RESEARCH ARTICLE

Editorial Process: Submission:01/17/2024 Acceptance:05/18/2024

Prognostic effect of *CTLA4/LAG3* Expression by T-Cells Subsets on Acute Myeloid Leukemia Patients

Wesam El Dosoky[1], Salah Aref[1]*, Nadia El Menshawy[1], Ahmed Ramez[2], Tarek Abou Zaid[3], Mohamed Aref[3], Doaa Atia[3]

Abstract

Background: Deregulation of immune checkpoint is an important point in cancer evolution as well as patients outcome. T-cells is an important arm in immunity against cancer. This study aimed to assess *CTLA4/LAG3* expression on different T-cell subsets and its effect on disease outcome. **Methods:** This study included 81 newly diagnosed Egyptian adult AML patients. For each one of the patients *CTLA4/ LAG3* expression on T-cell subsets was identified by flowcytometry before start of induction chemotherapy. **Results:** Total CD3 count in AML patients was lower than control. *LAG3* expression were significantly higher in total CD3, T-cell subsets (CD4, CD8) as compared to healthy control. Moreover, co-expression of *LAG3/CTLA4* on T-cell subsets were significantly higher in AML as compared to healthy control . NPM-/ FLT3+ was significantly associated with high *LAG3* expression in T-cells subsets as compared to other molecular subtypes. Shorter OS, DFS were significantly associated with higher expression of *LAG3* on T-cells subsets as compared to patients harbor low expression. COX regression analysis revealed that high expression of *CD3/LAG3, CD4/LAG3, CD8/LAG4, CD3/CTLA4/LAG3* were considered a poor prognostic risk factor. **Conclusion:** High *LAG3/CTLA4* expression could predict AML Patients' outcome **Conclusion:** Our findings indicated that high expression of *LAG3/CTL4* on T cells subsets identify a subgroup of AML patients with poor prognosis.

Keywords: CTLA4- LAG3- AML- T cell

Asian Pac J Cancer Prev, **25 (5)**, 1777-1785

Introduction

Acute myeloid leukemia (AML) long-term clinical remissions can be induced in 35–40% of adult AML patients who are 60 years or younger, as compared to 5–15% of AML patients above 60 years [1]. Besides patient age, survival rate also depends on the presence of cytogenetic or molecular genetic aberrations and patient performance status [2].

Immunological arm status gaining growing importance in response of AML to conventional chemotherapy. Chimeric antigen receptor (CAR) T-cell therapy is being developed for AML [3]. AML cells have been demonstrating to have reduced T-cell and NK-cell function and cytotoxicity [4-6]. Checkpoint receptors and their ligands play an important role in T-cell stimulation and exhaustion and are currently the focus of significant efforts in understanding and modulating antitumor immune responses [7, 8]. New kinds of AML treatment are the immune checkpoint inhibitors, which have shown impressive clinical efficacy in various solid cancers, including melanoma and lung cancer [9]. Tumor responsiveness to checkpoint blockers is dependent on different parameters, including mutational load of the tumor, pre-existing antitumor immunity and checkpoint ligand expression by the tumor [10].

LAG3 (lymphocyte activation gene 3) is co-inhibitory player of the Ig superfamily. It is a trans membrane protein with high structural homology to CD4, binds HLA-II molecules, though with higher affinity. LAG3 is expressed on activated T cells and Treg, as well as a subset of NK cells, B cells and plasmacytoid DC [5]. In resting T-cells, LAG3 is localized in endosomal compartments [10]. LAG3 has cell intrinsic and extrinsic mechanisms to impair T cell functionality by negatively regulates TCR- mediated signaling in effector T cells, proliferation and function [8]. blockade of LAG3 mediated signaling induces enhanced activation of human CD8 T cells [9] .

Data on *LAG3* expression in T cells is limited in hematological malignancies. In Hodgkin lymphoma, the presence of LAG3+ CD4 and Treg subsets in the tumor micro environment correlated with defective tumor-reactive T-cell responses. Subsequent deletion of the LAG3+ CD4+ T-cells augmented IFNc production

*[1]Hematology Unit, Mansoura University Oncology Center, Mansoura University, Egypt. [2]Mansoura University Oncology Center, Mansoura University, Egypt. [3]Mansoura University Oncology Center, Mansoura Faculty of Medicine, Mansoura University, Egypt. *For Correspondence: salaharef@yahoo.com*

and proliferation of tumor-reactive CD8+ T cells in vitro. Monoclonal antibodies directed against LAG3 (BMS-986016 and LAG525) are currently evaluated, particularly in combination with anti-PD-1, in early phase clinical trials in patients with lymphoma and multiple myeloma (NCT01968109 andNCT02061761) [11]. To date, no previous trial has been initiated to investigate the potential impact of LAG-3 T-cells exhaustion in AML outcome.

CTLA4, also known as CD152, is a protein encoded by the 4-exon CTLA4 gene, and a well-known co-inhibitory receptor expressed mostly on regulatory T cells (Treg) and activated CD4+ and CD8+ T cells. *CTLA4* is constitutively expressed in regulatory T cells, and in naïve resting T cells; it resides in the cytoplasm, until activation, when within 1 or 2 days, starts expressing on the surface. An even faster activation and expression are observed in memory T cells [12, 13].

The heterogeneity of exhausted T-cells may be a reason for the lower effects of PD-1 inhibitors as explained by Tan et al. [14]. Thus, the combinations of PD-1 inhibitors with standard ant- leukemic therapy are being rationally designed. Moreover, the combination of azacytidine and PD-1/PD- L1 inhibition was also used for AML treatment. More recently, it was reported that PD-1 (nivolumab) and CTLA-4 (ipilimumab) combined with azacytidine was used to treat multiple relapsed/refractory leukemia pediatric patients and improve clinical outcome [15, 16]. For this advanced in immune checkpointinhibitor, current study focusing on combination of two major negative regulator of T-cells on exhausted T-cells subsets.

This study aimed to identify the impact of *CTLA4/ LAG3* expression in T-cells subsets and AML patient's outcome.

