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The prospective prognostic value of the immune checkpoint *BTLA* expression in adult acute myeloid leukemia patients

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Abstract

Background: One of the crucial functions of the immune system is to prevent tumorigenesis, yet cancer occurs when malignant cells manage to evade immune surveillance via multiple strategies. Accordingly, this study aimed at assessing the potential significance of the novel immune checkpoint B and T lymphocyte attenuator (*BTLA*) as a prognostic marker in acute myeloid leukemia (AML), in addition to how it relates to response to treatment and patients' survival. Thus, mRNA expression of BTLA was investigated on peripheral blood in 60 AML patients and 15 healthy controls.

Results: *BTLA* expression was found to be significantly elevated ($p = 0.024$) in the tested AML cases in comparison with healthy controls. Moreover, *BTLA* was over-expressed in the CD13, CD33, and HLA-DR positive cases as compared to their negative counterparts ($p = 0.003$; $p < 0.001$, and $p = 0.001$, respectively), and cases showing *BTLA* over-expression had significantly poorer overall survival times ($p = 0.001$) as confirmed by Kaplan–Meier survival analysis.

Conclusion: These observations suggest that *BTLA* over-expression may be associated with reduced immunity against tumors and could be recommended as a promising biomarker for unfavorable prognosis in AML.

Keywords: Immune checkpoints, T-cells, B and T lymphocyte attenuator (*BTLA*), Acute myeloid leukemia

Background

Progress in understanding the pathophysiology and improving the therapy of acute myeloid leukemia (AML) is now occurring at a rapid pace [1]. AML is well known for including a wide range of gene mutations and chromosomal anomalies [2]. It may respond to high-dose chemotherapy in some patients; however, the mainstream succumb to resistance when eradication does not occur after induction chemotherapy [3]. Successful management of AML needs proper understanding of its pathophysiology at the cellular and molecular level, as well as its cytogenetic markers. It is essential to identify novel biomarkers that may provide better understanding of the molecular basis of AML. This could notably be helpful in diagnosis, prognosis, management and monitoring of patients [4].

Cancer involves the inability of the immune system to eradicate tumor cells. Dysfunctional antitumor T-cell responses actively participate in the development of cancer. Co-stimulatory and co-inhibitory mediators adjust T-cell proliferation, half-life, and cytokine release and enable effective T-cell responses to malignancy, yet controlling autoimmunity [5]. The balance between both these co-stimulatory and co-inhibitory signaling pathways acts as a molecular switch between activation and inhibition [6]. Nevertheless, this balance may be interrupted by continual antigen stimulation, as well as release of mediators, that suppress the immune response in the tumor microenvironment, causing T-cell dysfunction, called "T-cell exhaustion," leading to its failure of

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cancer eradication responses [7]. Tumor cells manage diverse approaches to escape the immune response; these include expressing co-inhibitory receptors which inhibits T-cell reactions and cytokine production. Thus, this up-regulated expression of co-inhibitory receptors is coupled to T-cell failure in malignancy [8]. The expanding immunotherapeutic approaches markedly offer several treatment options but should be engaged sensibly and cautiously.

AML cells develop a variety of mechanisms to evade T-cell-mediated immunity, leading to its progression and relapse. These mechanisms include activation of immune checkpoints pathways that interfere with effective T-cell antitumor immunity [9]. Recent reports demonstrated over-expression of cytotoxic T lymphocyte antigen-4 (*CTLA-4*) and lymphocyte activation gene-3 (*LAG-3*) as major immune checkpoints expressed on T-cells [10]. Moreover, it was lately reported that over-expression of programmed cell death protein- 1 (*PD-1*) and its ligands on leukemia cells is associated with more aggressive disease and AML relapse [9].

B and T lymphocyte attenuator (*BTLA*) or CD272 is a type I transmembrane co-signaling receptor that belongs to the CD28 superfamily and is mainly expressed on immune cells [5]. It is a ligand for tumor necrosis factor receptor superfamily member 14 (*TNFRSF14*), where their interaction inhibits T-cell immune responses. Besides, $BTLA^+$ T-cells have a less-differentiated phenotype, lower cytolytic function, and higher potential to proliferate compared with $BTLA^-$ T-cells [11]. Despite the critical role *BTLA* plays in immune tolerance and immune response, research is needed concerning its expression in AML. Accordingly, this study aimed at assessing the prognostic potential of *BTLA* in AML patients and how it relates to immune cell populations and response to therapy.

