



OPEN ACCESS

Edited by:

Mazdak Ganjalikhani Hakemi,
Isfahan University of Medical Sciences,
Iran

Reviewed by:

Matteo Molica,
S. Eugenio, Rome, Italy
Andrew Kelly,
Foundation Medicine Inc.,
United States

*Correspondence:

Yangqiu Li
yangqiu1@hotmail.com
Shaohua Chen
jnshaohuachen@163.com

*ORCID:

Yangqiu Li
orcid.org/0000-0002-0974-4036
Shaohua Chen
orcid.org/0000-0003-4945-5914
Jiaxiong Tan
orcid.org/0000-0002-5606-3962
Cunte Chen
orcid.org/0000-0003-3733-9174
Xianfeng Zha
orcid.org/0000-0002-4970-1305
Chaofeng Liang
orcid.org/0000-0002-0567-4739

†These authors have contributed
equally to this work

Specialty section:

This article was submitted to
Hematologic Malignancies,
a section of the journal
Frontiers in Oncology

Received: 13 July 2021

Accepted: 20 September 2021

Published: 08 October 2021

Citation:

Liang C, Zhao Y, Chen C, Huang S,
Deng T, Zeng X, Tan J, Zha X,
Chen S and Li Y (2021) Higher TOX
Genes Expression Is Associated With
Poor Overall Survival for Patients
With Acute Myeloid Leukemia.
Front. Oncol. 11:740642.
doi: 10.3389/fonc.2021.740642

Higher TOX Genes Expression Is Associated With Poor Overall Survival for Patients With Acute Myeloid Leukemia

Chaofeng Liang^{1†}, Yujie Zhao^{1†}, Cunte Chen^{1††}, Shuxin Huang¹, Tairan Deng¹,
Xiangbo Zeng¹, Jiaxiong Tan^{2†}, Xianfeng Zha^{3†}, Shaohua Chen^{1*†} and Yangqiu Li^{1*†}

¹ Key Laboratory for Regenerative Medicine of Ministry of Education, Institute of Hematology, School of Medicine, Jinan University, Guangzhou, China, ² Department of Hematology, First Affiliated Hospital, Jinan University, Guangzhou, China, ³ Department of Clinical Laboratory, First Affiliated Hospital, Jinan University, Guangzhou, China

Thymocyte selection-associated HMG box (TOX) is a transcription factor that belongs to the high mobility group box (HMG-box) superfamily, which includes four subfamily members: TOX, TOX2, TOX3, and TOX4. TOX is related to the formation of multiple malignancies and contributes to CD8+ T cell exhaustion in solid tumors. However, little is known about the role of TOX genes in hematological malignancies. In this study, we explored the prognostic value of TOX genes from 40 patients with *de novo* acute myeloid leukemia (AML) by quantitative real-time PCR (qRT-PCR) in a training cohort and validated the results using transcriptome data from 167 *de novo* AML patients from the Cancer Genome Atlas (TCGA) database. In the training cohort, higher expression of TOX and TOX4 was detected in the AML samples, whereas lower TOX3 expression was found. Moreover, both the training and validation results indicated that higher TOX2, TOX3, and TOX4 expression of AML patients (3-year OS: 0% vs. 37%, $P = 0.036$; 3-year OS: 4% vs. 61%, $P < 0.001$; 3-year OS: 0% vs. 32%, $P = 0.010$) and the AML patients with highly co-expressed TOX, TOX2, TOX4 genes (3-year OS: 0% vs. 25% vs. 75%, $P = 0.001$) were associated with poor overall survival (OS). Interestingly, TOX2 was positively correlated with CTLA-4, PD-1, TIGIT, and PDL-2 ($r_s = 0.43$, $P = 0.006$; $r_s = 0.43$, $P = 0.006$; $r_s = 0.56$, $P < 0.001$; $r_s = 0.54$, $P < 0.001$). In conclusion, higher expression of TOX genes was associated with poor OS for AML patients, which was related to the up-regulation of immune checkpoint genes. These data might provide novel predictors for AML outcome and direction for further investigation of the possibility of using TOX genes in novel targeted therapies for AML.

Keywords: TOX, prognosis, biomarker, immune checkpoint, acute myeloid leukemia

INTRODUCTION

In recent years with the improvement of chemotherapy regimens and the development of hematopoietic stem cell transplantation technology, acute myeloid leukemia (AML) patient treatment has achieved certain curative effects. However, there is still a high risk of relapse and a low disease-free survival rate (1, 2). The immune escape of tumor cells is a crucial cause of relapse and

refractory AML (3). It has been shown that in the tumor microenvironment, tumor cells induce the expression of immune checkpoint (IC) genes, such as programmed cell death protein 1 (PD-1), cytotoxic T lymphocyte-associated molecule-4 (CTLA-4), and lymphocyte-activation gene 3 (LAG-3), leading to T cell exhaustion and immune escape (4–9). Clinical trials of targeted inhibitory antibodies, such as anti-PD-1 and anti-CTLA-4, in solid tumors have demonstrated their significant effects (10). In contrast, the clinical effectiveness of such immune therapies appears to be relatively different for different AML cases and clinical trials with different outcomes (11–13). Therefore, it is worth exploring the immune biomarkers that may be related to the effects of immune checkpoint blockade and revision of T cell exhaustion as well as their association with clinical outcome in AML (14).

Thymocyte selection-associated HMG box (TOX), a transcription factor that can bind to DNA, belongs to the high mobility group box (HMG-box) superfamily. TOX includes four subfamily members (TOX1-4, TOX1 is also known as TOX) (15). TOX is a crucial transcription factor related to the development of malignancies and contributing to CD8+ T cell exhaustion in patients with solid tumors (16–18). For example, TOX is positively correlated with larger tumor size, lower differentiation, later tumor node metastasis (TNM) stage, and facilitating endocytic recycling of PD-1 (17). In tumor-infiltrating CD8+ T cells from human melanoma and non-small cell lung cancer (NSCLC), increased expression of TOX in CD8+ T cells is associated with high expression of PD-1 (19). In contrast, there are few studies on TOX genes in hematological malignancies. TOX is highly expressed in acute lymphoblastic leukemia (ALL), particularly in T cell - ALL (T-ALL). High expression of TOX inhibits the function of the repair factors KU70/KU80 causing abnormal non-homologous end joining (NHEJ) repair (17). Although TOX is positively expressed in almost all ALL cases, TOX deletion has also been detected in ALL patients (20). Therefore, the mechanism by which TOX plays a role in ALL remains to be investigated.