Materials and Methods

Patients

This is cross sectional prospective study, that was conducted on 81 newly diagnosed AML in Oncology center at Mansoura university. Their ages range from 18-78 years (55.6% males while 44.4% females), The AML patients' diagnosis was based on morphological examination of peripheral and bone marrow smear and Immunophenotyping by Flowcytometry. Control group comprised 20 healthy volunteers matched in age and sex. All investigated subject s gave gave informed consent. the study has been approved by the Mansoura Faculty of Medicine Local Ethics Committee (IRB). Follow up time was 24 months of AML patients.

The inclusion criteria included Newly diagnosed AML patients over 18 years and the exclusion criteria was patients with AML-M3 and if AML with combined with other malignancy or patient was under active treatment.

Four milliliters fresh bone marrow (BM) sample was taken for every one AML patient and distributed as follow: 2ml in EDTA tube for flowcytometry and molecular study and 2 ml into Na heparinized green capped tube for cytogenetic study.

Treatment protocol

Induction consisted of 90 mg/m2 of daunorubicin and 200 mg/m2 of Ara-C in patients, following the standard 7/3 protocol (ie. 7 days of Ara-C including 3 days of the associated drug). Bone marrow examination was scheduled on day 30; presence of persistent leukemia, defined as at least 5% blasts in the biopsy with at least 20% cellularity, called for a second course of induction therapy. Endpoints definitions: Overall survival is defined as the time from diagnosis until death or end of the study. DFS is defined as the time from remission until disease relapse, death or end of the study [17].

Detection of CTLA4/ LAG-3 antigens expression by T cells using Flowcytometry

The method of staining is in brief, 100 µL of whole blood was immune stained for 30 minutes in the dark, followed by lysis with 500 µL of erythrocyte lysis solution before 2 washes with 500 µL of PBS. After centrifugation, the supernatants were discarded, and cell pellets were suspended in 500 µL of PBS for analysis through FACS software. Before obtaining the target CD3+ cells, we gated mature lymphocytes (CD45bright SSC-A low) by CD45-BV510/SSC-A and subsequently eliminated dead and sticky, doublet cells by FSC-A/FSC-H (single-doublet discriminator scatter). The procedure was done multiparameter 8 color, 3 laser BD-FACS Canto II, V 33396202133 (USA). The data analysis was done using ADVIA software.

The following fluorochrome-conjugated monoclonal antibodies were used: LAG-3(CD223) Alexa FluorR BD Pharmingen TM (Clone: T47-530 BD Bioscience ,USA), CD3 PCY7 (Lot 737657 Beckman coulter), CD4 PCY5.5(Lot B16491 Beckman coulter), CD8 APCH7 / Alexa fluor 750 (REF A 94686 ,Lot 200060) , CD152 (CTLA-4) PerCP CY5.5 (Clone BNT3),(BD Bioscience), CD45-V500 (chrome orange (REF A96416,Lot 200130) purchased from Beckman coulter. Likewise erythrocyte lysis solution was purchased from BD Biosciences.

Molecular detection of of FLT3-ITD mutation by conventional PCR

DNA was extracted using Thermo scientific Gene JET Whole Blood Genomic DNA Purification Kit according to the protocol of manufacturer's instructions. The extracted DNA was stored frozen at -20°C. The DNA samples were quantified by Nano-Drop instrument, and the samples were measured 17-45 ng/µL. Apply 1 µl of extracted DNA with 12.5 µl red master mix (COSMO PCR red master mix Willowfort)(w1020300x) 0.1µl of 100 pmol of each primer (Forward: 5/ GCA ATT TAG GTA TGA AAG CCA GC 3/ Reverse: 5/CTT TCA GCA TTT TGA CGG CAA CC 3/ protocol of 95°C for 3 minutes followed with 35 cycles of 95°C for 30 seconds, 66°C for 30 seconds, 72°C for 30 seconds then final extension at 72 °C for 7 minutes.

Molecular detection of Nucleophosmin (NPM1) mutation

Real time PCR that depends on detection of wild type and mutant NPM by oligonucleotide hydrolysis principle uses specific primers and an internal double dye probe with a reporter and a quencher (FAM TM, TAMRATM)

with amplification thermal cycle protocols 50°C for 2 min, 95°C for 10 min, (95 °C for 15 sec, 60°C for 90 min for 40 cycles). Using Ipsogen NPM1 Muta Screen Kit (Qiagen, REF 677013, Germany) , reading on analyzer Using DT prime 4 /DNA technology (SN: ASD312, USA).

FISH analysis for Cytogenetic detection in AML patients

Pretreatment blood samples from all patients were studied by chromosome banding analysis to improve the accuracy of cytogenetic diagnosis. The specimens were also analyzed by fluorescence in situ hybridization for the presence of t(8;21) (q22;q22) for M2, inv(16) (p13q22) for M4e or 11q23 for M5,all was purchased from (Vysis, London, UK). Cell images were captured using a CCD camera (Photometrics SenSys camera) connected to a personal computer running using CytoVision system for image analysis (Applied Imaging).

Statistical Methods

The reordered data were revised, coded, tabulated and introduced to a PC using Statistical package for Social Science (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.). Data were presented and suitable analysis was done according to the type of data obtained for each parameter. Kolmogorov-Smirnov test was used as a test of normality, if the significance level is greater than 0.05, then normality is assumed. Student T test was used to assess the statistical significance of the difference between two study group means. Mann Whitney test (U test) was used to assess the statistical significance of the difference of a non-parametric variable between two study groups. The Kruskal-Wallis test is was used to assess the statistical significance of the difference between more than two study group non-parametric variables. Chi-Square test was used to examine the relationship between two qualitative variables

The ROC (receiver operating characteristic) curve provides a useful way to evaluate the sensitivity and specificity for quantitative diagnostic measures that categorize cases into one of two groups. The optimum cut off point was defined as that which maximized the AUC value. AUC is that a test with an area greater than 0.9 has high accuracy, while 0.7–0.9 indicates moderate accuracy, 0.5–0.7, low accuracy and 0.5 a chance result. Kaplan–Meier test was used for survival analysis and the statistical significance of differences among curves was determined by Log-Rank test. A P-value < 0.05 was considered statistically significant. Overall survival is defined as the time from diagnosis until death from any cause or end of the study. DFS is defined as the time from remission until disease relapse, death from any cause or end of the study.

