Annals of Hematology
https://doi.org/10.1007/s00277-021-04605-w

ORIGINAL ARTICLE

Expression of the immune checkpoint receptors *CTLA-4*, *LAG-3*, and *TIM-3* in β-thalassemia major patients: correlation with alloantibody production and regulatory T cells (Tregs) phenotype

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Received: 28 February 2021 / Accepted: 6 July 2021

Abstract

Alloimmunization is a serious complication in β-thalassemia major patients as a result of repeated blood transfusion. The immune checkpoint receptors play an important role in regulating immune system homeostasis and the function of the immune cells. This study aimed to evaluate the expression of cytotoxic T-lymphocyte–associated protein 4 (*CTLA-4*), lymphocyte activation gene 3 (*LAG-3*), and T-cell immunoglobulin and mucin domain-containing protein-3 (*TIM-3*) immune checkpoint molecules in β-thalassemia major patients with and without alloantibody. For this purpose, 68 β-thalassemia major patients with (34 patients) and without (34 patients) alloantibody as well as 20 healthy controls were enrolled. The expression of these genes was evaluated in different groups of patients by SYBR Green real-time PCR method. Our results showed that the mean expression of *LAG-3* was significantly increased in thalassemia patients compared to the control group ($^*P < 0.001$). However, there was no significant difference in expression of the *CTLA-4* and *TIM-3* as well as *LAG-3* genes between patients with and without alloantibody ($P > 0.05$). A positive correlation was observed between the level of *LAG-3* expression with markers associated with Treg function including *FOXP3* and *GDF-15* genes in β-thalassemia major patients. Taken together, the *LAG-3* molecule might have a more prominent role in the abnormality of the immune system in thalassemia patients especially the function of regulatory T cells (Tregs), prior to the *CTLA-4* and *TIM-3* genes.

Keywords Thalassemia · *CTLA-4* · *LAG-3* · *TIM-3* · Alloimmunization · Regulatory T cells (Tregs)

Introduction

β-Thalassemia major is one of the most common hematologic disorders, which is caused by reduced or absence of β-globin chain synthesis and is inherited as autosomal recessive [1]. Epidemiologic studies have shown that the carriers of β-thalassemia are about 1–20%, and each year, approximately 23,000 babies are born with β-thalassemia major in the world [2].

Different clinical phenotypes have been observed in β-thalassemia major patients such as pallor, jaundice, growth retardation, hepatosplenomegaly, skeletal changes, and defects in the function of organs, especially heart, liver, and endocrine glands due to the iron overload [3]. Regular blood transfusion, use of iron chelators, splenectomy, and hematopoietic stem cell transplantation are the usual treatments in these patients [4]. The most common complications associated with blood transfusion are viral infections, iron overload, and alloimmunization [5]. Alloimmunization is the production of alloantibodies against foreign red blood cell antigens, leading to the categorization of patients into two groups: responders (patients with alloantibodies) and nonresponders (patients without alloantibodies) [6]. Previous studies have shown that the most common alloantibodies among β-thalassemia major patients are anti-kell (50%), anti-D (15.8%), and anti-E (10.5%) [7]. Therefore, repeated

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Published online: 29 July 2021

blood transfusion in these patients causes antigenic stimulation and disruption of immune balance [8].

Naturally occurring regulatory T cells (nTregs) are a subset of T lymphocytes characterized by expression of the markers, CD4, CD25, and FoxP3, which play a crucial role in homeostasis by suppression of the immune cell activation [9]. Previous studies have indicated that Tregs have an important role in alloimmunization [10]. In addition to the FOXP3 gene, the function of Tregs is regulated by the expression of a group of cell surface receptors, also known as "immune checkpoint receptors". Under normal circumstances, the immune checkpoint receptors negatively regulate T-cell effector function and thus play a crucial role in maintaining immune homeostasis and abrogating autoimmunity [11]. The most common immune checkpoint molecules are cytotoxic T-lymphocyte–associated protein 4 (CTLA-4; CD152), lymphocyte activation gene-3 (LAG-3; CD223), T-cell immunoglobulin and mucin domain-containing protein-3 (TIM-3), programmed cell death protein 1 (PD-1), and PD-1 ligand (PD-L1; CD274) [11, 12].

