 cytokines Eur J Immunol, 2009;39(9):2492-2501 is expressed on activated human CD4+T.

27. Gorman, JV.; Colgan, JD. Regulation of Tcell responses by the receptor molecule Tim-3. Immunol Res,2014; 59(1-3):56-65.

28. Gautron ,AS.; Dominguez-Villar ,M.; de Marcken,M,.;Hafler DA. Enhanced suppressor function of TIM-3 FoxP3 regulatory T cells.*Eur J Immunol*, **2014**;*44(9)*:2703-2711.

29. Mastorodemos, V.; Ioannou, M.,;and Verginis, P. Cell-based modulation of autoimmune responses in multiple sclerosis and experimental autoimmune encephalomyelitis: therapeutic implications. Neuroimmunomodulation, 2015;22(3), 181-195.

30. Anderson, A. C., Lord, G. M.; Dardalhon, V.;Lee, D. H.; Sabatos-Peyton, C. A., Glimcher, L. H., and Kuchroo, V. K. T-bet, a Th1 transcription factor regulates the expression of Tim-3. **European journal of immunology**, **2010**;*40(3)*, 859-866.