Results

Patients' characteristics

The patients' characteristics data are shown in table 1. M1 and M2 FAB subtypes represent the majority of AML patients. The FLT3 and NPM1 mutations were detected in 32(39.5%) and in 46 (56.8%) respectively of the studied AML patients. Chemotherapy induction remission response was achieved in 67(82.8%) of AML cases, while relapse were detected in 21 (31.3%). The baseline characteristics are illustrated in Table 1.

Total CD3 was significantly lower in AML as compared to control. Moreover, *LAG3* expression in total CD3, and T cell subsets (CD4, CD8) were significantly higher as compared to control. Also, Co-expression of (*CTLA4/LAG3*) on CD3 was significantly higher in AML patients as compared to control (Table 2). Association between *LAG3/CTLA4* expression on T cell subsets and mortality, frequency of relapse, molecular study, cytogenetic risk.

No significant association was found between total *CD3, CD3/LAG3, CD4/LAG3, CD8/LAG3* and *CTLA4/LAG3* with CR or cytogenetic risk. No significant association was found between *CD3/LAG3, CD4/LAG3, CD8/LAG3* and *CTLA4/LAG3* with relapse except with total *CD3*. Significant association between total *CD3, CD3/LAG3, CD4/LAG3, CD8/LAG3* and *CTLA4/LAG3* and Positive FLT3, NPM1 and combined negative NPM1/FLT3 positive was detected (Table 3).

Impact of LAG3 /CTLA4 expression on T-cell subsets and OS, DFS

Total *CD3, CD3/LAG3, CD4/LAG3, CD8/LAG3* and *CTLA4/LAG3* were stratified into high and low according to median levels. Longer DFS was significantly associated with low *CD3/LAG3, CD4/LAG3, CD8/LAG3* and *CTLA4/LAG3* (Figure 1). Longer OS was significantly associated with low *CD3/LAG3, CD4/LAG3, CD8/LAG3* and *CTLA4/LAG3* (Figure 2).

Predictive value of T-cell subsets on AML patient's outcome

Cox regression analysis was conducted for prediction of OS, using age, gender, blasts cells count, *LDH, NPM1, FLT3*, Cytogenetic, *CD3, CD3/LAG3, CD4/LAG3,, CD8/LAG3, CTLA4/LAG3* as covariate. NPM was associated with longer OS, while *FLT3* mutation, higher Total *CD3, CD3/LAG3, CD4/LAG3, CD8/LAG3, CTLA4/LAG3* were associated with shorter OS in univariable analysis. However, in multivariable analysis, higher *CD3/LAG3, CD4/LAG3, CD8/LAG3, CTLA4/LAG3* were considered risk predictors for shorter OS (Table 4). Likewise, Cox regression analysis was conducted for prediction of DFS using age, gender, bone marrow blasts, LDH, NPM, *FLT3*, total *CD3, CD3/LAG3, CD4/LAG3, CD8/LAG4,* Intermediate risk group, *CTLA4/LAG3* expressions as covariate. High expression of *CD3/LAG3, CD4/LAG3, CD8/LAG3* and *CD3/LAG3/CTLA4* were considered risk predictors for shorter DFS in multivariable analysis (Table 5).

Discussion

T-cell exhaustion is one of currently theories of cancer development and progression, causing immune suppression based on alternative regulation of immune checkpoint proteins. *LAG3/CTLA4* negatively regulates T-cell function and contributes to cancer escape. Characterization of both expressions in different T-cell

Table 1. AML Patients Characteristics

			AML (n=81)
Age (years)		Mean ± SD	44.3±14.5
		range	18-78
Gender	Males	N (%)	45 (55.6%)
	Females	N (%)	36 (44.4%)
WBCs X 109/L		median (range)	46 (1.3-225)
Hemoglobin g/dl		median (range)	7.4 (4.1-13.4)
Platelet X 109/L		median (range)	40 (15-145)
BM blast %		median (range)	75 (25-90)
LDH (IU/L)		median (range)	490 (280-890)
FAB subtypes		N(%)	M0, 10 (12.3%); M1,8 (9.9%); M2, 14 (17.3%); M3, 9 (11.1%), M4, 14 (17.3%); M5, 14 (17.3%);M6, 3 (3.7%); M7, 9 (11.1%).
Performance status		N (%)	Grade 0,17 (21%)), Grade 1,27 (33.3%), Grade 2, 21 (25.9%), Grade3, 16 (19.8%).
FLT3	positive	N (%)	32 (39.5%)
NPM1	positive	N (%)	46 (56.8%)
Cytogenetic finding	Favorable	N (%)	33 (40.7%)
	Intermediate	N (%)	25 (30.9%)
	Unfavorable	N (%)	23 (28.4%)
Total CD3%		median (range)	8.2 (2.1-10.7)
CD3/LAG3 %		median (range)	3.4 (1.8-7.2)
CD4/LAG3%		median (range)	1.5 (0.7-4.1)
CD8/LAG3%		median (range)	1.9 (0.7-4.6)
CD3/ CTLA4/LAG3%		median (range)	0.8 (0.5-1.8)
Outcome	CR	N (%)	67 (82.7%)
	No CR	N (%)	14 (17.3%)
Relapse		N (%)	21 (31.3%)
Alive		N (%)	38 (46.9%)
Death		N (%)	43 (53.1%)

subsets, particularly co-occurrence with the exhausted phenotype in AML is lacking [18].

In this study, frequency of total *CD3* in bone marrow is lower than healthy volunteer. This finding is agreement with Brück et al. [19] reported in his study at BM biopsy that immune cell phenotypes are dissimilar AML BM and peripheral blood samples. The immunologic landscape considerably varies by leukemia subtype suggesting disease-specific immune regulation. Furthermore, the association of the AML immune microenvironment with clinical parameters suggests a rationale for including immunologic parameters to improve disease classification or even patient risk stratification. While disagree with Guo et al. [20] that reported total *CD3/CD56* is higher in AML than control with abnormal phenotype and genotype in peripheral blood sample study. Dysfunction of it may contribute to failure of the host immune response against leukemic blast.