In our previous study, we found higher TOX expression concurrent with PD-1, Tim-3, or CD244 in T cells from patients with B cell non-Hodgkin's lymphoma (B-NHL), which suggested that TOX may be involved in inducing CD8+ T cell exhaustion by co-regulation with immune checkpoint proteins (21).

In this study, we investigated the expression characteristics and prognostic value of the TOX genes and analyzed the correlation between TOX and IC genes in peripheral blood (PB) samples from AML patients in our clinical center. The results were further validated with high-throughput sequencing

Abbreviations: AML, acute myeloid leukemia; allo-HSCT, allogeneic hematopoietic stem cell transplantation; B-NHL, B-non-Hodgkin's lymphoma; BM, bone marrow; CI, confidence interval; CR, complete response; CTLA-4, cytotoxic T lymphocyte-associated molecule-4; ELN, European LeukemiaNet, HI, healthy individuals; HMG-box, high mobility group box; HR, hazard ratio; ICs, immune checkpoint genes; LAG-3, lymphocyte-activation gene 3; NHEJ, non-homologous end joining; NSCLC, non-small cell lung cancer; OS, overall survival; PB, peripheral blood; PBMCs, peripheral blood mononuclear cells; PD-1, programmed cell death protein 1; qRT-PCR, quantitative real-time PCR; RMST, restricted mean survival time; SD, standard deviation; SPSS, Statistical Product and Service Solutions; T-ALL, T cell - acute lymphoblastic leukemia; TCGA, The Cancer Genome Atlas; TNM, tumor node metastasis; TOX, thymocyte selection-associated HMG box; WBC, white blood cell.

data from The Cancer Genome Atlas (TCGA) database in a more significant number of patients.

MATERIALS AND METHODS

PB Sample Information

In this study, we collected PB mononuclear cells from 40 *de novo* AML patients with informed consent (15 males and 25 females) who ranged in age from 12 to 83 years and provided informed consent from March 2016 to March 2021. We also included 17 AML-complete response (CR) patients (11 males and 6 females) whose ages ranged from 12 to 62 years (**Figure 1**). In addition, we collected PB white blood cells (WBCs) from 25 healthy individuals (HIs), including 12 males and 23 females, whose ages ranged from 19 to 70 years as a control population. Overall survival (OS) was defined as the time from diagnosis to death or last follow-up. The clinical information of the patients in the training cohort was listed in **Table 1**. This study was approved by the Ethics Committee of the School of Medicine of Jinan University [The ethical committee study number: (2015) Lun Shen Pi Ke No. 9].

TCGA Dataset

The gene expression data and the clinical information of 167 *de novo* AML patients were obtained from the TCGA (<https://cancergenome.nih.gov/>) database by UCSC XENA (<https://xenabrowser.net/datapages/>) (6). The gene expression data from the TCGA database comprised the validation cohort for OS analysis and were used to validate the results of the training cohort.

Quantitative Real-Time PCR

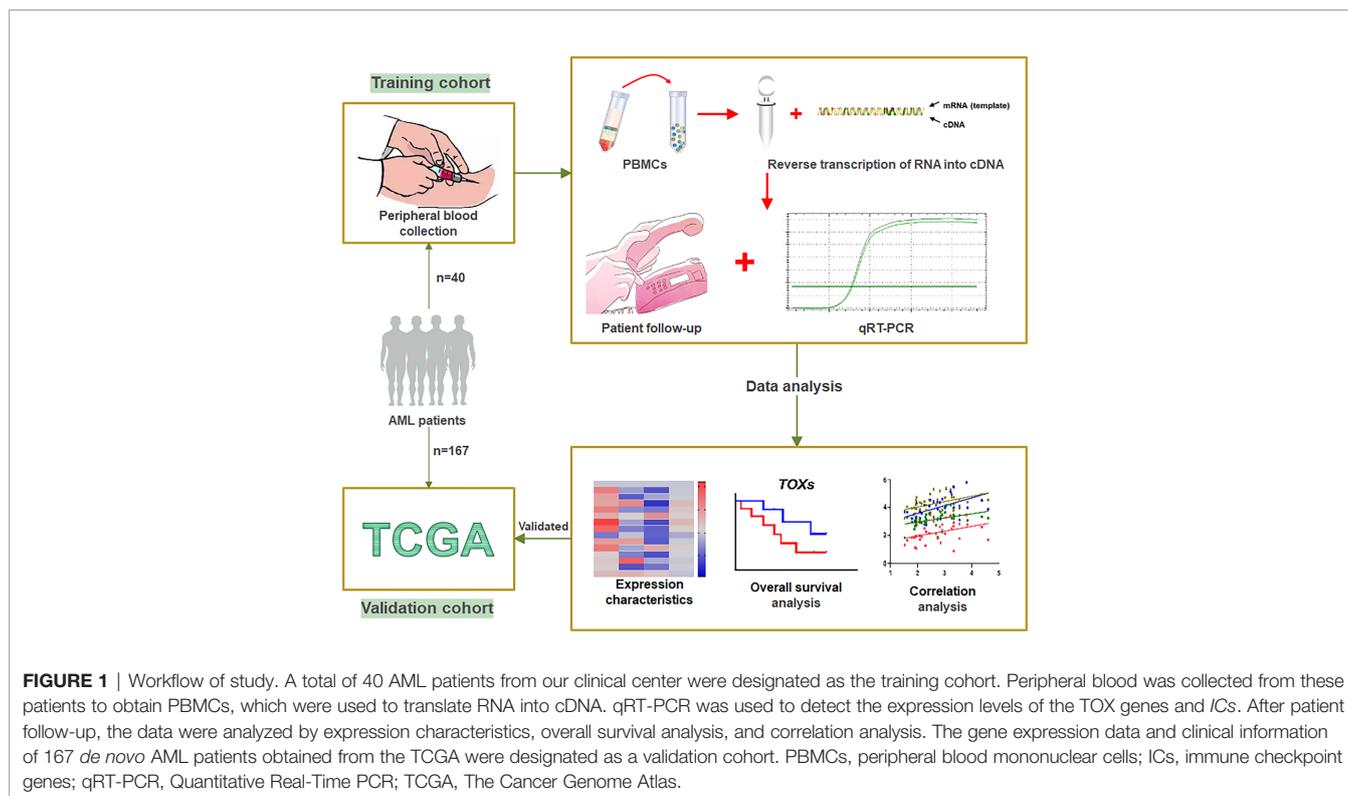
RNA isolation was performed using peripheral blood mononuclear cells (PBMCs) samples. Reverse transcription of RNA into cDNA was performed according to the manufacturer's instructions for the Reverse Transcription Kit (ABI, USA). The gene expression levels were quantified according to the manufacturer's instructions in the qRT-PCR kit (TIANGEN, China) (6), and $\beta 2M$ was used as an internal control. The sequences of the primers used for qRT-PCR are listed in **Supplementary Table 1**. The gene expression results are presented as the fold change $lg(2^{-\Delta\Delta CT} \times 100)$.

