

CD44 expression in bone marrow interstitial fluid of hematological malignancies and marrow blasts

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Abstract

The leukemic stem cell markers, CD44 and TIM-3, have been demonstrated to be prognostically significant with hematological malignancies. These findings were established with regard to histopathological or serum materials. The present study aimed to define the expression of CD44 and TIM-3 in bone marrow interstitial fluid with clinical relevance. The values of CD44 and TIM-3 were assayed from bone marrow interstitial fluid at diagnosis, including 68 cases with hematological malignancies and 28 non-neoplastic cases. Patients with hematological malignancies had higher levels of CD44 and TIM-3. The expressions of CD44 and TIM-3 increased significantly in hematological malignancies with bone marrow involvement compared with those without bone marrow involvement. Only CD44 expressions were correlated positively with blast percentages of bone marrow in acute myeloid leukemia and myelodysplastic syndrome. In conclusion, CD44 and TIM-3 increased in bone marrow from patients with hematological malignancies. CD44 expressions had a positive correlation with marrow blasts in acute myeloid leukemia and myelodysplastic syndrome.

Background

Acute myeloid leukemia (AML) consists of heterogeneous diseases which are characterized by a multitude of molecular abnormalities. There has been great improvements with current treatment strategies, however, half of these patients' relapses are still associated with grave survival. Cancer stem cells have been identified as a potential reason for therapeutic resistance in human malignancies. But acute myeloid leukemia is not the case of exception with a bundle of research focused on leukemic stem cells (LSC). The recognition of stemness features, including self-renewal, undifferentiated state, and drug resistance is not sufficient to distinguish leukemic stem cells from hematopoietic stem cells (HSC) in bone marrow (1). The existence of LSCs needs to meet the criteria first demonstrated 20 years ago with the ability to engraft and initiate human AML in immune-compromised mouse models. Thereafter, several lines of evidence suggest the CD34⁺/CD38⁻ phenotypes are the most relevant to LSCs (2). Based on the improvement of understanding of the true LSC, several markers have been investigated to define the different LSC phenotypes by new therapeutic targets and techniques, such as multicolor flow cytometry, side population assay, and ALDH assay. To improve the dismal outcome of AML, a better understanding of LSC is required to stratify the risk categories and to guide clinical decisions (3). Many surface markers have been demonstrated to be expressed on the LSCs' surface, such as CD33, CD123, CD44, and CD47 as well as more recently, T-cell immunoglobulin and mucin domain 3 (TIM-3) (4). But each marker can identify only a part of the AML patient population and only a few biomarkers can be detected in interstitial fluid. In this study, we evaluated the concentrations of CD44 and TIM-3 in bone marrow interstitial fluid to correlate clinical significance in patients with hematological malignancies, especially in AML and myelodysplastic syndrome (MDS).

Methods

Patients

The study focused on 60 patients with hematological malignancies and 28 matched patients with diagnoses other than hematological malignancies, such as aplastic anemia, idiopathic thrombocytopenic purpura, or viral infection.

There were 48 males and 40 females, aged from 21 to 93 years. They were recruited from Chi-Mei medical center, Tainan, Taiwan ROC. The study was approved by the research ethics committee and consent were obtained from patients. These patients were separated into three groups as follows: Group 1 (myeloid neoplasm group): included 16 patients with new diagnosis of AML and 12 patients with MDS. Group 2 (lymphoid neoplasm group) comprised 32 patients with lymphoma, including 13 diffuse large B cell lymphoma, 6 myeloma, 4 follicular lymphoma, 3 lymphoblastic lymphoma, 3 marginal zone lymphomas, 2 T cell lymphoma, and 1 Burkitt's lymphoma, who received bone marrow study for the staging of lymphoma disease (table 1). Among 32 patients with lymphoid malignancies, 16 patients with lymphoid malignancies had malignant involvement of bone marrow documented by marrow pathology. Group 3 (control group): consisted of 28 patients without hematological malignancies, but received diagnostic bone marrow aspiration for clinical indications of bone marrow exam. Most of them presented with anemia or thrombocytopenia. All the pathological reports from marrow in this group were normal cellular marrow without evidence of malignancy. The final diagnoses in group 3 included severe infection, autoimmune disease, renal disease and liver disease. This study was conducted in accordance with ethic principle of the Declaration of Helsinki, and study approval was obtained from the independent Ethic Committees of Chi Mei Medical Center (Institutional Review Board Serial No. 10302-003). Written informed consent was obtained from all participants.