Table 2. Comparison of Total *CD3, CD3/LAG3, CD4/LAG3, CD8/LAG3* and *CTLA4/LAG3* among Studied Groups

	Control median (range)	AML median (range)	p
Total CD3%	15.5 (10.5-20.6)	8.2 (2.1-10.7)	<0.001
CD3/LAG3 %	0.65 (0.3-0.9)	3.4 (1.8-7.2)	<0.001
CD4/LAG3%	0.35 (0.2-0.6)	1.5 (0.7-4.1)	<0.001
CD8/LAG3%	0.2 (0.1-0.5)	1.9 (0.7-4.6)	<0.001
CD3/CTLA4/LAG3%	0.045 (0-0.08)	0.8 (0.5-1.8)	<0.001

AML cases showed significantly lower total CD3%, significantly higher CD3/LAG3%, CD4/LAG3%, CD8/LAG3% and CD3/CTLA4/LAG3% when compared to control group.

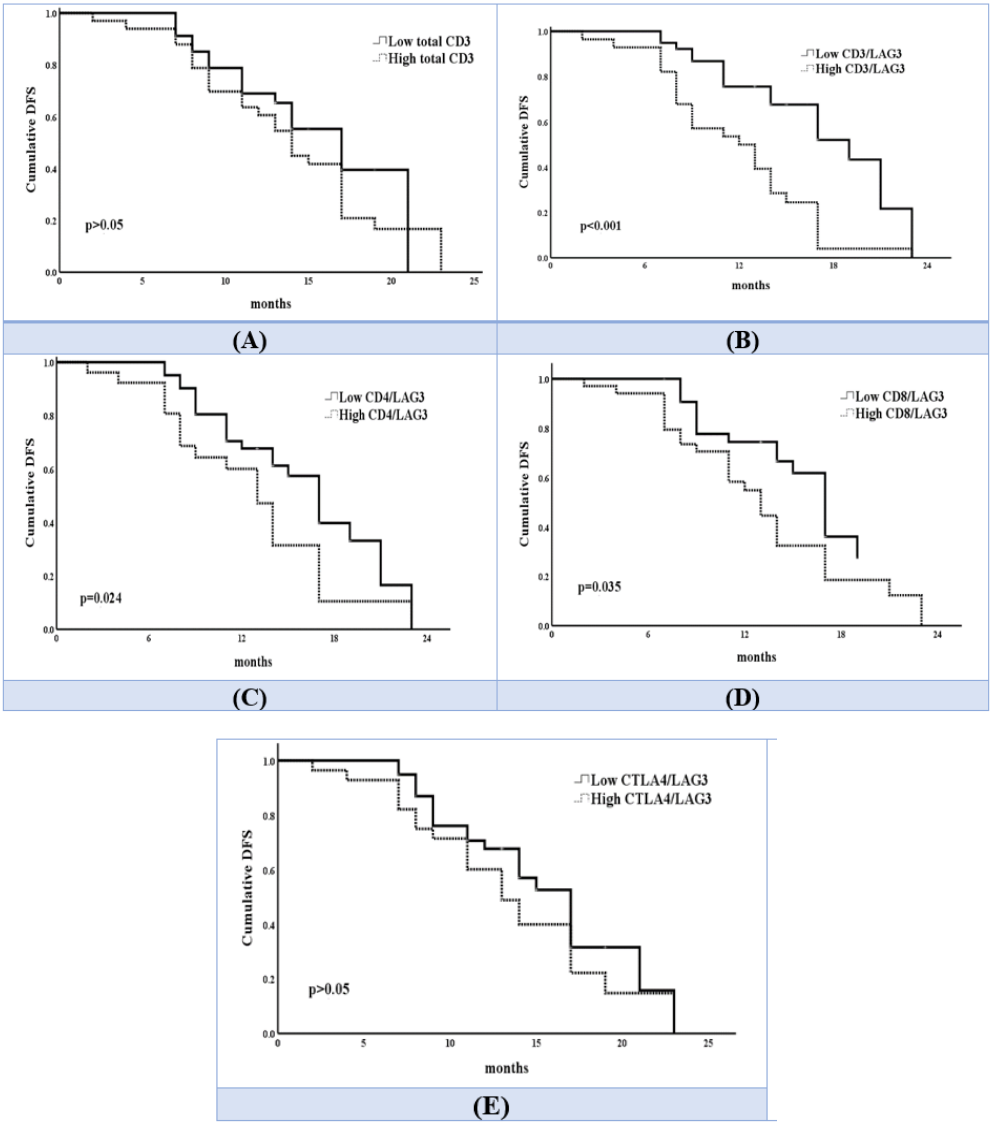

Figure 1. DFS According to Expression of (A) total *CD3* (B) *CD3/LAG3* (C) *CD4/LAG3* (D) *CD8/LAG3* and (E) *CD3/CTLA4/LAG3* in AML Cases

We analyzed the proportion of LAG3+ T-cells in AML. We found highly significant difference when compared to healthy volunteer. This finding is disagreement with the finding reported by Chen et al. [18] who did not find a statistically significant difference in the numbers of *LAG3+ CD3+ , CD4+* and *CD8+* T-cell subsets, a high proportion of LAG3+ T- cells was found in a few cases with AML. Likewise, Keane et al. [21] reported similar finding in Treg in aggregated lymph node in his study of diffuse large B-cell lymphoma. This controversy in the results could be attributed to the difference in sampling in our study which was bone marrow samples, while in the previous study it was peripheral blood. Better assessment of micro environmental condition of leukemic cells and immune status of bone marrow niches give valuable information regarding changes inthe immune system among patients with AML [22].

In the current study, we found a significantly higher proportion of co-expressed *CTLA4* and *LAG3* in the *CD3+* as compared to control. *CTLA4* is one of immunoglobulin related receptors that have dual stimulatory and inhibitory roles in T-cell immunity. *CTLA4* plays a critical negative regulatory role for T-cell function in the primary immune response [23]. This study is first one reported that the percentage of *CTLA4+ CD3+, CD4+* and *CD8+* T-cells was significantly higher in patients with AML compared to healthy individuals. These results are similar to findings from a study of immune checkpoint protein expression on T-cells from patients with chronic lymphocytic leukemia (CLL) in which higher numbers of *CD4+* and *CD8+* T-cells with intracellular *CTLA4* were observed in CLL [21]. Moreover, a previous study has also found that *CTLA4* is expressed on AML/MDS [24]. *CTLA4* was expressed in 25–85% of patients with AML and CML depending on the subtype [25]. Thus, it is thought that targeted *CTLA4* therapy may have dual effects in AML immunotherapy by either directly targeting on myeloid leukemia cells or restoring T-cell function.