Optimal Prognostic Cutoff Values

The Optimal prognostic cutoff values for TOX, TOX2, TOX3, and TOX4 were determined using the maximally selected rank statistics from the 'maxstat' R package, which was provided to the 'survminer' R package (22). This is an outcome-oriented method providing a value of a cut-point that corresponds to the most significant relationship with survival. According to the optimal cut-points of TOX genes, AML patients were divided into low- and high-expression groups to plot and compare Kaplan-Meier curves.

Statistical Analysis

All statistical analyses were performed using Statistical Product and Service Solutions (SPSS) (version 22.0, IBM, Armonk, NY,



USA), GraphPad Prism (version 8.4.2, CA, USA), and *R* (version 3.6.1, <https://www.r-project.org/>) as appropriate. Kaplan-Meier curves were plotted according to the optimal prognostic cutoff values (**Supplementary Figure S1**) for continuous variables, which were obtained using the “Survminer” package, and the log-rank test was used for comparison. A Correlation heatmap was generated using the “ggcorrplot” package, and it was analyzed using Spearman’s coefficient. Univariate and multivariate COX regression analyses were used to identify independent prognostic factors. The Mann-Whitney test was used for comparison between two groups, and the Kruskal-Wallis test was used to compare multiple gene expression groups. A two-tailed p -value < 0.05 was considered statistically significant.

RESULTS

Expression Characteristics of TOX Genes in AML

The expression level of four TOX genes was characterized for 40 AML patients, 17 AML-CR patients, and 25 HIs (**Figure 2A**). Compared with the HIs, *TOX* was highly expressed in AML (median: 2.48 vs. 1.93, $P = 0.010$) and AML-CR (median: 2.40 vs. 1.93, $P < 0.001$) patients (**Figure 2B**). The expression of *TOX2* in the AML-CR group was higher than that in the HI group (median: 2.45 vs. 2.00, $P = 0.016$); however, the expression of *TOX3* was lower than that in the HI group (1.53 vs. 1.97, $P = 0.016$). The expression characteristics of *TOX4* was as follows:

AML > HI > AML-CR (median: 2.10 vs. 1.95 vs. 1.81, AML vs AML-CR: $P < 0.001$; AML vs HI: $P = 0.014$; AML-CR vs HI: $P = 0.042$).

We further compared the expression level of the TOX genes in different AML subtypes in comparison to HIs (median: 1.93). The expression of *TOX* increased significantly in AML-M2 (median: 2.90, $P < 0.001$) and AML-M5 (median: 2.82, $P < 0.001$) patients. For *TOX*, the expression followed the pattern AML-M2 > AML-M5 > AML-M3 (AML-M2 vs. AML-M5: $P = 0.388$; AML-M3 vs. AML-M5: $P = 0.074$; AML-M2 vs. AML-M3: $P = 0.042$). There was no statistically significant difference for *TOX2* among the HI and AML subtypes ($P = 0.486$). *TOX3* expression in AML-M2 patients was lower than that in HIs (median: 1.01 vs. 1.96, $P = 0.028$). Interestingly, although *TOX3* was generally low in AML patients, its expression in AML-M5 patients (median: 2.04) was significantly higher than that in AML-M2 patients, and it had the following expression pattern: AML-M5 > AML-M3 > AML-M2 (AML-M5 vs. AML-M3: $P = 0.193$; AML-M3 vs. AML-M2: $P = 0.837$; AML-M5 vs. AML-M2: $P = 0.028$). The expression of *TOX4* in AML-M2 (median: 2.25) and AML-M5 (median: 2.13) patients maintained an upward trend compared with the HI group (median: 1.95, $P = 0.036$, $P = 0.034$); however, there was no statistically significant difference between the AML-M3 and HI groups (**Figure 2C**).

Higher Expression of TOX Genes Is Associated With Poor OS in AML Patients

To investigate the role of altered *TOX* expression in the clinical outcome of AML patients, we collected the clinical information of

TABLE 1 | Clinical characteristics of AML patients.

Variables	Patients (total n = 40)
Age, mean ± SD, years	55 ± 19
Gender, n (%)	
Female	25 (62.5)
Male	15 (37.5)
WBC (×10 ⁹ /L), mean ± SD	57.7 ± 100.8
BM blast cell, mean ± SD	70.0 ± 20.5
Risk stratification (ELN), n (%)	
Low	3 (7.5)
Intermediate	15 (37.5)
High	9 (22.5)
Unknown	13 (32.5)
Subtype, n (%)	
M2	11 (27.5)
M3	6 (15)
M4	3 (7.5)
M5	10 (25)
Unclassified	10 (25)
Gene mutation, n (%)	
FLT3	2 (5)
IDH2	2 (5)
NPM1	3 (7.5)
PML/RARA	6 (15)
RUNX1	2 (5)
WT1	4 (10)
No	4 (10)
Unknown	17 (42.5)
Cytogenetic abnormality, n (%)	
No	11 (27.5)
Yes	16 (40)
Unknown	13 (32.5)
Treatment, n (%)	
Chemotherapy	24 (60)
allo-HSCT	3 (7.5)
Other	13 (32.5)
Follow-up, median (range), days	316 (1-1608)
Status	
Alive	10 (25)
Dead	30 (75)

allo-HSCT, allogeneic hematopoietic stem cell transplantation; BM, bone marrow; ELN, European LeukemiaNet; SD, standard deviation; WBC, white blood cell.

