Original Article
Tim-3 as a leukemia stem cell specific marker expressing on bone marrow mononuclear cells is a factor for prognosis evaluation in patients with acute myelogenous leukemia

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Abstract: Background: T cell immunoglobulin-3 (Tim-3) is identified as a negative regulator of anti-tumor immunity. Recently, Tim-3 expression has been demonstrated in leukemic stem cells (LSCs) of acute myelogenous leukemia (AML), however, the alterations of Tim-3, as well as its marker and prognostic significance has yet to be evaluated in patients with AML. The present study is designed to investigate the potential association between Tim-3 expression and the poor prognosis of AML. Materials and methods: The expression of Tim-3 was evaluated by multicolor flow cytometry in 43 patients with AML and 6 normal samples. Correlations were analyzed between expression levels of Tim-3 protein and leukocyte count of newly diagnosed patients. Receiver operating characteristic (ROC) curve was applied to analyze the differentiating value of Tim-3 expression. Results: The expression of Tim-3 was significantly higher in AML than that in normal controls ($P=0.0011$). Increased Tim-3 expression was associated with leukocyte count and treatment cycles to complete remission (CR) ($P=0.0014$ and $P=0.0006$, respectively). ROC curve confirmed the value of Tim-3 expression in discriminating AML patients from healthy controls ($P<0.001$) and further distinguishing the refractory patients from all patients with AML ($P=0.003$). Moreover, correlation analysis shows that high Tim-3 expression were significantly positive associated with leukocyte count of newly diagnosed patients ($R=0.746$, $P<0.0001$). Conclusions: These results demonstrated that Tim-3 is expressed on $CD34^+CD38^-CD96^+$ cells of AML patients. The expression levels of Tim-3 showed significant correlation with patients' leukocyte count and treatment cycles to CR. Elevated Tim-3 expression may be a direct consequence of the molecular mutations presented in AML and a potential prognostic factor for patients with AML.

Keywords: Tim-3, negative co-stimulatory molecules, molecular markers, prognosis evaluation, acute myelogenous leukemia, leukemia stem cell

Introduction

Acute myeloid leukemia (AML) is an aggressive malignancy of the bone marrow with a 5-y overall survival between 30% and 40%, and much poorer outcomes for patients over age 65 [1, 2]. AML originates from a small number of self-renewing leukemic stem cells (LSCs) [3, 4], and Tim-3 as a surface molecule expressed on LSCs in most types of AML except for acute promyelocytic leukemia, but not on normal hema-topoietic stem cells (HSCs) [5, 6]. Tim-3 was identified as a negative regulator of antitumor immunity and some studies showed the expression of Tim-3 in tumor cells as an independent prognostic factor for patients with non-small cell lung cancers [7]. However, the relationship between the expression of Tim-3 and the prognosis for patients has not yet been investigated in AML. In most types of AML, LSCs are concentrated in the $CD34^+CD38^-$ fraction of AML cells [8, 9]. In addition, Yoshikane's research showed

Table 1. Clinicopathologic characteristics in AML patients in the present study

Clinicopathologic factor		n
Gender	Male	20
	Female	23
Age		15-72 (years)
Cancer type	WHO/M1	3
	WHO/M2	5
	WHO/M4	9
	WHO/M5	18
	WHO/M6	8
Risk stratification	Low-risk	26
	High-risk	17

the molecules Tim-3 expressed at high level specifically in CD34$^+$CD38$^-$ AML cells with cDNA microarray analysis such as previously identified LSC-specific molecules CD96 [6, 10]. Thus, in the present study, we investigated the expressions of Tim-3 on CD34$^+$CD38$^-$CD96$^+$ cells, and further analyzed whether the aberrant expression of TIM-3 is related to the prognosis for patients with AML.