Specimen collection

These bone marrow samples were collected during the first diagnostic aspiration. After harvesting, we used the centrifugation technique to obtain samples of bone marrow interstitial fluid (BMIF) from aspiration.

Measurement of CD44 and TIM-3

Using Enzyme linked Immuno Sorbent Assay (ELISA) method, these levels of CD44 and TIM-3 were estimated from bone marrow samples. ELISA was performed following the manufacturer's instructions (DuoSet ELISA development system, USA). On 96-well ELISA plates (GeneDireX), each well was coated with monoclonal capture antibody (antihuman CD44 and TIM-3) obtained from mouse (R&D System, USA) and incubated at 4°C overnight. The plates were blocked for 1 h and then incubated at 4°C overnight with plasma on the next day. Followed by the addition of corresponding biotinylated detection antibody obtained from goat (R&D system, USA), they were incubated for 2 hours. We used Streptavidin-Horseradish peroxidase as enzyme conjugate. After incubation, 3,3',5,5'-tetramethylbenzidine substrate (Clinical Science Products Inc., Mansfield, MA, USA) was added. The reaction was stopped using 2N HCl and optical density (OD) reading was taken at 450 nm in a microplate spectrophotometer (Epoch, BioTek,

Winooski, VT, USA). After obtaining a standard curve based on the standards provided by the manufacturer, the results were expressed as concentration (in pg/ml) read from the standard curve (concentration in range: minimum of 31.25 pg/ml, to maximum of 2000 pg/ml).

Statistical analysis

Qualitative data were described in the form of numbers and percentages. Quantitative data were described in the form of mean $\pm$ standard error of the mean (SEM). And we used two-sample t test when comparing quantitative data in hematological malignancy group and control group as well as subgroup analysis of quantitative data. The probability of being by chance (P value) was calculated for all parameters (P is significant if ≤ 0.05 at confidence interval 95%). Besides, considering the effect of extreme value, we used Winsorized means when analyzing the concentrations of CD44 and TIM-3. $g = [rn]$, $r = 0.2$, n = number of patients in each subgroup and g was the integer part in product of r and n .

Results

Demographics

Among patient demographics, the age of these patients were similar although the median ages in myeloid neoplasm group tend to be younger compared with other two groups. The median age of patients in myeloid neoplasm group was 60 years with the range between 27 and 90 years. 54% of patients in myeloid neoplasm group were male. The distribution of gender in myeloid neoplasm group was male dominant which was consistent with previous reports from an institute in Taiwan (5, 6).

CD44 and TIM-3 concentrations were measured in BMIF from patients in this study. Compared with non-malignancy group (control), the expression of CD44 in hematological malignancy group was higher (40.24 ± 5.45 ng/ml vs 16.36 ± 2.44 ng/ml) with significance statistically. The data in Table 2 were represented as mean $\pm$ SEM. The CD44 expression in myeloid malignancy group was 39.37 ± 5.02 ng/ml and 42.64 ± 9.97 ng/ml in lymphoid malignancy group, which were higher than those in control group (16.36 ± 2.38 ng/ml) (Fig. 1A and 1B). Among hematological malignancies, the TIM-3 expressions were 7.00 ± 1.32 ng/ml compared with 3.13 ± 0.43 ng/ml in control group. The TIM-3 expressions were 5.43 ± 1.41 ng/ml in myeloid malignancy group and 8.46 ± 2.24 ng/ml in lymphoid malignancy group separately. TIM-3 values in both groups were higher than the value in control group, 3.37 ± 0.55 ng/ml (table 2). But only TIM-3 expression in lymphoid malignancy group were higher than control group statistically ($p = 0.04$) (Fig. 2A and 2B).