the AML patients and analyzed the association between the TOX expression level and the OS of AML patients by Kaplan-Meier curves. The optimal prognostic cutoff value for TOX, TOX2, TOX3, and TOX4 was 2.26, 1.32, 1.25, and 2.49, respectively (**Supplementary Figures S1A–D**). Using these values, we divided the patients into high and low expression groups (**Figures 3A–H**). The results demonstrated that AML patients with high TOX expression were associated with short survival time and poor OS in the training cohort, but there was no statistically significant difference (3-year OS 23% vs. 32%, $P = 0.269$, **Figure 3A**). The 3-year restricted mean survival time (RMST) of the high expression group was 397 days, and the 3-year RMST of the low expression group was 578 days (**Supplementary Figure S2A**). Similarly, TOX expression in the TCGA data had no statistically significant difference ($P = 0.080$, **Figure 3E**). In the training cohort, AML patients with high TOX2, TOX3, and TOX4 expression had shorter survival time and more inferior OS (3-year OS: 0% vs. 37%, $P = 0.036$; 3-year OS: 4% vs. 61%, $P < 0.001$; 3-year OS: 0% vs. 32%, $P = 0.010$, **Figures 3B–D**). The 3-year RMST of the high expression

group was 412, 270, 67 days, respectively, and the 3-year RMST of the low expression group was 727, 776, 531 days, respectively (**Supplementary Figures S2B–D**). These findings were confirmed in the validation cohort (3-year OS: 24% vs. 44%, $P = 0.021$; 3-year OS: 9% vs. 35%, $P = 0.018$; 3-year OS: 27% vs. 60%, $P = 0.011$, **Figures 3F–H**).

Considering an additive effect on the outcome if multiple TOX genes are aberrantly elevated, we characterize the predictive value of co-expression of TOX genes in AML. Using the co-expression of TOX genes to evaluate the OS, we found that lower OS was observed in TOX2^{high}TOX4^{high} AML patients in comparison with TOX2^{high}TOX4^{low} or TOX2^{low}TOX4^{high} AML patients and TOX2^{low}TOX4^{low} AML patients (3-year OS: 0% vs. 25% vs. 56%, $P = 0.002$, **Figure 4A**). In addition, TOX^{high}TOX2^{high}TOX4^{high} AML patients are also related to the poor prognosis of patients (3-year OS: 0% vs. 25% vs. 75%, $P = 0.001$, **Figure 4B**). The same results were also confirmed in the validation cohort (3-year OS: 21% vs. 38% vs. 100%, $P < 0.001$; 3-year OS: 22% vs. 36% vs. 100%, $P = 0.008$, **Figures 4C, D**).

To better understand the relationship between TOX, TOX2, TOX3, and TOX4 and other impact factors, COX regression analysis was used. When age, gender, AML subtype, ELN risk group, CBF rearrangements, hematologic parameters, treatment, TOX, TOX2, TOX3, and TOX4 were included in univariate COX regression analysis, only age, TOX2, TOX3, and TOX4 were significantly associated with poor overall survival in AML patients. Therefore, age was used for adjusting TOX2, TOX3, and TOX4 in AML patients. Importantly, we found that high expression of TOX2, TOX3, and TOX4 is an independent factor affecting survival. Compared with patients with low expression of TOX2, TOX3, and TOX4, those with high expression are at higher risk of death than those with low expression: TOX2: $P = 0.005$, hazard ratio (HR) = 1.03 (95% confidence interval (CI): 1.01-1.05); TOX3: $P = 0.037$, HR = 1.02 (95% CI: 1.00-1.04); TOX4: $P = 0.032$, HR = 1.03 (95% CI: 1.00-1.05). However, in the univariate COX regression model, the expression level of TOX was not significantly associated with the OS of AML patients (HR = 1.60, 95% CI: 0.77-3.35, $P = 0.210$, **Table 2**).

Correlation of TOX and IC Genes Expression in AML

Based on the previous finding of TOX expression concurrent with that of PD-1 and Tim-3 in T cells from patients with lymphoma (21), we analyzed the correlation of the gene expression level of the TOX genes and IC genes in AML patients (**Figures 5A–C**). Significantly, TOX2 has a positive correlation with TIGIT, PD-1, CTLA-4, and PDL2 ($r_s = 0.43$, $P = 0.006$; $r_s = 0.43$, $P = 0.006$; $r_s = 0.56$, $P < 0.001$; $r_s = 0.54$, $P < 0.001$). Moreover, the expression levels of TOX and TOX4 had a positive correlation ($r_s = 0.41$, $P = 0.008$), while the expression level of TOX and TOX2 demonstrated a trend toward a negative correlation ($r_s = -0.133$, $P = 0.412$). Interestingly, there was a significantly negative correlation between TOX and TOX2 expression in the TCGA dataset ($r_s = -0.23$, $P = 0.003$). These results were confirmed in the validation cohort ($r_s = 0.34$, $P < 0.001$; $r_s = 0.29$, $P < 0.001$; $r_s = 0.44$, $P < 0.001$; $r_s = 0.26$, $P < 0.001$, **Figure 5B**).