Materials and methods

Patients

The bone marrow samples of 43 adult AML cases diagnosed according to French-American-British (FAB) and WHO criteria were enrolled. Control adult bone marrow and peripheral blood cells were obtained from 6 healthy donors. Informed consent was obtained from all patients and controls in accordance with the Helsinki Declaration of 1975 that was revised in 1983. The study protocol was approved by the ethics committee of the Qilu Hospital of Shandong University. Patient's characteristics are shown in **Table 1**.

Flow cytometry analysis

In brief, for the analyses, cells were stained with FITC-conjugatedanti-CD34, APC-conjugated anti-CD38, Percp-conjugated anti-CD96 and PE-conjugatedanti-TIM-3 (ebiosciences). Appropriate isotype-matched, irrelevant control monoclonal antibodies were used to determine the level of background staining. The cells were analyzed by flow cytometer (Gallios; Beckman Coulter).

Statistical analysis

All values are expressed as mean ± SD. To determine statistical differences between 2 groups, unpaired t-tests were used. Pearson correlational analysis was run on comparisons of Tim-3 expression vs. leukocyte count and Tim-3 expression vs. age of newly diagnosed patients. Receiver operating characteristic (ROC) curve and area under the ROC curve (AUC) were used to assess the value of Tim-3 expression in discriminating AML patients from healthy controls and further distinguishing the refractory patients from all patients with AML. All statistical tests were two-tailed and *P*-values less than 0.05 were considered to be statistically significant. All analyses were performed using the SPSS software package 18.0 (SPSS Inc. USA). The GraphPad Prism software (GraphPad Software, LaJolla, CA) was used for all statistical analyses.

Results

Tim-3 expression was significantly higher in AML compare to those in healthy controls

Similar as previous studies [6], we found that human Tim-3 was expressed in the vast majority of CD34$^+$CD38$^-$ LSCs in AML of most FAB types. As shown in **Figure 1**, Tim-3 protein was highly expressed in CD34$^+$CD38$^-$CD96$^+$ AML cells but not in normal HSCs (*P*=0.0011).

Correlation of Tim-3 expression with clinicopathologic parameters

As shown in **Figure 2**, the expression of Tim-3 was no statistical differences between male and female patients (*P*=0.3131) as well as between patients older than 60 years of age and less than 60 years of age (*P*=0.547). The significantly higher expression of Tim-3 was found in patients with leukocyte count more than 100×10^9/L and in patients with treatment more than 2 cycles to CR (*P*=0.0014, *P*=0.0006, respectively). The clinical characteristics of subjects are summarized in **Table 2** in detail.

Positive Tim-3 expression were significantly associated with leukocyte count in newly diagnosed patients