An interesting finding in current study is the presence of association between *LAG3, CTLA4* expression in

Table 3. Association of total *CD3, CD3/LAG3, CD4/LAG3, CD8/LAG3* and *CD3/CTLA4/LAG3* with AML Outcome, Mortality, Cytogenetic Risk, NMP and FLT3.

		Total *CD3*	*CD3/LAG3*	*CD4/LAG3*	*CD8/LAG3*	*CD3/CTLA4/LAG3*
Induction Remission Response	CR	6.8 (2.1-10.7)	3.4(1.8-6.8)	1.5 (0.7-4.1)	1.9 (0.7-3.7)	0.8 (0.5-1.8)
	Non CR	8.55 (2.6-8.8)	3.95(1.8-7.2)	1.6 (0.7-3.9)	1.9 (0.8-4.6)	0.9 (0.5-1.5)
	p	0.555	0.612	0.556	0.661	0.247
Relapse	No relapse	4.05 (2.6-10.7)	3.2(1.8-6.8)	1.4 (0.7-4.1)	1.85 (0.7-3.7)	0.8 (0.5-1.5)
	Relapse	8.8 (2.1-8.8)	3.5(1.8-6.8)	1.5 (0.8-3.8)	1.9 (0.8-3.7)	0.8 (0.5-1.8)
	p	0.044	0.07	0.26	0.719	0.769
Mortality	Alive	3.75 (2.1-10.7)	2.5 (1.8-6.4)	1.3 (0.7-3.8)	1.4 (0.7-3.2)	0.8 (0.5-1.5)
	Died	8.8 (2.6-10.7)	4.5 (1.8-7.2)	1.7 (0.7-4.1)	2.2 (0.9-4.6)	0.9 (0.5-1.8)
	p	0.003	<0.001	<0.001	<0.001	0.004
Cytogenetic findings	Favorable	4.2 (2.1-10.7)	3.2 (1.8-6.8)	1.4 (0.7-4.1)	1.9 (0.8-3.7)	0.8 (0.5-1.5)
	Intermediate	8.8 (2.6-10.7)	3.4 (1.8-6.8)	1.6 (0.7-3.2)	1.8 (0.7-3.7)	0.9 (0.5-1.5)
	Unfavorable	8.4 (2.6-10.7)	3.5 (1.8-6.8)	1.5 (0.7-3.2)	1.9 (0.8-3.7)	0.8 (0.5-1.5)
	p	0.154	0.265	0.59	0.758	0.655
NPM1	negative	8.3(2.6-8.8)	4.5 (1.9-7.2)	2.1 (0.7-4.1)	2.2 (0.7-4.6)	0.9 (0.5-1.8)
	positive	4.8(2.1-10.7)	2.7 (1.8-5.2)	1.3 (0.7-3.2)	1.4 (0.8-3.2)	0.8 (0.5-1.5)
	p	0.324	<0.001	<0.001	<0.001	0.004
FLT3	negative	3.9 (2.1-10.7)	2.6 (1.8-4.5)	1.3 (0.7-2.2)	1.4 (0.8-2.8)	0.8 (0.5-1.5)
	positive	8.25 (2.9-8.8)	4.9 (2.7-7.2)	2.5 (1.3-4.1)	2.2 (0.7-4.6)	1.1 (0.6-1.8)
	p	0.158	<0.001	<0.001	<0.001	<0.001
NPM1, FLT3	Both negative	3.9 (2.6-8.8)	3.4 (1.9-4.5)	1.3 (0.7-1.7)	1.9 (0.9-2.8)	0.8 (0.5-1.1)
	NPM+FLT3-	3.9 (2.1-10.7)	2.4 (1.8-3.6)	1.3 (0.7-2.2)	1.3 (0.8-2.8)	0.8 (0.5-1.5)
	NPM- FLT3+	8.35 (2.9-8.8)	5.05 (2.7-7.2)	2.5 (1.3-4.1)	2.25 (0.7-4.6)	1.1 (0.6-1.8)
	Both positive	5.7 (4.2-8.8)	3.7 (3.6-5.2)	1.8 (1.5-3.2)	1.8 (1.4-3.2)	0.75 (0.7-1.2)
	p	0.559	<0.001	<0.001	<0.001	0.002

CR, Complete remission

T- cell subsets with settled prognostic molecular markers namely *FLT3, NPM1*. This proof their clinical value in risk stratification of AML patients. AML patient harbored *LAG3, CTLA4* co-expression on T-cell subsets did not achieved complete remission (CR) after one cycle of chemotherapy, associated with high frequency of mortality. To the best of our knowledge this finding is the first one study co expression of both immune checkpoint on T-cell subsets with prognosis and outcome of AML patients'. Further investigation may be needed to evaluate whether assessment of *CTLA4* and *LAG3* co-expression in T-cells could be considered a factor for immune suppression-related poor prognosis.

High expression of *LAG3* on *CD3/CD4/CD8* T-cells was significantly associated with shorter overall survival, disease-free survival. These findings were in agreement

Table 4. Cox Regression Analysis to Identify the Independent Predictor of AML OS.

	Univariable				Multivariable			
	p	HR	95% CI		p	HR	95% CI	
Age (years)	0.169	1.018	0.999	1.039				
Gender	0.153	0.638	0.344	1.181				
Baseline BM blasts%	0.974	1.002	0.986	1.015				
LDH (IU/L)	0.62	1.033	0.979	1.098				
NPM1+ mutation	0.005	0.4	0.21	0.759	0.109	0.507	0.221	1.163
FLT3+ mutation	<0.001	3.185	1.711	5.927	0.663	1.222	0.496	3.013
Cytogenetic grade	0.163	0.624	0.322	1.211				
Total *CD3%*	0.046	1.129	1.002	1.272	0.595	1.046	0.885	1.237
CD3/LAG3%	<0.001	1.642	1.351	1.996	0.037	1.385	1.177	2.835
CD4/LAG3%	<0.001	2.074	1.539	2.794	0.015	1.252	1.087	2.669
CD8/LAG3%	<0.001	1.935	1.34	2.796	0.028	1.489	1.146	2.658
CD3/CTLA4/LAG3%	0.028	3.114	1.129	8.586	0.027	1.755	1.133	4.294