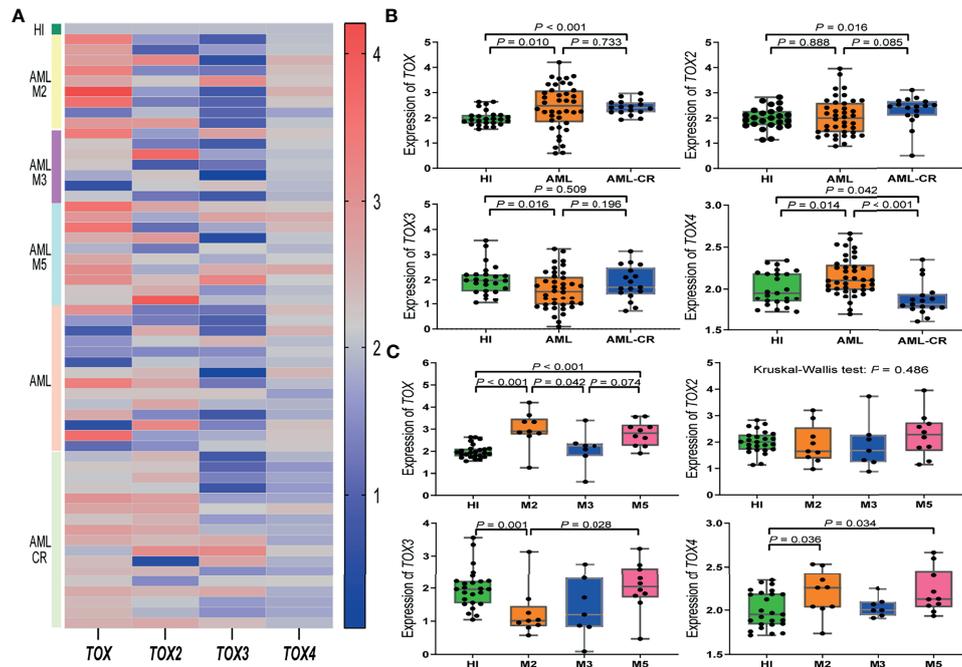


FIGURE 2 | TOX gene expression levels in AML patients. **(A)** Heatmap of the expression levels of the TOX genes in AML patients from different subtypes and periods compared to a HI. **(B)** Expression levels of TOX genes in AML (orange) and AML-CR (blue) patients compared to HIs (green). **(C)** Expression levels of TOX genes in the M2 (orange), M3 (blue), and M5 (pink) subtypes in AML patients compared to HIs (green). CR, complete response; HI, healthy individuals.

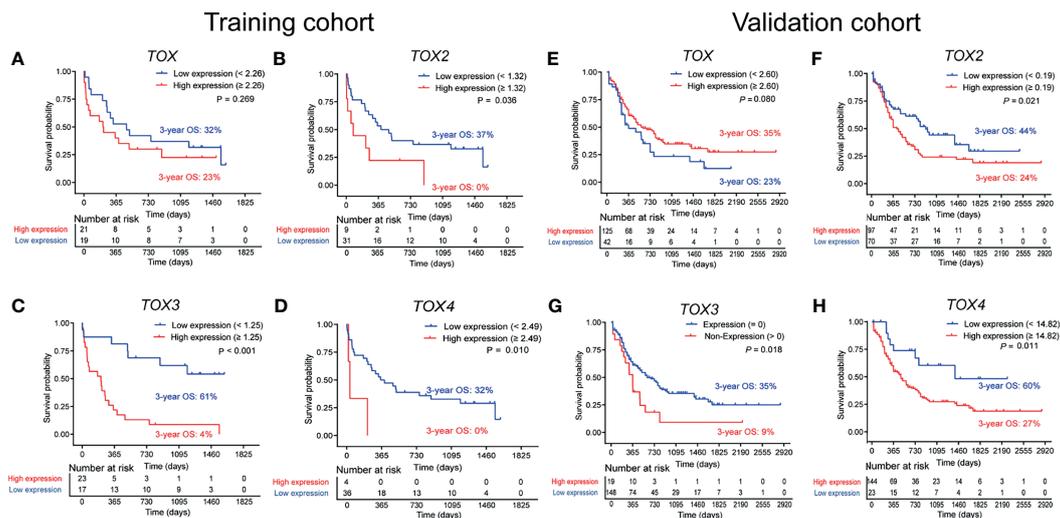


FIGURE 3 | Overall survival (OS) analysis of TOX, TOX2, TOX3, and TOX4 from training **(A–D)** and validation **(E–H)** cohort. According to optimal cutoff values, the TOX genes were divided into High expression (red line) and Low expression (blue line) groups, which were plotted in Kaplan-Meier curves (top) with the number at risk AML patients (bottom).

DISCUSSION

The biomarkers for AML outcome, particularly those related to immune suppression, which often occurs in cancer immune

escape, are far from clear. Recent studies have shown that TOX is a crucial transcription factor that contributes to T cell exhaustion and is involved in tumor development (16, 23). However, how TOX is altered in AML remains unclear. In this

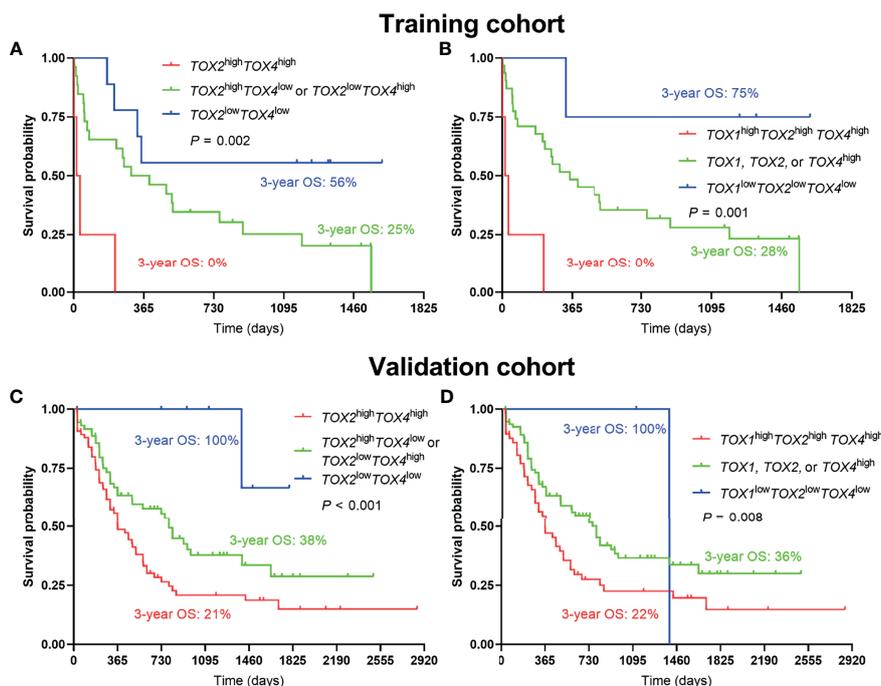


FIGURE 4 | Co-expression of TOX genes in predicting poor OS of AML patients. Kaplan-Meier curves are shown for co-high expression, single high expression, and co-low expression of *TOX2/TOX4* (A) and *TOX1/TOX2/TOX4* (B) in training and validation cohort (C, D).

study, we explored the expression characteristics of the four TOX family members in AML samples. Interestingly, the expression patterns of the TOX genes were different with the expression of *TOX* and *TOX4* significantly increased, *TOX3* was decreased, and *TOX2* demonstrated an increasing expression trend. These differences may be due to the functional differences of TOXs in the AML subtypes and may be involved in clinical outcome. It is well known that AML includes eight subtypes and is a heterogeneous disease (24). For instance, AML-M3 is a particular subtype with a favorable outcome. Indeed, the expression of TOX genes in patients of different AML subtypes i.e., AML-M2, M3, and M5, was different. Overall, *TOX* and *TOX4* had low expression in the M3 group and high expression in the M2 and M5 groups, while *TOX3* was low in AML-M2 and high in AML-M5. Therefore, the study of the TOX family as a biomarker may provide particular predictive value for the study of patients with different types.