As shown in **Figure 3**, the Tim-3 expression levels were positively correlated with leukocyte
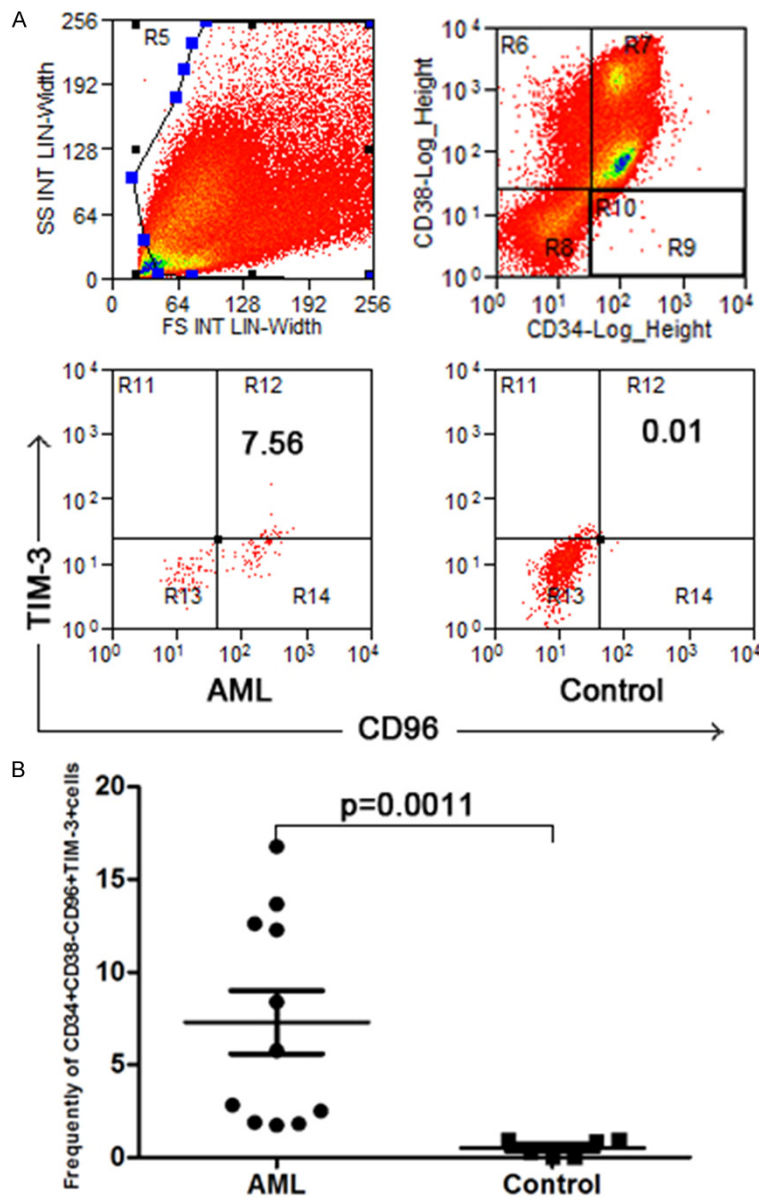
Figure 1. Tim-3 expression was significantly higher in AML compare to those in healthy controls. The CD34$^+$CD38$^-$ adult bone marrow HSCs and CD34$^+$CD38$^-$ AML LSCs were purified and tested for their Tim-3 expression. A. The expression of representative surface proteins in HSCs and AML LSCs on FACS. B. Summarized data of Tim-3 expression in FAB types of AML and healthy controls. Differences between two groups were compared using the unpaired t-tests.

ROC curve was applied to analyze the differentiating value of Tim-3 expression

As shown in **Figure 4**, ROC curve analysis revealed that Tim-3 expression could serve as a promising biomarker for discriminating AML patients from healthy controls with an AUC of 1 (95% CI: 1-1; P< 0.001). When the cutoff value was set at 1.08, the sensitivity was 100% and the specificity was 100%. Furthermore, this observation also indicated that Tim-3 level yielded an AUC of 0.808 in differentiating patients with treatment more than 2 cycles to CR from all patients with AML (95% CI: 0.648-0.968; P=0.003). When the cutoff value was set at 12.35, the sensitivity and the specificity were 64.7% and 100%, respectively.

Discussion

Acute myeloid leukemia (AML) is a clonal malignant disorder derived from a small number of leukemic stem cells (LSCs). Despite the improvements in chemotherapy, about 60% of AML remission patients are still difficult to overcome relapse and drug resistance. LSCs are the main cause of refractoriness because of its uncontrollable proliferation, apoptosis blocked, and differentiation obstacle caused by the malignant clonal disorder and tumor immune escape.

count (Pearson r=0.746; *P*<0.0001, n=32). According to leukocyte count, higher expression of Tim-3 was observed in patients whose leukocyte count more than 100×10^9/L (*P*=0.043). No significant relationship of Tim-3 expression and age was observed in the newly diagnosed patients (Pearson r=-0.019; *P*= 0.916, n=32).