Subgroup analysis of CD44 and TIM-3

Next, we analyzed the expression of CD44 in these hematological malignancies grouped by bone marrow involvement of malignant cells. Hematological malignancies with bone marrow involvement had higher expression of CD44 compared with those without bone marrow involvement from BMIF ($51.25 \pm$

6.86ng/ml vs 14.57 ± 1.61 ng/ml, $p < 0.01$). CD44 had been correlated with poor prognosis of AML by immunohistochemical analysis of bone marrow samples from a previous study (7). Therefore, we further analyzed the expression of CD44 in AML/MDS and control groups. CD44 expressions in AML/MDS were higher than these in control group (46.42 ± 7.39 ng/ml vs 16.36 ± 2.44 ng/ml, $p < 0.01$ (Fig. 1C and 1D)).

TIM-3 and its ligand, Gal-9, are expressed on AML blasts and LSC (8). Among patients with hematological malignancies, the expression of TIM-3 increased in those malignancies with bone marrow involvement compared with those without bone marrow involvement (8.59 ± 1.75 ng/ml vs 2.97 ± 0.32 ng/ml, $p < 0.01$). But patients with AML/MDS disease did not have high values of TIM-3 in bone marrow (Fig. 2C and 2D)).

To determine the clinical relevance of CD44 and TIM-3 in AML/MDS blasts, we analyzed the correlation between these factors and AML/MDS blasts. The blast count was calculated by microscopic exam. The expressions of CD44 in BMIF were positively correlated with AML/MDS blasts in Fig. 3A, but TIM-3 failed to demonstrate the correlation with AML/MDS blasts from our analysis (Fig. 3).

Discussion

Cancer stem cells have been described as solid and hematopoietic cells. All tumor cells could be biologically equivalent and heterogeneity derives from extrinsic and intrinsic influences. The clinical relevance of CSCs beyond experimental models is extensively investigated(9). An early study in the pathogenesis of lymphoid malignancies showed that high expression of the CD44 molecule in the earlier phases of T or B cell development suggests that CD44 may be involved in the migration of bone marrow derived lymphoid precursors to the site of maturation (10). In this study, we demonstrated the clinical significance of CD44 expression in hematological malignancies from bone marrow interstitial fluid. Elevation of serum CD44 along with another stem cell factor, osteopontin, had been disclosed from a study of 54 patients with non-Hodgkin lymphoma (11). As a prognostic aspect, elevation of serum CD44 was associated with worse outcome in patients with aggressive lymphoma as well as indolent lymphoma (12, 13). In murine chronic myeloid leukemia, CD44 in leukemia cells and E-selection on bone marrow endothelium are essential mediators for the engraftment of leukemic stem cells (14). Many studies have disclosed that elevated levels of serum CD44 can predict an unfavorable outcome in MDS and have been associated with advanced disease in B-cell chronic lymphocytic leukemia (15, 16). Besides prognostic impact of CD44 in leukemia, the serum CD44 levels also correlated well with the absolute numbers of leukemia cells in peripheral blood, which implied that CD44 could be a reliable tumor marker of acute leukemia (17). The exploratory functions of CD44 have been connected with certain biological functions of AML. An interaction between hyaluronic acid and CD44 triggers inside-out activation of VLA-4 resulting in enhanced leukemic cell adhesion on VCAM-1 (18). CD44 is also involved in CXCL12 promotion of resistance to venetoclax-induced apoptosis in human AML cell lines and AML patient samples which provided evading evidence of leukemia cancer stem cells to therapeutic refractoriness (19). The correlation between CD44 expression and blasts in marrow provided potential clinical implications. Notably, CD34, CD44, and CD49f expression were significantly increased in high risk

MDS and AML associated metaclusters from a recent report using high-dimensional mass cytometry (20).

We also demonstrated the elevation of TIM-3 expression in bone marrow from patients with hematological malignancies, including lymphoid and myeloid entities. However, TIM-3 expression failed to correlate with blasts number in marrow fluid of AML/MDS from our study. But the expression of TIM-3 measured by multicolored-flow cytometry in bone marrow samples was significantly higher, compared to peripheral blood in AML patients with treatment failure from a previous report (21). The expression of TIM-3 detected by reverse transcription-quantitative PCR was significantly up-regulated in patients with AML compared with that in healthy individuals. High TIM-3 expression was also correlated with poor overall survival rates in AML patients (22). As a potential therapeutic target in AML, bispecific and split chimeric antigen receptor cells targeting TIM-3 and CD13 eradicated acute myeloid cells in vitro and animal models with reduced toxicity to human bone marrow stem cells and peripheral myeloid cells in mouse models (23).

