

Study of T-cell immunoglobulin and mucin domain-3 expression profile in peripheral blood and bone marrow of human acute lymphoblastic leukemia patients

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Background: Acute lymphoblastic leukemia (ALL) is a malignancy with aggressive tumors of immature lymphocytes. T-cell immunoglobulin and mucin-domain 3 (TIM-3) is a Type I transmembrane glycoprotein which is involved in cell proliferation. The objective of this research is to determine the TIM-3 expression in peripheral blood (PB) and bone marrow (BM) of 80 samples of normal and ALL patients. **Materials and Methods:** The amount of mRNA and protein of TIM-3 measured in the BM and PB the mononuclear layer of samples by real-time polymerase chain reaction and Western blotting. **Results:** Our findings indicated that relative mRNA expression of TIM-3 in PB and BM of the mononuclear layer of ALL patients was 1.7 and 5 times higher than normals, respectively. We also reported that the protein level of TIM-3 in mononuclear cells of ALL patients was 3.2-fold in BM and two-fold in PB more than normals. **Conclusion:** In conclusion, this study shows that TIM-3 increases in ALL patients, thus the expression of TIM-3 in tumor cells may be considered as a potential predictive factor in ALL patients, which needs to be explored in future.

Key words: Gene expression profiling, precursor cell lymphoblastic leukemia-lymphoma, real-time polymerase chain reaction, T-cell immunoglobulin and mucin-domain 3

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INTRODUCTION

The prevalence of acute lymphoblastic leukemia (ALL) is more than other leukemia in childhood. This malignancy occurs at any age, but approximately 60% of patients are <20 years old with an incidence peak between 2 and 5 years of age.^[1,2] In the last few years, significant advances obtained in ALL patients' treatment, but one of the main issues in ALL treatment is the lack of a uniform biomarker for ALL targeting. The discovery of molecular mechanisms of the development and progression of ALL has led to the identification of molecular aims of cancer cells. Therefore, the findings

show that cancer cells can be destroyed by targeting critical molecules in cancer cells.

T-cell immunoglobulin and mucin domain (TIM) proteins located in the cell membrane and have three domains that include: an extracellular region contains immunoglobulin V domain, mucin-like domain followed by a transmembrane region and a cytoplasmic tail. Eight genes present on the mouse (TIM1-8) and three genes in humans (TIM-1, -3, -4) are constitutive members of the TIM gene family.^[3] The human TIM-1, -3, -4 proteins have higher similarity to the mouse TIM-1, -3, -4, respectively.^[4,5] All TIM proteins have tyrosine-kinase phosphorylation motif in the cytoplasmic area except

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for the TIM-4 protein. TIM-3 is a factor for identifying Th1 and Th2 cells in mice and humans. TIM-3 is also presented in several cells, such as CD8 T-cells, monocytes, and dendritic cells.^[6] Moreover, TIM-3 has significant roles in metastasis in malignancy.^[7] Recent studies have revealed highly relationship between TIM-3 expression and immune suppression associated with tumors.^[8] Huang *et al.* found that TIM-3 acts as a suppressor of CD4+ T lymphocytes activity through the activation of the interleukin-6/STAT3 pathway.^[7,9]

Previous studies found that TIM-3 overexpressed in many malignancies such as lung, cervical, prostate cancer, and leukemia.^[10-13] Previous studies have been shown the presence of TIM-3 on leukemia stem cell (LSCs) in most samples of various acute myeloid leukemia (AML).^[14,15] A recent study suggested that TIM-3 is one of the diagnosis factors of AML that could be a hopeful solution to the improvement of leukemia treatment.^[11] Another researcher reported that TIM-3 has a high expression on AML leukemic stem cells than in normal bone marrow (BM) hematopoietic cells.^[14] Furthermore, cervical cancer patients with higher expression levels of TIM-3 had tremendous potential in metabolic metastatic, progressive cancer grades, and shorter overall survival in these people.^[10] The protein TIM-3 is a possible indicative marker in prostate tumors and lung cancers.^[12,13] These findings persuaded us to investigate the TIM-3 gene and protein expressions in ALL for the first time.