CTLA-4 is one of the inhibitory molecules, which regulates the effector function of T lymphocytes. CTLA-4 is expressed on activated T cells as well as Tregs, where it competes with CD28 for binding to the ligands CD80 and CD86 [13].

LAG-3 is another inhibitory molecule, which is expressed on different cells such as activated B and T cells, plasmacytoid dendritic cells, natural killer T cells (NKT cells), and Tregs [14]. In combination with PD-1, LAG-3 impairs the function of the T lymphocytes and is known as a hallmark of T-cell exhaustion status [15].

TIM-3 is one of the regulators of the immune system, expressed by T-helper 1 (Th1), T-helper 17 (Th17), $CD8^+$ cells, Tregs, natural killer cells (NK cells), monocytes, and dendritic cells [16]. TIM-3 plays an important role in inhibiting Th1 responses and the production of cytokines such as INF-γ and TNF, and impaired regulation of TIM-3 gene expression can lead to autoimmune diseases [17].

There is not enough information about the expression of these inhibitory molecules and the association with the development of alloimmunization in β-thalassemia major patients. Accordingly, this study aimed to evaluate whether the change in the expression of the *CTLA-4*, *LAG-3*, and *TIM-3* is associated with the development of alloantibodies in β-thalassemia major patients.

Material and methods

Patient selection

A case–control study was performed in 68 β-thalassemia major patients who were admitted to our referral hospital for hemoglobinopathy disorders in southern Iran during April and September 2018. These β-thalassemia major patients were divided into two groups; 34 patients with alloantibodies and 34 without alloantibodies. Besides, 20 healthy sex-/age-matched individuals were considered as the control group. Patients were matched in age, sex, age of first blood transfusion, and status of splenectomy. All of them had been transfused every 2 to 4 weeks and received appropriate chelation therapy. Consumption of deferiprone (L1), hydroxyurea and immunosuppressive drugs, and having infections such as HIV, HBV, and HCV were considered as exclusion criteria.

The study was approved by the local ethics committee of Shiraz University of Medical Sciences, and informed consent was obtained from all patients or their legal guardians.

Laboratory parameters

Before transfusion, 2 ml of fresh whole blood in ethylenediaminetetraacetic acid (EDTA)-containing tubes was collected from all patients. To determine hematologic parameters, complete cell blood count (CBC) of all patients and control group was performed using Sysmex-KX-21 N (Sysmex Corporation, Japan).

RNA isolation and cDNA synthesis

In order to evaluate the expression of *CTLA-4*, *LAG-3*, and *TIM-3* genes, total RNA was extracted from cells using RNX plus TM solution (CinnaGen, Tehran, Iran). For determination, the quality of extracted RNA (260/280 nm ratio), nanodrop (Thermo Fisher Scientific, USA) was used. After that, the cDNA was synthesized using PrimeScript® 1st strand cDNA synthesis kit (Takara Bio, Japan) according to the manufacturer's instructions.

Real-time PCR

Real-time PCR was performed using SYBR Green PCR master mix (SYBR Premix Ex Taq™II, Tli RNaseH Plus, Yektatajhiz, Iran) in iQ5 thermocycler (Bio-Rad Laboratories, USA) as described previously [18]. The specific forward and reverse primers were designed for each gene using AlleleID version 6, Oligo software version 7, and Molecular Beacon software and rechecked with Blast software. β-Actin (ACT-β) was used as housekeeping gene, and the relative expression of *CTLA-4*, *LAG-3*, and *TIM-3* was normalized to it. Table 1 shows the primer sequences and PCR product size for all genes. The relative expression of targeted genes was finally calculated using $2^{-\Delta\Delta CT}$ method.