It has been reported that both *TOX* and *TOX2* are correlated with CD8⁺ T cell exhaustion (25). In this study, we also found that *TOX2* is positively correlated with *CTLA-4*, *PD-1*, *TIGIT*, and *PDL-2* in AML samples from either our center or from the TCGA dataset. Previous studies have indicated that higher *PD-1*, *PD-L1*, or *CTLA-4* expression is associated with poor OS in AML (6). Thus, *TOX2* may be one more biomarker for predicting the T cell immune suppression related to the clinical outcome of AML. However, we did not find any association between the expression of *TOX* and the immune checkpoint genes. The reason for this discrepancy may be the level of *TOX* expression in different cells.

It is possible that the association between TOX and PD-1 or CTLA-4 co-expression only occurs in T cells (21) and not PBMCs, which include a high percentage of AML cells. We found that TOX genes are also expressed in AML cell lines and primary AML cells (data not shown), which suggests that there are different patterns of expression for TOX genes in T cells and AML cells, which may play a different role. *TOX3* is an essential protective transactivator in neurons (26) and plays different roles in various tumors (27–29). Moreover, *TOX4* regulates the cell cycle and fate (30, 31), but there is no information regarding the expression characteristics of *TOX4* in cancer or hematological malignancies. In this study, we characterized the expression patterns of *TOX3* and *TOX4* in AML. Interestingly, the expression of *TOX3* was significantly decreased and different from the other TOX genes, while *TOX4* was highly expressed. Whether these genes play different regulatory roles in AML requires further investigation. We also considered whether there is any correlation or complementation between the expression of the TOX genes. From our results, we could find a positive correlation between *TOX* and *TOX4* in the training and validation groups, while a negative correlation or correlation trend between *TOX* and *TOX2* expression was found. Whether this is complementary regulation remains an open question.

To further discuss the role of the altered expression of the TOX genes in AML, we explored the association between the expression of the TOX genes and the OS of AML patients. Our results demonstrated that AML patients with high *TOX2* expression have an inferior OS. Combined with the finding

TABLE 2 | Uni- and multivariate regression analysis of *TOX2*, *TOX3*, and *TOX4* in AML patients.

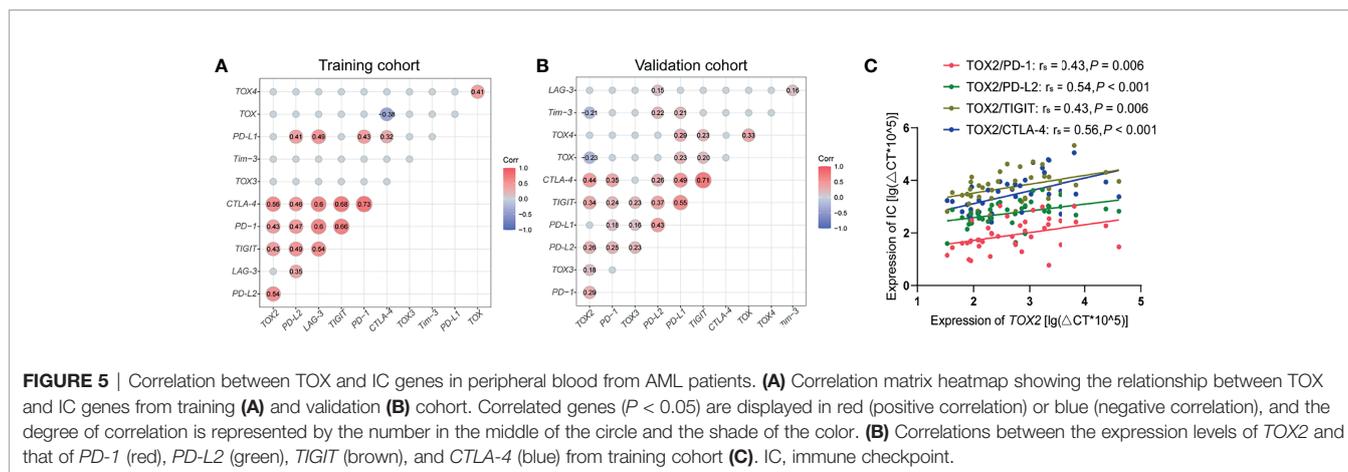
Variables	Univariate regression		Multivariate regression						
	HR (95% CI)	P value	<i>TOX2</i> /Age		<i>TOX3</i> /Age		<i>TOX4</i> /Age		
			HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value	
Gender									
Female	Reference								
Male	0.68 (0.32, 1.45)	0.319							
Age, years	1.03 (1.01, 1.05)	0.007	1.03 (1.01, 1.05)	0.005	1.02 (1.00, 1.04)	0.037	1.03 (1.00, 1.05)		0.032
Subtype									
M2	Reference								
M3	0.15 (0.02, 1.12)	0.075							
M5	1.91 (0.69, 5.25)	0.212							
WBC, 10 ⁹ /L	1.00 (1.00, 1.00)	0.888							
BM blast cell, %	0.99 (0.97, 1.01)	0.402							
Risk stratification (ELN)									
Low	Reference								
Intermediate	2.17 (0.48, 9.79)	0.314							
High	1.38 (0.27, 7.21)	0.700							
Treatment									
allo-HSCT	Reference								
Chemotherapy	4.23 (0.56, 31.98)	0.162							
Other	4.47 (0.57, 34.86)	0.153							
<i>TOX</i>									
Low expression	Reference								
High expression	1.60 (0.77, 3.35)	0.210							
<i>TOX2</i>									
Low expression	Reference		Reference						
High expression	2.98 (1.03, 8.59)	0.043	3.22 (1.10, 9.49)	0.034					
<i>TOX3</i>									
Low expression	Reference				Reference				
High expression	4.47 (1.89, 10.59)	0.001			3.92 (1.61, 9.58)	0.003			
<i>TOX4</i>									
Low expression	Reference						Reference		
High expression	5.79 (1.83, 18.35)	0.003					3.50 (1.04, 11.72)	0.043	

allo-HSCT, allogeneic hematopoietic stem cell transplantation; BM, bone marrow; CI, confidence interval; ELN, European LeukemiaNet; HR, hazard ratio; WBC, white blood cell.