Numerous studies were performed to reveal the characteristics and function of LSCs. One of the most prevalent aims focused on the CD34$^+$CD38$^-$LSC$^-$ enriched cells, which had been proposed as an important factor in drug resistance. Studies had shown that LSCs and hematopoietic stem cells (HSCs) have the same cellular immune phenotype CD34$^+$CD38$^-$.
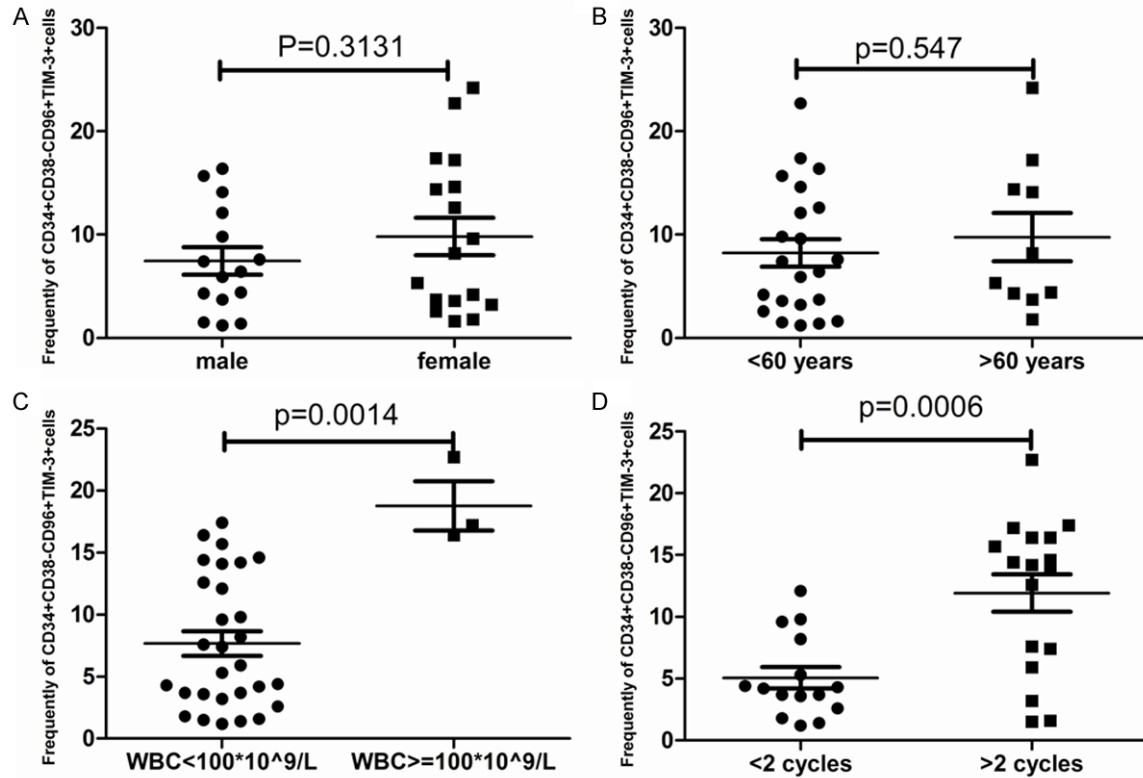
Figure 2. Tim-3 expressions in AML according to clinicopathologic parameters. The CD34$^+$CD38$^-$ AML LSCs were purified and tested for their Tim-3 expression. A, B. Summarized data of Tim-3 expression in AML LSCs on FACS between male and female patients as well as older and younger patients. C. Summarized data of Tim-3 expression in AML LSCs on FACS between patients with leukocyte count more than 100×10^9/L or not. D. Summarized data of Tim-3 expression in AML LSCs on FACS between patients with treatment cycles to CR more than two times or not. Differences between two groups were compared using the unpaired t-tests.