Table 1 The primer sequences and PCR products of immune checkpoint receptors

Gene	Primer sequences (5′–>3′)	PCR product length (bp)
CTLA-4 forward primer	5′-TTCTTCTCTTCATCCCTGTCTTCT-3′	130
CTLA-4 reverse primer	5′-CGGACCTCAGTGGCTTTG-3′	
LAG-3 forward primer	5′-CTTCTTGGAGCAGCAGTG-3′	133
LAG-3 reverse primer	5′-AAAGGAGCAGAGAAAGGAC-3′	
TIM-3 forward primer	5′-GTCATCAAACCAGCCAAGG-3′	113
TIM-3 reverse primer	5′-AGTGTCTGTGTCTCTGCT-3′	
β-Actin forward primer	5′-ATCGTGCGTGACATTAAGGAG-3′	177
β-Actin reverse primer	5′-GAAGGAAGGCTGGAAGAGTG-3′	

Statistical analysis

All statistical analysis was performed using GraphPad Prism version 8 and Statistical Package for the Social Sciences (SPSS) version 25. For comparison of quantitative data between different groups, a one-way ANOVA test was used using post hoc Bonferroni multiple correction method. Pearson correlation test was performed for the correlations between targeted genes. *P* values less than 0.05 were considered to be statistically significant.

Results

The mean age of patients was 27.18 ± 7.25 (range 8–42 years) and 27.44 ± 6.54 (range 16–42 years) in responders (with alloantibodies) and nonresponders (without alloantibodies), respectively. Also, the mean age of the healthy control group was 26.16 ± 7.03 years (range 8–33 years). The demographic, laboratory, and clinical characteristics of patients and controls are described in Table 2.

Analysis of *CTLA-4*, *LAG-3*, and *TIM-3* expression

To evaluate the relative expression of genes, the expression of *CTLA-4*, *LAG-3*, and *TIM-3* genes was normalized to

Table 2 Demographic, laboratory, and clinical features of β-thalassemia major patients and controls

Groups	Allo (+) (*n* = 34)	Allo (-) (*n* = 34)	Controls (*n* = 20)
Age (mean ± SD)	27.26 ± 7.33	27.65 ± 6.65	24.85 ± 9
Sex (male/female)	14/20	14/20	9/11
Laboratory and clinical characteristics			
WBC ($\times 10^3$/mL)	9.76 ± 5.91	10.82 ± 9.53	5.82 ± 2.01
Lymphocyte (%)	37.08 ± 10.76	39.33 ± 9.86	36.35 ± 12.26
Hb (g/dL)	9.66 ± 0.92	9.72 ± 0.98	13.75 ± 3.67
Serum ferritin (ng/mL)	1621.01 ± 443.68	2056.03 ± 399.39	44.81 ± 9.69
Serum folate (ng/mL)	10.01 ± 7.72	11.88 ± 8.15	11.18 ± 4.79
Folic acid consumption			
Yes	24	18	
No	10	12	
Splenomegaly			
Positive	10	10	
Negative	24	24	
Age of first transfusion (month)	12.53 ± 7.3	9.79 ± 4.7	-
Number of blood transfusions (year)	21.85 ± 6.88	20.52 ± 3.54	-
Volume of blood requirement (year)	267 ± 86.1	246.3 ± 44.02	-
Age of diagnosis (month)	8.12 ± 4.68	6.71 ± 2.2	-

Data are presented as men ± SD. *Allo (+)*, patients with alloantibody; *Allo (-)*, patients without alloantibody; *WBC*, white blood cells; *Hb*, hemoglobin

β-actin as a housekeeping gene by subtracting β-actin Ct from Ct of targeted genes and defined as dCt. After that, the dCt of targeted genes was compared between different groups of β-thalassemia major patients and control group.