HR, Hazard ratio; CI, confidence interval

Table 5. Cox Regression Analysis to Identify the Independent Predictor of AML DFS

	Univariable				Multivariable			
	p	OR	95% CI		p	OR	95% CI	
Age (years)	0.189	1.018	0.997	1.039				
Gender	0.135	0.627	0.34	1.157				
Baseline BM blasts%	0.337	1.008	0.992	1.024				
LDH (IU/L)	0.269	1.001	0.999	1.003				
NPM1+ mutation	0.355	0.755	0.416	1.37				
FLT3+ mutation	0.084	1.699	0.931	3.099				
Intermediate cytogenetic	0.037	0.488	0.249	0.959				
Total *CD3 %*	0.108	1.1	0.979	1.236				
CD3/LAG3%	<0.001	1.45	1.196	1.758	0.049	1.952	1.001	3.803
CD4/LAG3%	<0.001	1.813	1.335	2.462	0.036	1.838	1.38	2.845
CD8/LAG3%	0.031	1.517	1.039	2.214	0.029	1.661	1.304	1.839
CD3/CTLA4/LAG3%	0.224	1.921	0.67	5.505	0.015	0.415	0.204	0.842

HR, Hazard ratio; CI, confidence interval

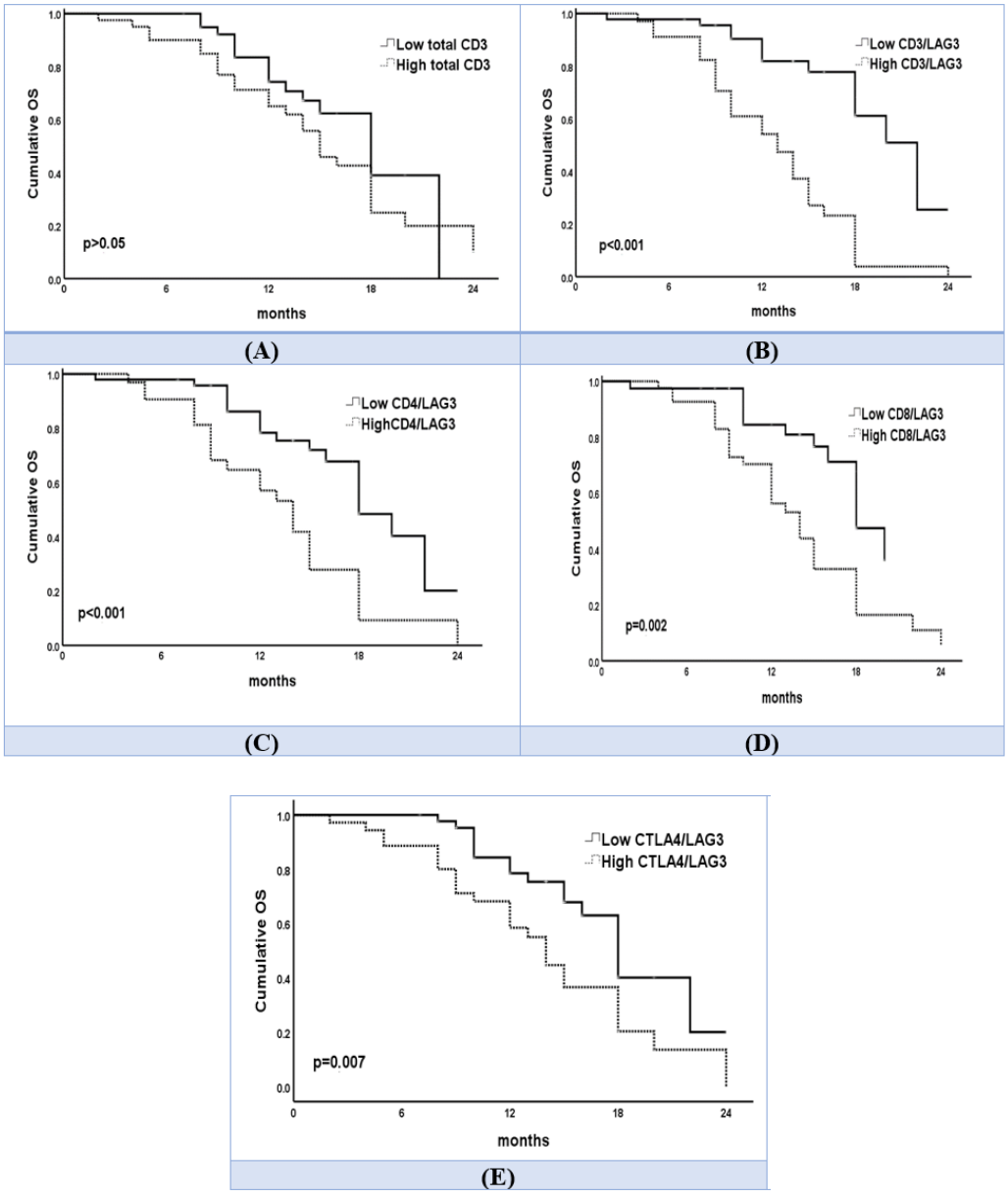

Figure 2. OS According to Expression of (A) total *CD3*, (B) *CD3/LAG3,* (C) *CD4/LAG3,* (D) *CD8/LAG3* and (E) *CD3/CTLA4/LAG3* in AML Cases.

with other studies in other malignant disorders [26, 27]. However, in AML studies William et al. [28] did not observe any clinical meaningful association between survival and the distribution of T-cell subsets. Furthermore, Chen et al. [18] in his study found insignificant difference in the number of *CTLA4/LAG3 /CD4* T-cells except one case only with poor outcome and genetic DNMT3A alteration. This conflict in outcome observation may relate to characterization of patients study or performance status.

Finally, COX regression analyses were done to address which one of the studied parameters is independent predictor for AML patients overall survival. The analyses revealed that higher expression of total *CD3, CD3/LAG3, CD4/LAG3, CD8/LAG3*, and *CTLA4/LAG3* were associated with shorter OS in univariable analysis. However, in multivariable analysis, higher *CD3/LAG3, CD4/LAG3, CD8/LAG3*, and *CTLA4/LAG3* expression were considered risk predictors for shorter OS. Furthermore, higher *CD3/LAG3, CD4/LAG3*, and *CD8/LAG3* expression were considered risk predictors for shorter DFS in univariable and multivariable analyses. This interesting statistical finding is not done by other studies.