MATERIALS AND METHODS

Patients and samples

Altogether, 80 BM and peripheral blood (PB) specimens collected from 40 normals (20 normal BM, 20 normal PB) and 40 ALL patients (20 BM and 20 PB) attended to Seyed Al-Shohada Hospital affiliated to Isfahan University of Medical Sciences and Imam Khomeini Hospital affiliated to Tehran University of Medical Sciences. Patients were between 2 and 10 years of age (median age = 6 years). All patients newly diagnosed with ALL by the oncologist. About 70% of the samples were pre-B-ALL and 30% were T-ALL [Table 1]. Normal samples collected from children who did not have any leukemia or any inflammatory, autoimmune, and allergic diseases. Normal PB samples collected from persons who had given a blood sample for an annual check-up. Normal BM samples were obtained from patients referred for the diagnosis of other diseases other than leukemia and did not have any inflammatory disease. This study confirmed by the research ethics committee (ethical code number MUI393484) of the Isfahan University of Medical Sciences, and the informed consent was obtained from all patients.

Table 1: Demographic characteristic of the study samples

Characteristics	Median (range)	n (%)
Age (years)	6 (2-10)	40 (100)
Sex		
Male		27 (67.5)
Female		13 (32.5)
WBC×10 ³ /μl	9.1 (3-23)	
RBC×10 ⁶ /μl	3.5 (2-5)	
Hemoglobin (g/dL)	9.4 (5-11)	
Lymphocyte PB %	42.5 (15-100)	
Blast BM %		
15-35		16 (40)
36-50		8 (20)
51		16 (40)
The dominant type of ALL (Pre-B ALL)		28 (70)
T-ALL		12 (30)
TIM-3 expression in PB ≥1.7 (fold change)		13 (65)
TIM-3 expression in BM ≥5 (fold change)		14 (70)

TIM-3=T-cell immunoglobulin and mucin-domain 3; ALL=Acute lymphoblastic leukemia; PB=Peripheral blood; BM=Bone marrow; WBC=White blood cell; RBC=Red blood cell

Quantitative reverse transcriptase-polymerase chain reaction analysis

TIM-3 mRNA expression level was examined in the PB and BM mononuclear layers of all specimens by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Initially, mononuclear cells were isolated from PB and BM using Ficoll (CEDERLANE, Netherlands).^[16] RNX-Plus kit (CinnaGen Co, Iran), with following the manufacturer's instruction, was used to extract total RNA from mononuclear cells. First-strand cDNA was synthesized by M-MuLV reverse transcriptase enzyme Fermentas kit. The TIM-3 primers (Forward, 5'-TCCAAGGATGCTTACCACCAG-3, Reverse, 5'-GCCAATGTGGATATTTGTGTTAGATT-3') were applied to accompany with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control (forward, 5'-CTCCCGCTTCGCTCTCTG-3', reverse, 5'-TCCGTTGACTCCGACCTTC-3') of transcript levels.^[17] The following protocol was carried out for real-time PCR: (12.5 μl master mix, 2 μl cDNA, 1 μl specific primer for each of TIM-3 or GAPDH, 8.5 μl RNase and DNase free water) initial denaturation at 95°C for 10 min, then denaturation at 95°C for 15 s, annealing at 60°C for 30 s and extensions at 72°C for 20 s for 40 cycles. Amplification product specificity was verified by melting curve analysis at the end of every PCR reaction. Corbett Rotor gene 6000 system with Syber Green Master Mix (AMPLIQON, Denmark) was used to real-time quantitative PCR. The 2^{-ΔΔCt} method was accomplished for relative quantitative analysis of TIM-3 expression.^[18]

Western blotting

The expression profile of TIM-3 protein in the obtained samples was also checked using Western blotting. Forty

specimens of the PB and BM mononuclear cells of ALL and normals consist of 20 normal BM, 20 normal PB, 20 ALL BM, and 20 ALL PB was lysed in a lysis buffer (Roche Applied Science, Germany) composed of aprotinin (1 mg/ml), pepstatin (1 mg/ml), PMSF (0.05 mmol/ml), sodium orthovanadate (1 mg/ml), NaF (500 mmol/ml), and EDTA (500 mmol/ml). The protein samples and loading buffer mixed and heated for 5 min at 95°C. The protein separation was made possible by SDS-PAGE on 10% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane using electroblotting. The membrane was placed in blocking solution containing 5% fat-free milk and phosphate-buffered saline with Tween-20 (PBS-T) for 1 h at room temperature; the blot was incubated overnight at 4°C in TIM-3 polyclonal antibody (Abcam, USA) and then rinsed with PBS-T. The membrane was exposed to horseradish peroxidase-labeled secondary antibody (Sigma, Germany) for 1 h at room temperature. Finally, the detection of protein bands on the membrane was accomplished using enhanced chemiluminescence a detection system (BioRad, USA). Anti- β -actin antibody (Acris-Antibody, Germany) was used as the internal control at the appropriate concentration.^[19]