The results showed that the relative expression of *CTLA-4* and *TIM-3* was not significantly different between thalassemia patients and control group (mean 9.17 ± 1.45 vs. 9.99 ± 2.29; $P = 0.152$ and 8.71 ± 1.37 vs. 8.87 ± 1.35; $P = 0.694$, respectively). Although, the expression of *LAG-3* gene was significantly higher in patients compared to the control group (mean 6.81 ± 2.03 vs. 10.41 ± 1.87; $^*P < 0.001$) (Fig. 1A).

Comparison of responders and nonresponders showed that the expression of *CTLA-4*, *LAG-3*, and *TIM-3* was not significantly different between these two groups ($P > 0.05$). However, the expression of *LAG-3* was significantly decreased in control groups compared to both responders (mean 10.41 ± 1.87 *vs.* 7.17 ± 1.88; $^*P < 0.001$) and also the nonresponder ones (mean 10.41 ± 1.87 vs. 6.5 ± 2.12; $^*P < 0.001$) (Fig. 1B).

There was a positive correlation between the relative expression of *CTLA-4* and *LAG-3* ($r = 0.346$,$^*P = 0.002$) and *TIM-3* ($r = 0.674$,$^*P < 0.001$) (Fig. 2).

In addition, the level of *CTLA-4*, *LAG-3*, and *TIM-3* gene expression was not different between patients who had undergone splenectomy compared to the non-splenectomized ones ($P > 0.05$).

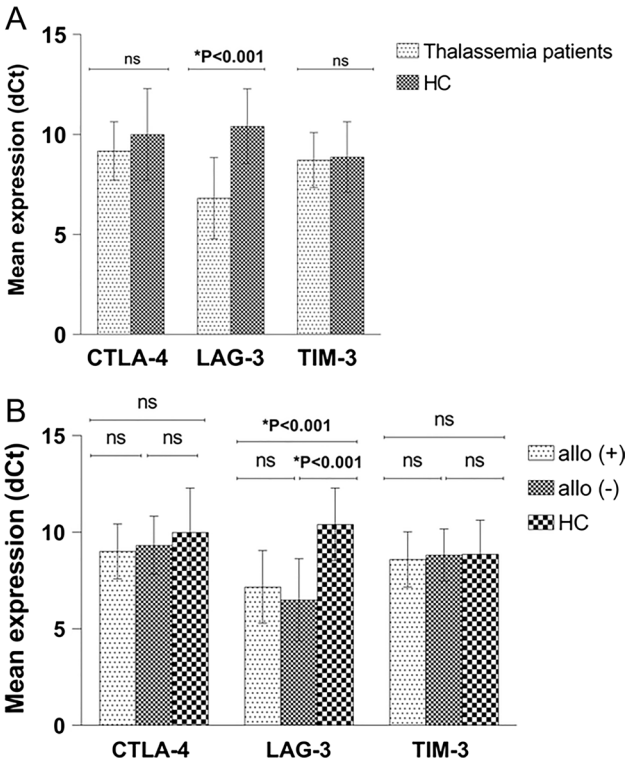

Fig. 1 Expression of the CTLA-4, LAG-3, and TIM-3 genes in ß-thalassemia major patients and healthy controls. Data are represented as mean ± SD of dCt for each gene. Graphs are created by GraphPad Prism 8. *P* value < 0.05 is considered statistically significant. *Allo (+)*, patients with alloantibody; *Allo (-)*, patients without alloantibody; *HC*, healthy controls

Correlation between *CTLA-4*, *LAG-3*, and *TIM-3* gene expression and laboratory parameters

The relationship between *CTLA-4*, *LAG-3*, and *TIM-3* gene expression and laboratory characteristics including white blood cell (WBC) and red blood cell (RBC) count, serum hemoglobin (Hb), folate, and ferritin level was evaluated in thalassemia patients. Accordingly, no positive correlation was observed between the expression of *CTLA-4*, *LAG-3*, and *TIM-3* genes and these laboratory parameters ($P > 0.05$).