Table 2. Tim-3 expressions in AML according to clinicopathologic parameters

	Tim-3 (mean ± SD)
Gender	
Male (n=15)	7.460±1.323
Female (n=17)	9.818±1.814
Age (years)	
<60 (n=22)	8.236±1.320
≥60 (n=10)	9.760±2.322
Leukocyte count of newly diagnosed patients	
WBC<100×10^9/L (n=3)	7.672±0.9878
WBC≥100×10^9/L (n=29)	18.77±1.980
Treatment cycles to CR	
<2 cycles (n=15)	5.06±0.8594
≥2 cycles (n=17)	11.94±1.508

Although lack of certain HSCs phenotypes, such as Thy-1 (CD90), c-Kit (CD117) and HLA-DR, LSCs have their unique characteristics in cell phenotype, including CD123, CD44, CLL-1, CD96, CD47, CD32 and CD25. Of note, CD96 was proved to be an efficient identical marker of LSCs in CD34$^+$CD38$^-$ groups [11]. So, in our present study, we distinguished and purified LSCs from the CD34$^+$CD38$^-$ enriched cells fraction using CD96. But, in the latest study, Tim-3 as a surface molecule was also found to be highly expressed on LSCs in most types of AML, with the exception of acute promyelocytic leukemia (APL), but not on normal HSCs. And its specificity for targeting AML LSCs was significantly higher than those relatively specific molecular markers of LSCs, such as CD44, CLL-1, CD96, and CD47, which had been reported in previous studies [12, 13]. Yet it is still unclear whether Tim-3 shows a predictive value for clinical evaluation and prognosis.

Through in-depth studies of tumor immune escape and tumor microenvironment, a group of negative co-stimulatory molecules mediating immune regulation, including CTLA-4, PD-1, Tim-3, and so on were found, which were called negative immune checkpoints. Those mole-
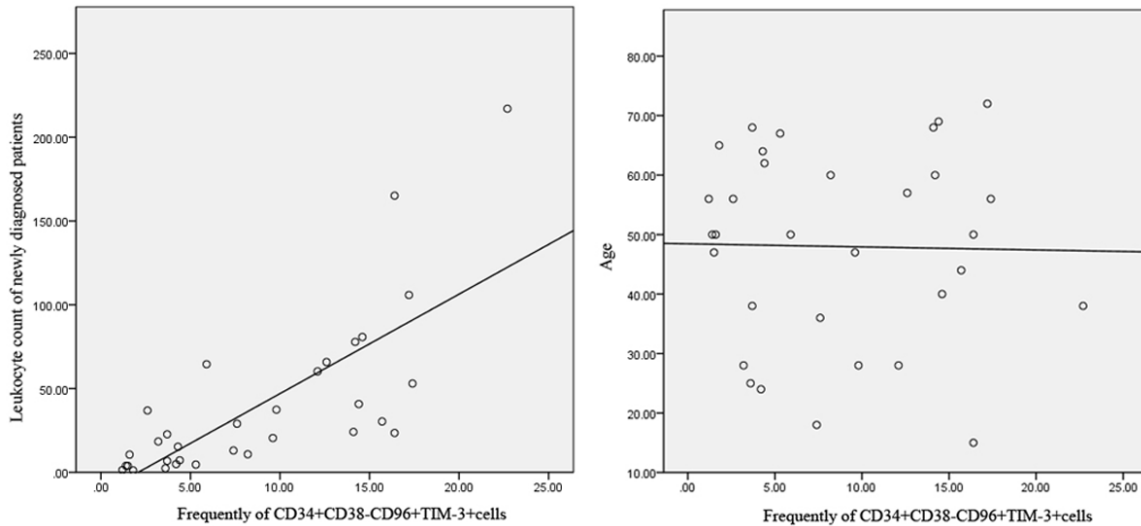
Figure 3. Relationship of Tim-3 expression and leukocyte count/age of newly diagnosed patients. Pearson correlation analysis shows a significant positive correlation of Tim-3 expression and leukocyte count (Pearson r=0.746; *P* < 0.0001, *n*=32). No significant relationship of Tim-3 expression and age was observed in the newly diagnosed patients (Pearson r=-0.019; *P*=0.916, *n*=32).