To the best of our knowledge, *LAG3* was not only up regulated in T-cells but in leukemia cells as well. It has been reported that CLL cells could express and secrete LAG3, which is associated with shorter time to the first treatment. Soluble *LAG3* enhances leukemic cell activation and inhibits leukemic cell apoptosis through its engagement with MHC class II and induces immune exhaustion on the CLL microenvironment both *CTLA4/LAG3* may be a potential novel prognostic marker for CLL [29]. To overcome the varieties immune suppression observed in AML, patients may need combinations of immune checkpoint antibodies, checkpoint antibodies with BiTE (bi-specific T-cell engager) antibodies [30] or HMAs, or strategies that include external supplementation with activated T cells, such as chimeric antigen receptor (CAR)-T cells (35). One such strategy that has been evaluated in the clinic with early encouraging results is combining HMAs with immune checkpoint inhibitors in patients who have MDS/AML. especially refractory or frequent relapsed [31].

The limitation of our study is that the data focused on checkpoint receptor and ligand expression in T lymphocytes and not in AML blasts. Possible contributions by natural killer cells and other myeloid subsets, such as macrophages, monocytes, myeloid-derived suppressor cells, or other components of the tumor microenvironment, in addition, the patients received various different therapies on different clinical trials ongoing at our institution in that timeframe. Thus, as we learn more about the associations between the immune microenvironment and AML biology, the information gained may help guide treatments and potentially allow for the personalized selection of immune checkpoint pathways to target special high-risk AML.

In conclusion, our findings indicated that high expression of *LAG3/CTLA4* in T cells subsets identify a subgroup of AML patients with poor prognosis. Moreover, both *CTLA-4* and *LAG-3* may be promising prognostic markers in AML patients.

Author Contribution Statement

Conception: Salah Aref, Nadia El Menshawy; Interpretation and analysis of data: Wesam El Dosoky, Doaa Atia, Mohamed Aref; Preparation of the manuscript: Tarek Abou Zaid, Mohamed Aref, Wesam El Dosoky; Revision for important intellectual content: Ahmed Ramez; Supervision: Salah Aref, Nadia, El Menshawy

Acknowledgements

The authors acknowledge the patients who participate in this study. Also; we acknowledge technicians in the Mansoura University Oncology Center laboratories.

Funding statement

This study funded from the authors.

Ethical aspects

This study was approved by the local Mansoura Faculty of Medicine Ethics Committee. Informed consent from all included subjects were taken prior to recruitment.

Conflict of interest

The authors declare that there is no conflict of interest Availability of data The data available upon request to the corresponding author

References

1. Burnett AK. Treatment of acute myeloid leukemia: Are we making progress? Hematology Am Soc Hematol Educ Program. 2012;2012:1-6. https://doi.org/10.1182/asheducation-2012.1.1.
2. Aref S, Al Khodary T, Zeed TA, El Sadiek A, El Menshawy N, Al Ashery R. The prognostic relevance of baalc and erg expression levels in cytogenetically normal pediatric acute myeloid leukemia. Indian J Hematol Blood Transfus. 2015;31(1):21-8. https://doi.org/10.1007/s12288-014-0395-z.
3. Lichtenegger FS, Krupka C, Haubner S, Köhnke T, Subklewe M. Recent developments in immunotherapy of acute myeloid leukemia. J Hematol Oncol. 2017;10(1):142. https://doi.org/10.1186/s13045-017-0505-0.
4. Tan J, Chen S, Xu L, Lu S, Zhang Y, Chen J, et al. Increasing frequency of t cell immunosuppressive receptor expression in cd4+ and cd8+ t cells may related to t cell exhaustion and immunosuppression in patients with aml. Blood. 2016;128:5166-. https://doi.org/10.1182/blood.V128.22.5166.5166.
5. Jamal E, Azmy E, Ayed M, Aref S, Eisa N. Clinical impact of percentage of natural killer cells and natural killer-like t cell population in acute myeloid leukemia. J Hematol. 2020;9(3):62-70. https://doi.org/10.14740/jh655.
6. Aref S, Khaled N, Al Gilany AH, Ayed M, Abouzeid T, Attia D. Impact of bone marrow natural killer cells (nk); soluble tnf-α and il-32 levels in myelodysplastic syndrome patients. Asian Pac J Cancer Prev. 2020;21(10):2949-53. https://doi.org/10.31557/apjcp.2020.21.10.2949.
7. Menter T, Bodmer-Haecki A, Dirnhofer S, Tzankov A. Evaluation of the diagnostic and prognostic value of pdl1 expression in hodgkin and b-cell lymphomas. Hum Pathol. 2016;54:17-24. https://doi.org/10.1016/j.humpath.2016.03.005.