Statistical analysis

The outcomes of this study are based on mean \pm standard error of the mean Student's *t*-test was applied to compare the relative mRNA and protein expression of TIM-3 in patients and normal samples. All experiments were evaluated in triplicate. The software package SPSS version 21 (Statistical Package ver. 18.0; SPSS Inc., Chicago, IL, USA) was used for the statistical analyses. The difference at $P < 0.05$ was considered statistically significant.

RESULTS

Comparison of T-cell immunoglobulin and mucin-domain 3 mRNA level in acute lymphoblastic leukemia patients and normal subjects

Analysis of real-time PCR data revealed that the level of TIM-3 mRNA was higher (1.7 folds) in PB mononuclear cells of ALL patients, compared to normal patients ($P < 0.001$). Furthermore, relative mRNA expression of TIM-3 gene in BM mononuclear cells of patients with ALL group was five times more than in the normal group ($P < 0.001$) [Figure 1a and b]. The mean relative expression of TIM-3 in the BM of patients with ALL was greater (2.9 folds) than the expression of this gene in the PB of patients with ALL ($P < 0.001$) [Figure 1c]. Interestingly, in 69.7% of PB samples of ALL patients, TIM-3 expression was 1.5 times higher than normals. Furthermore, the TIM-3 level was significantly higher in the BM of ALL patients compared to normal samples. In this regard, 85.1% of BM samples of ALL patients showed over three folds expression of TIM-3 in comparison to normal BM samples [Figure 1a and b].

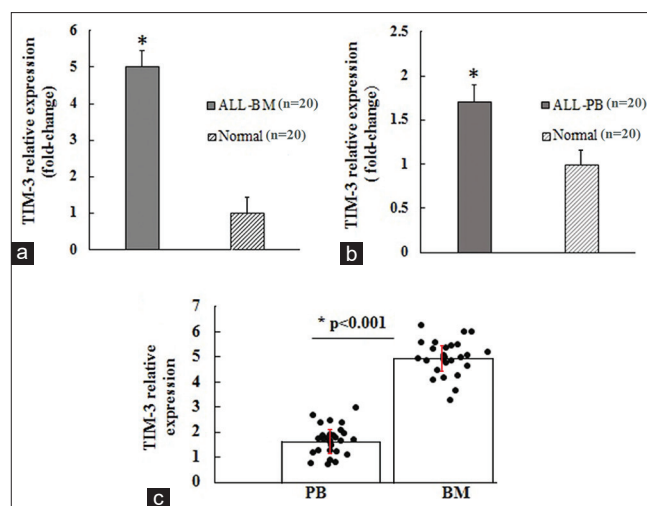


Figure 1: TIM-3 relative transcript expression, measured by real-time PCR in PB (a) and BM (b) mononuclear cells of 80 ALL patients and normal subjects. (c) A graph representing the distribution range of TIM-3 relative expression in PB ($n = 20$) and BM ($n = 20$) of ALL patients compared to normals, based on $2^{-\Delta\Delta Ct}$. Results are obtained from the mean of triplicate tests. Differences were considered as statistically significant ($*P < 0.001$). TIM-3 mRNA expression was normalized to GAPDH housekeeping in each sample. PB = Peripheral blood; BM = Bone marrow; ALL = Acute lymphoblastic leukemia; PCR = Polymerase chain reaction; TIM-3 = T-cell immunoglobulin and mucin-domain 3; GAPDH = Glyceraldehyde-3-phosphate dehydrogenase

Comparison of T-cell immunoglobulin and mucin-domain 3 protein expression in acute lymphoblastic leukemia patients and normal subjects

The TIM-3 protein levels in PB and BM mononuclear cells of ALL and normal groups were determined by Western blot. A qualitative higher level of TIM-3 protein was seen in the PB and BM mononuclear cells of ALL patients than PB and BM of normal groups [Figure 2a]. Image J software (National Institutes of Health, Bethesda, MD, USA) was used to determine the TIM-3 protein expression level. The results of the quantification of protein expression by Western blotting revealed that TIM-3 protein expression in BM and PB of ALL patients is about 3.2 and 2 times higher than in the normal group, respectively [Figure 2b].