Cutoff value	1.08
Sensitivity (%)	100
Specificity (%)	100

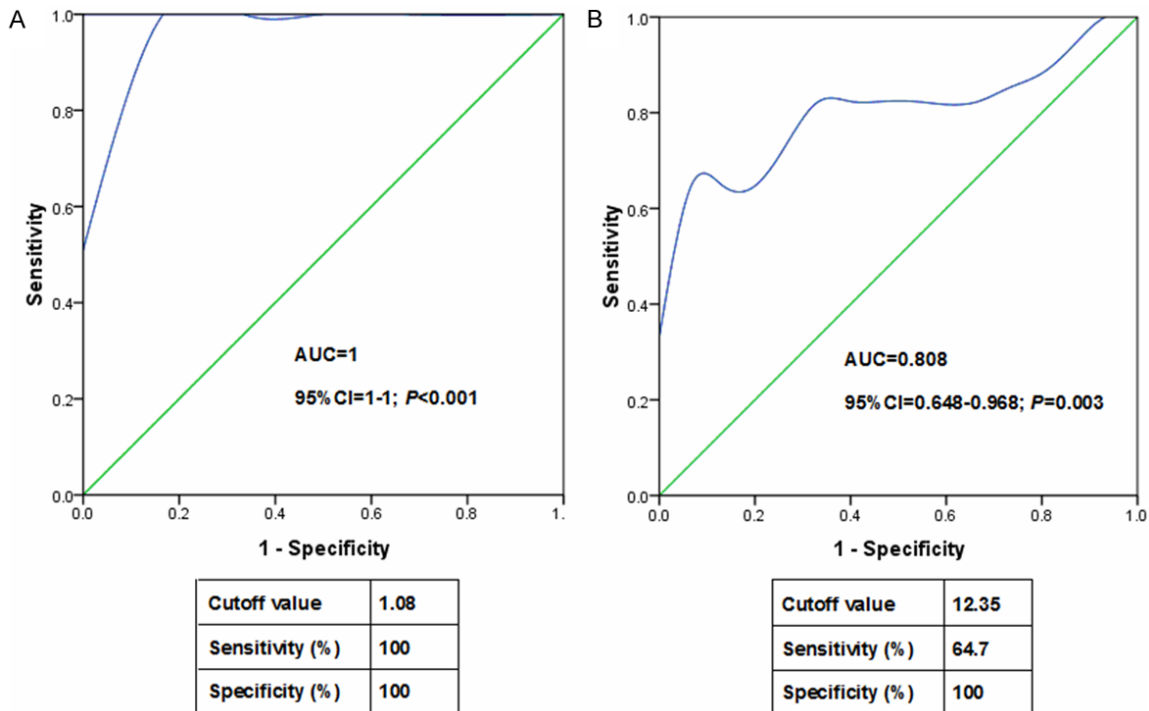| Cutoff value	12.35
Sensitivity (%)	64.7
Specificity (%)	100

Figure 4. ROC curve analysis using Tim-3 for discriminating AML patients. A. ROC curve analysis discriminating AML patients from healthy controls; B. ROC curve analysis differentiating patients with treatment more than 2 cycles to CR from all patients with AML.

cules expressed abnormally on a range of tumor tissue and/or infiltrating immune cells involving in tumor immune escape, constituted an important component of tumor microenvironment, and closely related with clinicopathology and prognosis. In the previous studies, Tim-3 was found mainly expressed in lots of mature immune cells such as Th1 cells, CD8^{+} T

cells, monocytes, regulatory T cells (Treg) and also considered to be an important marker of CD8+ T cell exhaustion/anergy [14, 15]. Tim-3 was believed to play a critical role in inhibiting Th1 responses through Treg expressing galectin-9, which was evidenced by its blockade administration resulted in the less tolerogenic effects of donorderived Treg cell transfusion during the allogeneic transplantation [16]. As Tim-3 associated with its ligand galectin-9 could evidently down-regulate Th1 responses and promote the peripheral tolerance, its role in tumor immunity is getting more and more attention [17].