that *TOX2* positively correlates with IC genes and is highly expressed in AML-CR patients, overexpression of *TOX2* together with T cell exhaustion may be a reason why AML patients have poor OS. It is worth further investigating the value in predicting OS for AML by evaluating the co-expression of *TOX* with immune checkpoint genes (32). Although the expression of *TOX3* is decreased in AML, patients with higher *TOX3* expression have an inferior 3-year OS, the function of *TOX3* is needed to further characterize. Moreover, we found that patients with increased *TOX3* expression are primarily AML-M5 patients. These findings may provide a precise and valuable predictor of

OS for AML. Similarly, higher expression of *TOX4* is also related to poor prognosis. An interesting finding is that when the *TOX*, *TOX2*, *TOX4* genes co-expressed highly in AML patients, the prognosis of these patients is significantly poor. This finding indicates that *TOX* genes play a negative role in AML patients to a large extent. Overall, *TOX* genes may be potential biomarkers for predicting clinical outcomes in AML, and their blockade may be considered a new direction for the treatment of AML patients.

In summary, in this study, we characterized the altered expression of *TOX* genes in AML and defined their different roles. We also demonstrated that *TOX2* is positively correlated



with the *CTLA-4*, *PD-1*, *TIGIT*, and *PDL-2* genes. Moreover, higher expression of *TOX2*, *TOX3*, and *TOX4* of AML patients and the AML patients with highly co-expressed *TOX*, *TOX2*, *TOX4* genes were associated with poor OS for AML patients, which may be related to the upregulation of immune checkpoint genes. These data indicate that TOX genes may be novel predictors for clinical outcomes in AML. Moreover, TOX, as the upstream molecule of immune checkpoint proteins, is not only expressed in AML cells but also associated with T cell exhaustion, which might provide direction for future investigations of the possibility of the dual effect of TOX targeted inhibition, inhibiting proliferation of AML cells and revising T cell exhaustion and restoring anti-AML T cell function.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the School of Medicine of Jinan University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

REFERENCES

- Döhner H, Weisdorf DJ, Bloomfield CD. Acute Myeloid Leukemia. *N Engl J Med* (2015) 373(12):1136–52. doi: 10.1056/NEJMra1406184
- Stein EM, Tallman MS. Emerging Therapeutic Drugs for AML. *Blood* (2016) 127(1):71–8. doi: 10.1182/blood-2015-07-604538

AUTHOR CONTRIBUTIONS

SC and YL contributed to the concept development and study design and edited the manuscript. CL performed the experiments, interpreted the data, and wrote the manuscript. YZ and CC helped write the manuscript. SH, TD, and XBZ supported reviewing the references and preparing figures. JT and XFZ collected the clinical information and provided clinical peripheral blood samples. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from the National Natural Science Foundation of China (Nos. 82070152, 81770152, and 81570143), the Guangzhou Science and Technology Project (Nos. 201807010004 and 201803040017), and the Training Program of Innovation and Entrepreneurship for Undergraduates of Jinan University (No. CX20150).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fonc.2021.740642/full#supplementary-material>

Supplementary Table 1 | Primers for qRT-PCR.

Supplementary Figure 1 | Optimal cutoff values for *TOX*, *TOX2*, *TOX3*, and *TOX4* from training **(A–D)** and validation **(E–H)** cohort.

Supplementary Figure 2 | Relationship between RMST and the expression levels of AML patients. RMST, restricted mean survival time.

- Mougiakakos D. The Induction of a Permissive Environment to Promote T Cell Immune Evasion in Acute Myeloid Leukemia: The Metabolic Perspective. *Front Oncol* (2019) 9:1166. doi: 10.3389/fonc.2019.01166
- Kursunel MA, Esendagli G. A Co-Inhibitory Alliance in Myeloid Leukemia: TIM-3/Galectin-9 Complex as a New Target for Checkpoint