CTLA-4, *LAG-3*, and *TIM-3* gene expression and Treg markers

In our previously published paper in the same population, we showed a positive relationship between *FOXP3* and *GDF-15*: two important genes associated with Treg function in these β-thalassemia major patients [19]. Regarding this, a positive correlation was observed between the level of *LAG-3* expression with *FOXP3* ($r = 0.658$, $^*P < 0.001$) and *GDF-15* genes ($r = 0.642$, $^*P < 0.001$) (Fig. 3). The expression of the *CTLA-4* gene was positively correlated with the total Treg number ($r = 0.299$, $^*P = 0.016$).

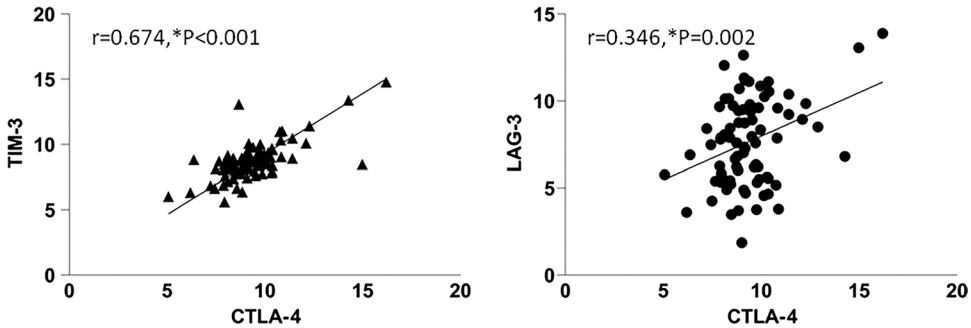

Fig. 2 Correlation between CTLA-4, LAG-3, and TIM-3 gene expression in β-thalassemia major patients. Graphs are created by GraphPad Prism 8. *r* = Pearson correlation co-efficient

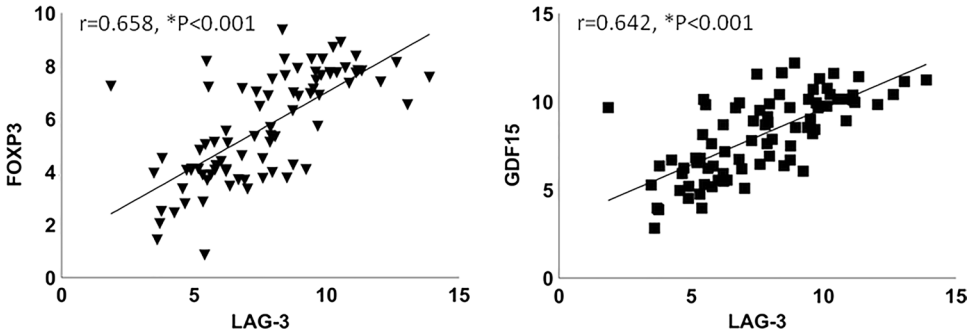

Fig. 3 Correlation between LAG-3 and FOXP3/GDF15 gene expression in β-thalassemia major patients. Graphs are created by GraphPad Prism 8. *r* = Pearson correlation co-efficient

Discussion

Beta-thalassemia major is a hematologic disorder of red blood cells characterized by defective hemoglobin (Hb) production as a result of a mutation in β-globin chains of the Hb gene. Blood transfusion is widely used to replenish abnormal red blood cells in these patients. However, frequent blood transfusion causes various complications such as iron overload and alloimmunization in these patients [20, 21]. Iron overload is known to be one of contributing factors that alter the immune balance [22]. On the other hand, alloimmunization can happen as a result of the activation of the immune system following exposure to the foreign antigens during a repeated blood transfusion. Although genetic differences between "responders" and "nonresponders" might be a key factor triggering alloimmunization, the immunologic signature diversity could also be so important [23, 24].

The immune checkpoint receptors are among the most fundamental molecules that regulate the activation of the immune system. Until now, there is not enough information about the role of inhibitory checkpoint receptors like *CTLA-4*, *LAG-3*, and TIM-3 gene expression and alloimmunization in β-thalassemia major patients.