DISCUSSION

A tumor microenvironment is a place that tumor cells and immune cells meet each other. In this milieu tumor, cells try to survive and proliferate via different immunosuppressive mechanisms largely through inhibitory signals and recruiting suppressive inhibitory cells.^[20] In this context, molecules such as the cytotoxic T-lymphocyte-associated protein 4 and programmed cell death-1 are among well-known immune checkpoint targets that play pivotal roles in damping the anti-tumor response.^[21]

TIM-3 has been studied in many solid tumors and cell lines from a different origin. Cao *et al.* assessed the level of TIM-3 mRNA and protein by quantitative RT-PCR and immunoblot, respectively, in cervical tumor cells (HeLa,

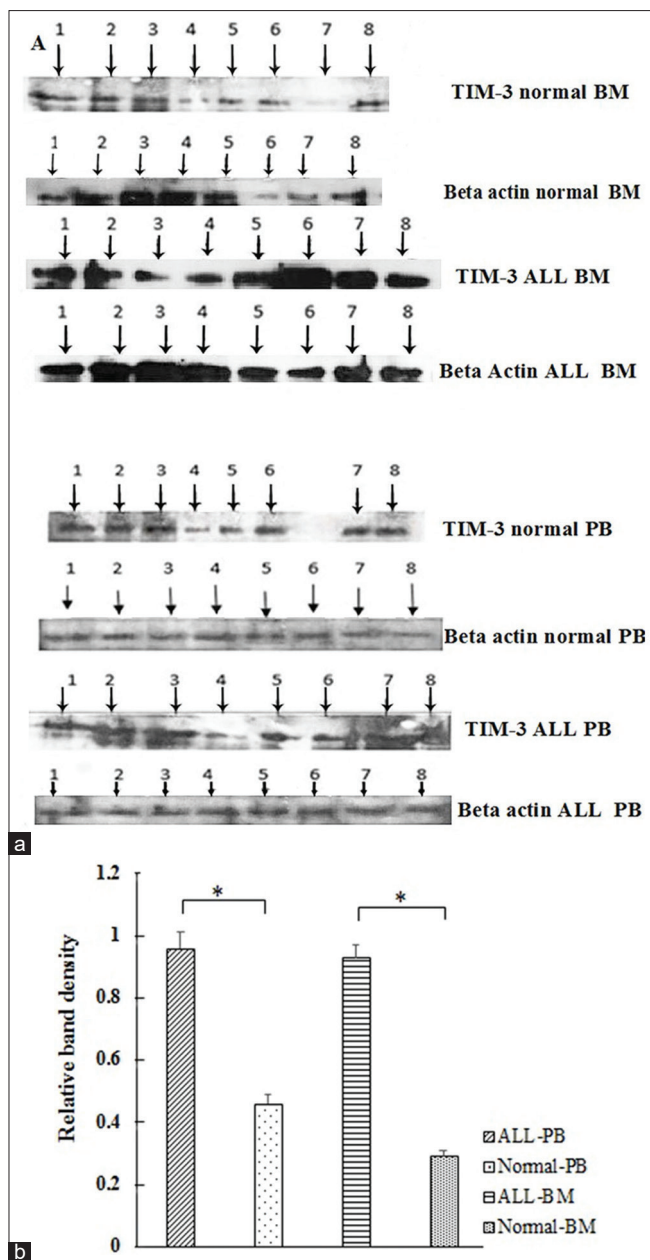


Figure 2: TIM-3 protein expression level from Western blot analysis in PB ($n = 8$) and BM mononuclear cells of ALL and normal samples. The numbers on the figure indicate 8 protein bands for each of normal, PB and BM samples for both housekeeping (β -actin) and TIM-3 genes. (a) TIM-3 protein and β -actin protein expression profile in ALL and normal samples ($*P < 0.05$). The indicated numbers on figure represent the protein band observed for eight samples. (b) Relative TIM-3/ β -actin protein band densitometry data were performed using Image J quantification software. Results are reported as mean \pm SD of triplicate tests ($*P < 0.05$). PB = Peripheral blood; BM = Bone marrow; ALL = Acute lymphoblastic leukemia; TIM-3 = T-cell immunoglobulin and mucin-domain 3; SD = Standard deviation