In conclusion, we detected the elevation of CD44 and TIM-3 expression in bone marrow fluid from patients with myeloid and lymphoid neoplasm. Only the expressions of CD44 in bone marrow correlated with the amount of blast in marrow fluid from patients with AML/MDS disease. The therapeutic implications and clinical impact required further studies to define the role of cancer stem cell markers, including CD44 and TIM-3.

Declarations

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The authors do not disclose any potential conflicts of interest.

This study was conducted in accordance with ethic principle of the Declaration of Helsinki, and study approval was obtained from the independent Ethic Committees of Chi Mei Medical Center (Institutional Review Board Serial No. 10302-003). Written informed consent was obtained from all participants.

Author Contribution

Feng YH, Wu HC, Tung CL, Chang WT, Chen ZC, Chang CP, Kang NW, Kuo YH, Chen WY, Huang CT organized the concept initiation and data review; Feng YH, Tung CL wrote the main manuscript test; , Feng YH, Wu HC, Tung CL, Chang CP, Kang NW, Kuo YH, Chen WY, Huang CT for samples collection; Feng YH, Tung CL analyzed the data and plotted figures; Feng YH, Wu HC, Tung CL, Chang WT, Chen ZC, Chang CP, Kang NW, Kuo YH, Chen WY, Huang CT involed data discussion. All authors reviewed the manuscript.

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Tables

Tables 1 and 2 are available in the Supplementary Files section.