In this study, we evaluated the expression of immune regulatory checkpoint receptors including *CTLA-4*, *LAG*-3, and *TIM-3* genes in β-thalassemia major patients and their association with alloantibody production in these patients. Our results showed that the expression of the *LAG-3* gene was significantly increased in β-thalassemia major patients compared to the control group, but there was no difference between patients with and without alloantibody considering *CTLA-4*, *LAG-3*, and *TIM-3* genes. A positive correlation was observed between the level of *LAG-3* expression with *FOXP3* and *GDF-15* genes; two essential markers that regulate Treg function.

Recent studies have shown a wide range of abnormalities in several components of the immune system in β-thalassemia major patients affecting both numbers as well as the function of the immune cells [22]. These include abnormal change of $CD4^+$ and $CD8^+$ T cells (both numbers and function), defective natural killer (NK) cell activity, impaired immunoglobulin secretion, as well as defective chemotaxis and phagocytosis function by neutrophils and macrophages [22, 25–27]. Therefore, it seems that the immune system might be dysregulated in β-thalassemia major patients.

CTLA-4, LAG-3, and TIM-3 are immune checkpoint molecules expressed on the surface of Tregs which suppress the immune system in different diseases [28]. Previous studies have shown that monoclonal antibodies, which target these molecules can be used for immunotherapy in cancers [29]. Tregs play a key role in maintaining immune homeostasis by suppressing the activation and function of other immune cells. These cells are usually characterized by the transcription factor FoxP3, and dysregulation of this factor can cause autoimmune diseases [30, 31]. Consistent with our results, our previous study demonstrated that the frequency of Tregs and the expression of FoxP3 gene were increased in β-thalassemia major patients compared to the control group, but no difference was observed between responders and nonresponders [19]. Various studies have shown that LAG-3 is essential for the function of regulatory T cells in order to control the T-cell homeostasis and, inhibiting the function of LAG-3 using its antagonists, can increase the activity of the immune system which has been widely used for the treatment of different cancers [32–34]. Also, in a study by Camisaschi et al., LAG-3 was expressed in an active form of regulatory T cells with high expression of FOXP3 which was associated with the production of immunosuppressive cytokines [35].

Accordingly, our results may explain that the LAG-3 molecule might have a more prominent role in the abnormality of the immune system in thalassemia patients, prior to the *CTLA-4* and *TIM-3* genes. Besides, it seems that high expression of *LAG-3* along with FOXP3/GDF-15 genes might be implicated in Treg dysfunction in thalassemia patients. Whether these markers are linked to alloimmunization is not clear and needs to be evaluated in a larger population. In this regard, functional assessment of Tregs especially in those thalassemia patients with and without alloantibody along with the evaluation of these

immune checkpoint receptors at the protein level by Tregs can provide helpful information about the clinical significance of Tregs and the expression of the immune checkpoint receptors in these patients.

Conclusion

To the best of our knowledge, we showed for the first time that the expression of immune checkpoint receptor *LAG-3* is dysregulated in β-thalassemia major patients which was positively correlated with Treg functional markers: the *FOXP3* and *GDF-15* genes. The study of a larger population along with the evaluation of *LAG-3* as well as other checkpoint receptors at the protein level especially on the surface of Tregs might be so informative and is highly recommended.

Authors' contribution Negin Shokrgozar contributed to performing the research and writing the paper; Mehran Karimi, Hossein Golmoghaddam, and Sedigheh Sharifzadeh contributed to the performing the research and critically revision of the manuscript; and Nargess Arandi contributed to study design, analysis, interpretation of data, writing paper, and performing the research.

Funding This study was financially supported by a grant provided by Shiraz University of Medical Sciences (grant number 18902).

Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval This study was performed according to the ethical standards of the local Ethics Committee of Shiraz University of Medical Sciences (ethical code IR.SUMS.REC.1398.1387) and in compliance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent to participate Informed consent was obtained from all individual participants included in the study.

Conflict of interest The authors declare no competing interests.

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