SiHa) and tissue. They found the existence of TIM-3 mRNA and protein in HeLa and SiHa cells. They showed that the level of TIM-3 in cervical tumors was more than the control groups. Moreover, they mentioned that higher metastasis and progressive grades and lower survival were found in patients with high levels of TIM-3. In addition, the downregulation of TIM-3 protein has a direct relationship

with inhibiting the metastasis of HeLa cells. Therefore, TIM-3 may have the potential to be a prognostic marker for cervical tumors.^[10] Other study revealed that the TIM-3 levels in prostate tumors are higher than to their normal tissues.^[12] Moreover, Zhuang *et al.* showed that TIM-3 levels are greater in patients with NSCLC (non-small-cell lung cancer) than normals. They showed that 70.69% (82 of 116) malignant patients presented TIM-3. Overall, they confirm that the TIM-3 protein level related to prostate cancer poor survival and might be a target for this cancer.^[13] Wiener, *et al.* found that TIM-3 was expressed in two HT168-M1 and WM35 melanoma cells, and this expression was higher than in normal cells.^[22] Other investigations indicated that TIM-3 expression was clearly higher in both CD4+ and CD8+ T-cells in malignant ovary tissues in comparison with normal tissues.^[23] Zheng *et al.*'s study showed that the mRNA levels of TIM-3 in the PB mononuclear cells of systemic lupus erythematosus are significantly higher than in the control groups.^[24] Two current studies found that TIM-3 level in 786-O/Caki-2 Clear Cell Renal Cell (ccRCC) cells were 4 and 3 folds higher than in HK-2 cells, respectively. As well as they suggested, TIM-3 can be used as a clinical response prediction factor and a potential target for ccRCC therapy.^[24,25] Li *et al.*'s data showed that the TIM-3 level in T-cells of hepatocellular carcinoma (HCC) was higher than in normal cells.^[26] Regarding leukemia, TIM-3 over-expression studies have been mostly focused on AML, myelodysplastic syndromes (MDS), chronic myeloid leukemia, and chronic myelomonocytic leukemia.^[15,27] Zhu *et al.* demonstrated that the levels of TIM-3 were high in follicular helper T-cells in breast tumors.^[28]

As far as we know, there is no report on the TIM-3 expression profile in ALL patients. To find the TIM-3 expression profile and its role in ALL, we designed and performed the current study. Our findings demonstrated that the relative mRNA level of TIM-3 gene in both PB and BM ALL were 1.7 and 5 folds higher than normal counterparts, respectively ($P < 0.001$). Compatible with transcript findings, TIM-3 protein is overexpressed in BM and PB mononuclear cells. Compared to normal samples, TIM-3 had a 3.2- and 2-fold increase in BM and PB of ALL patients, respectively. There is no report about TIM-3 expression in ALL; however, it seems that TIM-3 is overexpressed in acute leukemia from both lymphoid and myeloid origins. TIM-3 protein was also found on many of CD34⁺/CD38⁻ LSCs and CD34⁺/CD38⁺ leukemic cells in all FAB types of AML, excluding M3 type that promotes self-renewal as well as AML development.^[11,29] Interestingly, TIM-3 has not existed in CD34⁺ CD38⁻ normal HSCs and CD34⁺ CD38⁺ normal progenitor cells.^[11,30]

CONCLUSION

The study demonstrated that TIM-3 is over-expressed 1.7 and 5 times, respectively, in both PB and BM of ALL patients

compared to normal samples. Similarly, the TIM-3 protein level in PB of ALL patients was 2-fold and in BM of these patients 3.2-fold higher than normal samples. According to this research, determine the expression pattern and function of TIM-3 may be used as a prognostic or diagnostic factor for ALL, which needs further studies in this context.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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