- Blockade Therapy. *EBioMedicine* (2017) 23:6–7. doi: 10.1016/j.ebiom.2017.08.002
5. Kasakovski D, Xu L, Li Y. T Cell Senescence and CAR-T Cell Exhaustion in Hematological Malignancies. *J Hematol Oncol* (2018) 11(1):91. doi: 10.1186/s13045-018-0629-x
 6. Chen C, Liang C, Wang S, Chio CL, Zhang Y, Zeng C, et al. Expression Patterns of Immune Checkpoints in Acute Myeloid Leukemia. *J Hematol Oncol* (2020) 13(1):28. doi: 10.1186/s13045-020-00853-x
 7. Yasinska IM, Sakhnevych SS, Pavlova L, Teo Hansen Selno A, Teuscher Abeleira AM, Benlaouer O, et al. The Tim-3-Galectin-9 Pathway and Its Regulatory Mechanisms in Human Breast Cancer. *Front Immunol* (2019) 10:1594. doi: 10.3389/fimmu.2019.01594
 8. Martínez-López M, Soto M, Iborra S, Sancho D. Leishmania Hijacks Myeloid Cells for Immune Escape. *Front Microbiol* (2018) 9:883. doi: 10.3389/fmicb.2018.00883
 9. Chen C, Zeng C, Li Y. The Importance of Genomic Predictors for Clinical Outcome of Hematological Malignancies. *Blood Sci* (2021) 3(3):93–5. doi: 10.1097/bs9.0000000000000075
 10. Wang J, Yang T, Xu J. Therapeutic Development of Immune Checkpoint Inhibitors. *Adv Exp Med Biol* (2020) 1248:619–49. doi: 10.1007/978-981-15-3266-5_23
 11. Gale RP. Will Immune Therapy Cure Acute Myeloid Leukemia? *Blood Sci* (2019) 1(1):2–3. doi: 10.1097/bs9.0000000000000024
 12. Zhang X, Yang Y, Fan D, Xiong D. The Development of Bispecific Antibodies and Their Applications in Tumor Immune Escape. *Exp Hematol Oncol* (2017) 6:12. doi: 10.1186/s40164-017-0072-7
 13. Zhu R, Tao H, Lin W, Tang L, Hu Y. Identification of an Immune-Related Gene Signature Based on Immunogenomic Landscape Analysis to Predict the Prognosis of Adult Acute Myeloid Leukemia Patients. *Front Oncol* (2020) 10:574939. doi: 10.3389/fonc.2020.574939
 14. Salik B, Smyth MJ, Nakamura K. Targeting Immune Checkpoints in Hematological Malignancies. *J Hematol Oncol* (2020) 13(1):111. doi: 10.1186/s13045-020-00947-6
 15. O'Flaherty E, Kaye J. TOX Defines a Conserved Subfamily of HMG-Box Proteins. *BMC Genomics* (2003) 4(1):13. doi: 10.1186/1471-2164-4-13
 16. Liang C, Huang S, Zhao Y, Chen S, Li Y. TOX as a Potential Target for Immunotherapy in Lymphocytic Malignancies. *Biomark Res* (2021) 9(1):20. doi: 10.1186/s40364-021-00275-y
 17. Lobbardi R, Pinder J, Martinez-Pastor B, Theodorou M, Blackburn JS, Abraham BJ, et al. TOX Regulates Growth, DNA Repair, and Genomic Instability in T-Cell Acute Lymphoblastic Leukemia. *Cancer Discov* (2017) 7(11):1336–53. doi: 10.1158/2159-8290.Cd-17-0267
 18. Dulmage BO, Akilov O, Vu JR, Falo LD, Geskin LJ. Dysregulation of the TOX-RUNX3 Pathway in Cutaneous T-Cell Lymphoma. *Oncotarget* (2019) 10(33):3104–13. doi: 10.18632/oncotarget.5742
 19. Kim K, Park S, Park SY, Kim G, Park SM, Cho JW, et al. Single-Cell Transcriptome Analysis Reveals TOX as a Promoting Factor for T Cell Exhaustion and a Predictor for Anti-PD-1 Responses in Human Cancer. *Genome Med* (2020) 12(1):22. doi: 10.1186/s13073-020-00722-9
 20. Bardet V, Couque N, Cattolico L, Hetet G, Devaux I, Duprat S, et al. Molecular Analysis of Nonrandom 8q12 Deletions in Acute Lymphoblastic Leukemia: Identification of Two Candidate Genes. *Genes Chromosomes Cancer* (2002) 33(2):178–87. doi: 10.1002/gcc.10014
 21. Huang S, Liang C, Zhao Y, Deng T, Tan J, Lu Y, et al. Increased TOX Expression Concurrent With PD-1, Tim-3, and CD244 in T Cells From Patients With Non-Hodgkin Lymphoma. *Asia Pac J Clin Oncol* (2021). doi: 10.1111/ajco.13545
 22. Laska E, Meisner M, Wanderling J. A Maximally Selected Test of Symmetry About Zero. *Stat Med* (2012) 31(26):3178–91. doi: 10.1002/sim.5384
 23. Guo R, Lü M, Cao F, Wu G, Gao F, Pang H, et al. Single-Cell Map of Diverse Immune Phenotypes in the Acute Myeloid Leukemia Microenvironment. *biomark Res* (2021) 9(1):15. doi: 10.1186/s40364-021-00265-0
 24. Chen C, Chio CL, Zeng H, Li Y. High Expression of CD56 May Be Associated With Favorable Overall Survival in Intermediate-Risk Acute Myeloid Leukemia. *Hematol (Amsterdam Netherlands)* (2021) 26(1):210–4. doi: 10.1080/16078454.2021.1880734
 25. Seo H, Chen J, González-Avalos E, Samaniego-Castruita D, Das A, Wang YH, et al. TOX and TOX2 Transcription Factors Cooperate With NR4A Transcription Factors to Impose CD8(+) T Cell Exhaustion. *Proc Natl Acad Sci USA* (2019) 116(25):12410–5. doi: 10.1073/pnas.1905675116
 26. Yuan SH, Qiu Z, Ghosh A. TOX3 Regulates Calcium-Dependent Transcription in Neurons. *Proc Natl Acad Sci USA* (2009) 106(8):2909–14. doi: 10.1073/pnas.0805555106
 27. Han YJ, Zhang J, Zheng Y, Huo D, Olopade OI. Genetic and Epigenetic Regulation of TOX3 Expression in Breast Cancer. *PLoS One* (2016) 11(11):e0165559. doi: 10.1371/journal.pone.0165559
 28. Zeng D, Lin H, Cui J, Liang W. TOX3 is a Favorable Prognostic Indicator and Potential Immunomodulatory Factor in Lung Adenocarcinoma. *Oncol Lett* (2019) 18(4):4144–52. doi: 10.3892/ol.2019.10748
 29. He Y, Liu H, Chen Q, Shao Y, Luo S. Relationships Between SNPs and Prognosis of Breast Cancer and Pathogenic Mechanism. *Mol Genet genomic Med* (2019) 7(9):e871. doi: 10.1002/mgg3.871
 30. Lee JH, You J, Dobrota E, Skalnik DG. Identification and Characterization of a Novel Human PP1 Phosphatase Complex. *J Biol Chem* (2010) 285(32):24466–76. doi: 10.1074/jbc.M110.109801
 31. Vanheer L, Song J, De Geest N, Janiszewski A, Talon I, Provenzano C, et al. Tox4 Modulates Cell Fate Reprogramming. *J Cell Sci* (2019) 132(20):jcs232223. doi: 10.1242/jcs.232223
 32. Chen C, Xu L, Gao R, Wang S, Zhang Y, Wang C, et al. Transcriptome-Based Co-Expression of BRD4 and PD-1/PD-L1 Predicts Poor Overall Survival in Patients With Acute Myeloid Leukemia. *Front Pharmacol* (2020) 11:582955. doi: 10.3389/fphar.2020.582955
- Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
- Publisher's Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.
- Copyright © 2021 Liang, Zhao, Chen, Huang, Deng, Zeng, Tan, Zha, Chen and Li. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.