8. Schumacher TN, Schreiber RD. Neoantigens in cancer immunotherapy. Science. 2015;348(6230):69-74. https://doi.org/10.1126/science.aaa4971.
9. Workman CJ, Rice DS, Dugger KJ, Kurschner C, Vignali DA. Phenotypic analysis of the murine cd4-related glycoprotein, cd223 (lag-3). Eur J Immunol. 2002;32(8):2255-63. https://doi.org/10.1002/1521-4141(200208)32:8<2255::Aid-immu2255>3.0.Co;2-a.
10. Bae J, Lee SJ, Park CG, Lee YS, Chun T. Trafficking of lag-3 to the surface on activated t cells via its cytoplasmic domain and protein kinase c signaling. J Immunol. 2014;193(6):3101-12. https://doi.org/10.4049/jimmunol.1401025.
11. Woo SR, Turnis ME, Goldberg MV, Bankoti J, Selby M, Nirschl CJ, et al. Immune inhibitory molecules lag-3 and pd-1 synergistically regulate t-cell function to promote tumoral immune escape. Cancer Res. 2012;72(4):917-27. https://doi.org/10.1158/0008-5472.Can-11-1620.
12. Zhang H, Dai Z, Wu W, Wang Z, Zhang N, Zhang L, et al. Regulatory mechanisms of immune checkpoints pd-l1 and ctla-4 in cancer. J Exp Clin Cancer Res. 2021;40(1):184. https://doi.org/10.1186/s13046-021-01987-7.
13. De Silva P, Aiello M, Gu-Trantien C, Migliori E, Willard-Gallo K, Solinas C. Targeting ctla-4 in cancer: Is it the ideal companion for pd-1 blockade immunotherapy combinations? Int J Cancer. 2021;149(1):31-41. https://doi.org/10.1002/ijc.33415.
14. Tan J, Chen S, Huang J, Chen Y, Yang L, Wang C, et al. Increased exhausted cd8(+) t cells with programmed death-1, t-cell immunoglobulin and mucin-domain-containing-3 phenotype in patients with multiple myeloma. Asia Pac J Clin Oncol. 2018;14(5):e266-e74. https://doi.org/10.1111/ajco.13033.
15. Krupka C, Kufer P, Kischel R, Zugmaier G, Lichtenegger FS, Köhnke T, et al. Blockade of the pd-1/pd-l1 axis augments lysis of aml cells by the cd33/cd3 bite antibody construct amg 330: Reversing a t-cell-induced immune escape mechanism. Leukemia. 2016;30(2):484-91. https://doi.org/10.1038/leu.2015.214.
16. Daver N, Garcia-Manero G, Basu S, Boddu PC, Alfayez M, Cortes JE, et al. Efficacy, safety, and biomarkers of response to azacitidine and nivolumab in relapsed/refractory acute myeloid leukemia: A nonrandomized, open-label, phase ii study. Cancer Discov. 2019;9(3):370-83. https://doi.org/10.1158/2159-8290.Cd-18-0774.
17. Gourgou-Bourgade S, Cameron D, Poortmans P, Asselain B, Azria D, Cardoso F, et al. Guidelines for time-to-event end point definitions in breast cancer trials: Results of the datecan initiative (definition for the assessment of time-to-event endpoints in cancer trials)†. Ann Oncol. 2015;26(5):873-9. https://doi.org/10.1093/annonc/mdv106.
18. Chen Y, Tan J, Huang S, Huang X, Huang J, Chen J, et al. Higher frequency of the ctla-4(+) lag-3(+) t-cell subset in patients with newly diagnosed acute myeloid leukemia. Asia Pac J Clin Oncol. 2020;16(2):e12-e8. https://doi.org/10.1111/ajco.13236.
19. Brück O, Dufva O, Hohtari H, Blom S, Turkki R, Ilander M, et al. Immune profiles in acute myeloid leukemia bone marrow associate with patient age, t-cell receptor clonality, and survival. Blood Adv. 2020;4(2):274-86. https://doi.org/10.1182/bloodadvances.2019000792.
20. Guo W, Xing C, Dong A, Lin X, Lin Y, Zhu B, et al. Numbers and cytotoxicities of cd3+cd56+ t lymphocytes in peripheral blood of patients with acute myeloid leukemia and acute lymphocytic leukemia. Cancer Biol Ther. 2013;14(10):916-21. https://doi.org/10.4161/cbt.25938.
21. Keane C, Law SC, Gould C, Birch S, Sabdia MB, Merida de Long L, et al. Lag3: A novel immune checkpoint expressed by multiple lymphocyte subsets in diffuse large b-cell lymphoma. Blood Adv. 2020;4(7):1367-77. https://doi.org/10.1182/bloodadvances.2019001390.
22. Liao D, Wang M, Liao Y, Li J, Niu T. A review of efficacy and safety of checkpoint inhibitor for the treatment of acute myeloid leukemia. Front Pharmacol. 2019;10:609. https://doi.org/10.3389/fphar.2019.00609.
23. Broglie L, Gershan J, Burke MJ. Checkpoint inhibition of pd-l1 and ctla-4 in a child with refractory acute leukemia. Int J Hematol Oncol. 2019;8(1):Ijh10. https://doi.org/10.2217/ijh-2018-0009.
24. Boddu P, Kantarjian H, Garcia-Manero G, Allison J, Sharma P, Daver N. The emerging role of immune checkpoint based approaches in aml and mds. Leuk Lymphoma. 2018;59(4):790-802. https://doi.org/10.1080/10428194.2017.1344905.
25. Rowshanravan B, Halliday N, Sansom DM. Ctla-4: A moving target in immunotherapy. Blood. 2018;131(1):58-67. https://doi.org/10.1182/blood-2017-06-741033.
26. Shapiro M, Herishanu Y, Katz BZ, Dezorella N, Sun C, Kay S, et al. Lymphocyte activation gene 3: A novel therapeutic target in chronic lymphocytic leukemia. Haematologica. 2017;102(5):874-82. https://doi.org/10.3324/haematol.2016.148965.
27. Zeng H, Zhou Q, Wang Z, Zhang H, Liu Z, Huang Q, et al. Stromal lag-3(+) cells infiltration defines poor prognosis subtype muscle-invasive bladder cancer with immunoevasive contexture. J Immunother Cancer. 2020;8(1). https://doi.org/10.1136/jitc-2020-000651.
28. Williams P, Basu S, Garcia-Manero G, Hourigan CS, Oetjen KA, Cortes JE, et al. The distribution of t-cell subsets and the expression of immune checkpoint receptors and ligands in patients with newly diagnosed and relapsed acute myeloid leukemia. Cancer. 2019;125(9):1470-81. https://doi.org/10.1002/cncr.31896.
29. Palma M, Gentilcore G, Heimersson K, Mozaffari F, Näsman-Glaser B, Young E, et al. T cells in chronic lymphocytic leukemia display dysregulated expression of immune checkpoints and activation markers. Haematologica. 2017;102(3):562-72. https://doi.org/10.3324/haematol.2016.151100.
30. Kraman M, Faroudi M, Allen NL, Kmiecik K, Gliddon D, Seal C, et al. Fs118, a bispecific antibody targeting lag-3 and pd-l1, enhances t-cell activation resulting in potent antitumor activity. Clin Cancer Res. 2020;26(13):3333-44. https://doi.org/10.1158/1078-0432.Ccr-19-3548.
31. Lecocq Q, Keyaerts M, Devoogdt N, Breckpot K. The next-generation immune checkpoint lag-3 and its therapeutic potential in oncology: Third time's a charm. Int J Mol Sci. 2020;22(1). https://doi.org/10.3390/ijms22010075.