Figures

Figure 1

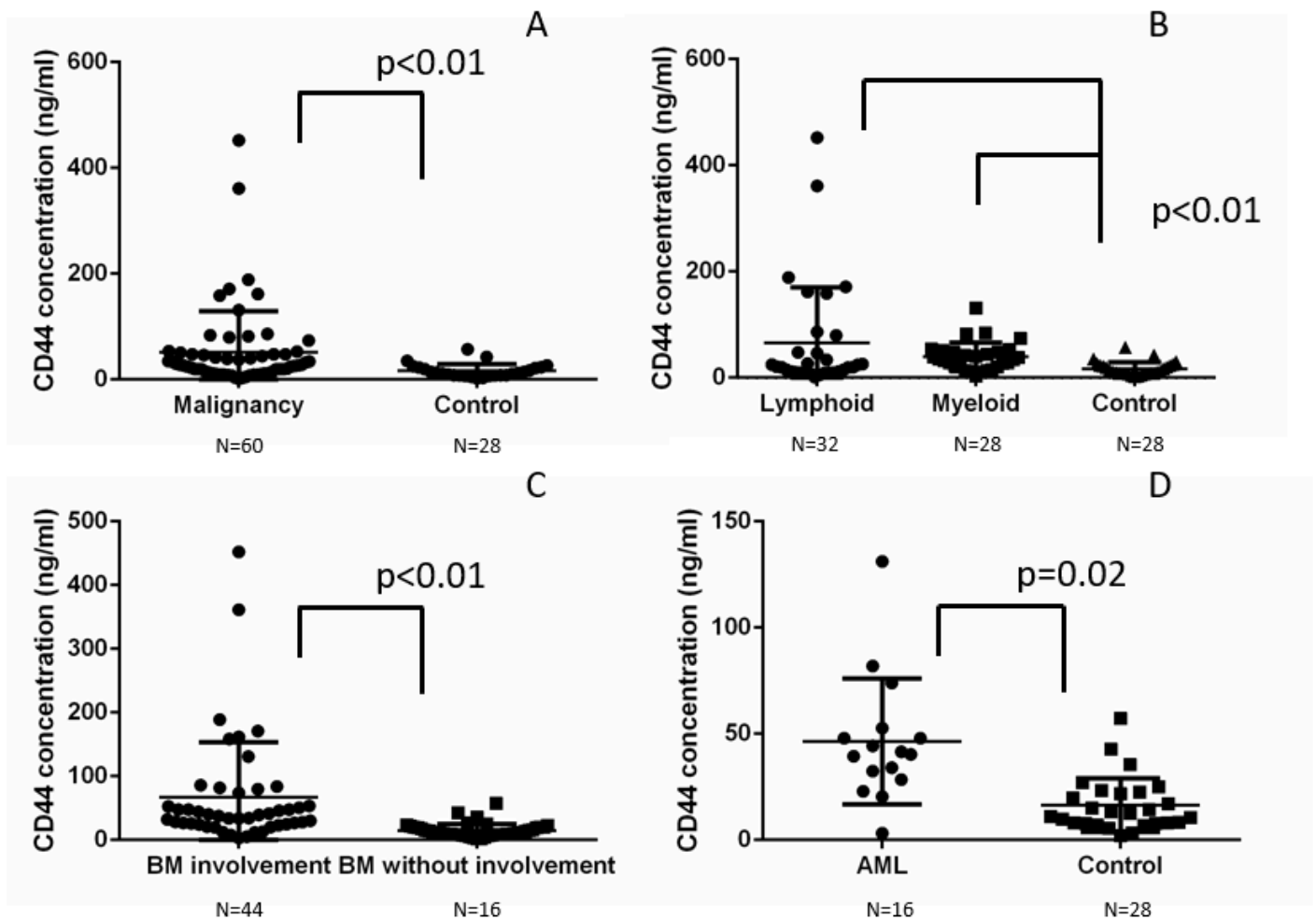


Figure 1

Expression of CD44 in different subtypes of hematological malignancies. A. Expression of CD44 in hematological malignancies and control groups. B. Expression of CD44 in lymphoid, myeloid, and control groups. C. Expression of CD44 in hematological malignancies with or without bone marrow involvement. D. Expression of CD44 in AML/MDS and control groups.