In our present study, we explored the data of 43 adult AML cases (except APL) and 6 healthy donors through bone marrow mononuclear cell analysis, and further analyzed the correlations between expression levels of Tim-3 protein and leukocyte count of newly diagnosed patients. By using flow cytometry, we demonstrated that Tim-3 protein was highly expressed in CD34+CD38-CD96+ cells of AML patients, which showed that human Tim-3 was expressed in the vast majority of CD34+CD38- LSCs in AML of most FAB types but not in normal HSCs. The very significant up-regulation of Tim-3 in AML patients was also confirmed by ROC curve analysis at the same time, which showed that Tim-3 expression could serve as a promising diagnostic biomarker for discriminating AML from controls. And our result indicated that Tim-3 detection in combination with CD96 could propose a simple and effective method for rapid preliminary screening to evaluate the LSCs proportion in newly diagnosed AML patients. Meanwhile, in our study, increased Tim-3 expression was uncovered to be associated with leukocyte count and treatment cycles to complete remission. Furthermore, our correlation analysis showed that high Tim-3 expression was significantly positive associated with leukocyte count of newly diagnosed patients. In addition to a newly discovered molecular marker of LSCs, our study showed that elevated Tim-3 expression might be a direct consequence of the molecular mutations present in AML and be suggested as an independent prognostic predictor for patients with AML, and further be indicated the clinical value to predict recurrence by regular monitoring minimal residual disease. Importantly, the significantly higher expression of Tim-3 was found in patients with

treatment more than 2 cycles to CR. Meanwhile, ROC curve analysis also revealed that Tim-3 level could be available for differentiating patients with treatment more than 2 cycles to CR from all patients with AML. The results strongly indicated that a higher expression of Tim-3 may promote a poor response for chemotherapy, which may be closely associated with primary resistant, and supported the hypothesis that Tim-3 level might be used as a potential predictor of chemotherapeutic sensitivity and prognosis in AML patients.

As Kikushige Y research showed that in xenograft models reconstituted with human AML LSCs or HSCs, an anti-human Tim-3 mouse IgG2a antibody with cytotoxic activities eradicates AML LSCs in vivo, but does not affect normal human hematopoiesis [18], we confirmed Tim-3 expressed on LSCs was not only the molecular marker, but also played an important role in maintaining the functional leukemia stem cells survival. And the conceptual understanding of its biological role is required before consideration of this protein for therapeutic settings. A series of previous studies revealed the detailed mechanism of action underlying the biological responses mediated by Tim-3 receptor, which demonstrated that Tim-3 receptor molecules were distributed largely on the surface of primary AML cells, whereas in healthy leukocytes Tim-3 protein was mainly expressed intracellularly. Tim-3 triggered growth factor type responses in AML cells by activating PI3 kinase/mTOR pathway. This was in line with increased accumulation of HIF-1α and secretion of VEGF and TNF-α, and that could activate hypoxic signalling pathways upregulating glycolysis and proangiogenic responses [19, 20].

Thus, TIM-3 is a promising therapeutic target for the eradication of AML LSCs. TIM-3 is frequently expressed in the CD34+CD38- LSCs population in AML patients and significantly associated with leukocyte count and treatment cycles to complete remission, which strongly suggested that TIM-3 may be a marker of LSCs, candidate therapeutic target and prediction factor in AML patients. For the limit of adherence and follow-up of the patients, only the outcomes in 43 AML patients with completed clinical data were analyzed. In the following research, further follow-up and survival analy-

sis should be performed in order to identify the role of Tim-3 in the prognosis of AML. On the other hand, we would try to filter specific antigen of LSCs using Tim-3 as a marker targeting for the active immunotherapy to treat acute myeloid leukemia, which would also explore a beneficial way for removal of the minimal residual disease and improvement of the prognosis of AML.

Disclosure of conflict of interest

None.

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