Figure 2

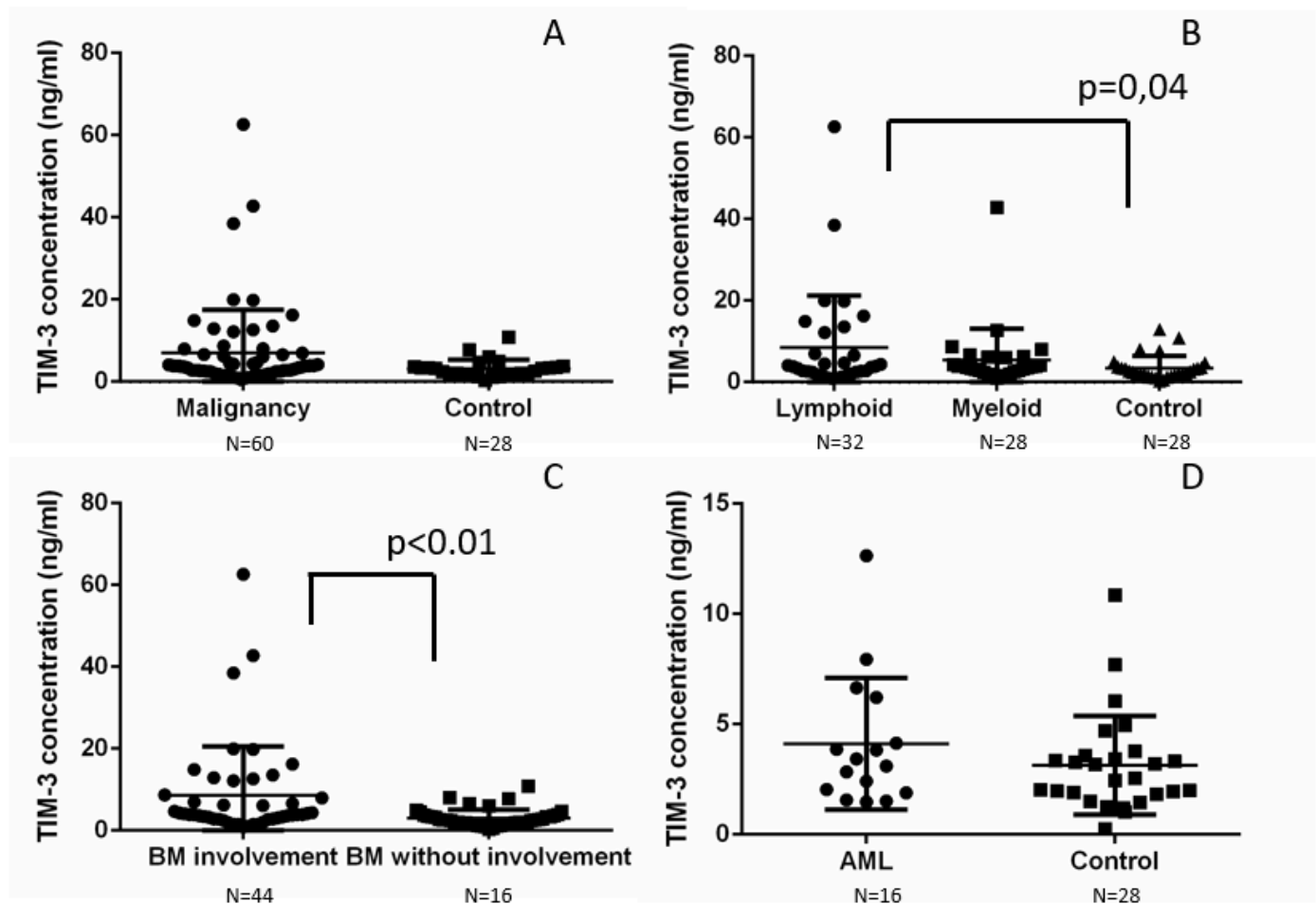


Figure 2

Expression of TIM-3 in different subtypes of hematological malignancies. A. Expression of TIM-3 in hematological malignancies and control groups. B. Expression of TIM-3 in lymphoid, myeloid, and control groups. C. Expression of TIM-3 in hematological malignancies with or without bone marrow involvement. D. Expression of TIM-3 in AML/MDS and control groups.

Figure 3

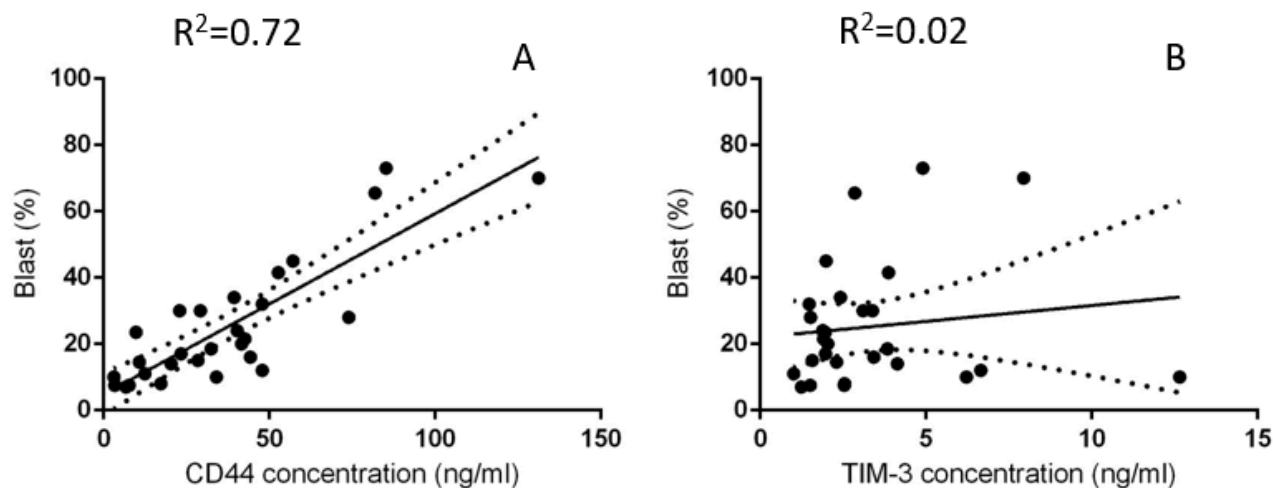


Figure 3

The correlation between blasts in bone marrow with CD44 or TIM-3. A. The CD44 expression was correlated positively with blasts in AML/MDS ($R^2=0.72$). B. The correlation between blast and TIM-3 in AML/MDS ($R^2=0.02$).

Supplementary Files

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- [Tablev19.pptx](#)