Materials and methods

Subjects

Sixty recently diagnosed AML patients from the Internal medicine Department, Clinical Hematology and Stem Cell Transplantation Unit, [Ain Shams] University Hospitals, Cairo, Egypt were recruited in this study. This AML patients group included 24 males and 36 females with a mean age of 53.4 ± 12.9 years. Morphologic findings from Wright–Giemsa-stained smears of bone marrow aspirates and immunophenotype characterization of leukemic cells were used for diagnosis. According to the FAB classification, this AML group included 8 M0, 4 (M1-M2), 12 M2, 12 M3, 24 M4 patients. Prior to receiving any treatment, peripheral blood (PB) samples were collected in vaccutainer tubes containing Na_2EDTA (1.5 mg/ml final concentration) for full blood count, immunophenotype characterization and for total RNA extraction. All patients were followed up for 12 months. Overall survival (OS) referred to the time between date of diagnosis and death. Subjects who survived till the end of the 12 months were censored. A healthy control group, consisting of 15 healthy, sex and age matched volunteers, was also involved in the study. This healthy control group included 7 males and 8 females with mean age of 46.9 ± 6.3 years.

Treatment plan

All patients in this study have received standard induction chemotherapy as specified by The National Comprehensive Cancer Network (NCCN) 2019 recommendations for AML (3 + 7 protocol (anthracycline + cytarabine) for all types of AML except M3 subtype who received PETHEMA protocol) [12]. On day 28, assessments were done for all patients who have survived the induction, according to which patients were classified into responders, who achieved Complete Remission (CR), and non-responders, who were refractory to chemotherapy. CR was defined as an absolute neutrophilic count > 1000/μl, a platelet count ≥ 100,000/μl, and BM blasts < 5% with no evidence of extramedullary disease.

Methods

Immunophenotypic characterization

Flow cytometry technique was used for immunophenotypic characterization using diagnostic kits supplied by Beckman Coulter, Fullerton, CA, USA [13].

Cytogenetic analysis

Fluorescence in situ hybridization (FISH) was applied as illustrated by Pinkel et al. [14] and Anastasi et al. [15] to verify cytogenetic abnormalities. Additionally, as stated by Fischer et al. [16] and Frohling et al. [17], fluorescence microscopy was carried out in order to determine the site of mutation or chromosomal defect.

Gene expression analyses

Total RNA extraction and purification from whole blood: using The QIAamp RNA blood mini kit (Qiagen, Hilden, Germany) according to the protocol specified by the manufacturer.

Synthesis of complementary DNA (cDNA): High capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) was used, and synthesized cDNA was stored at − 20 °C till use.

Gene expression analyses: using Rotor-Gene Q® Real-Time PCR cycler (Qiagen, Hilden, Germany) using standard thermal cycling conditions and Taqman assays specific for *BTLA* (Hs00699198_m1). *GAPDH* (Hs02786624_g1) was utilized as an endogenous control

for data normalization. The expression levels were normalized and analyzed using the $2^{-\Delta\Delta Ct}$ method.

Statistics

Data analysis was done using IBM SPSS Statistics for Windows, version 23 (IBM© Corp., Armonk, NY) and MedCalc© version 18.2.1 (MedCalc© Statistical Software, Ostend, Belgium). For skewed numerical data, Mann–Whitney *U* test (for two-group comparison) was used for comparing between-group differences. Correlations were examined using Spearman rank correlation. Low or high *BTLA* fold expression was concluded depending on cutoff values, and survival curves for both low and high *BTLA* expression were plotted by the Kaplan–Meier method and compared by the log-rank test. Cox proportional hazards regression was used to examine the relation between *BTLA* expression and overall survival after adjustment for the effect of age and gender. Statistical significance was considered at p values < 0.05.

Results

Baseline characteristics of study participants

Of the 60 patients, 20 (33.3%) patients had cytogenetic profile suggesting favorable prognosis, exhibiting low risk cytogenetic abnormalities such as t(15;17), t(8;21) and inv(16) while 40 (66.7%) patients had cytogenetic profile of unfavorable prognosis, exhibiting intermediate or high risk cytogenetic abnormalities such as (3q), del(5q), −5/−7 or complex karyotype. All demographic features are shown in Table 1.

Table 1 Baseline characteristics of study participants

Characteristics	Control group	AML group
Age (years) (mean ± SD)	46.9 ± 6.3	53.4 ± 12.9
Gender (male/female)	7/8	24/36
FAB classification		
M0	–	8 patients
M1–M2	–	4 patients
M2	–	12 patients
M3	–	12 patients
M4	–	24 patients
Prognosis according to cytogenetic studies (favorable/unfavorable)	–	20/40

CD13, CD33 and HLA-DR expression in AML patients

CD13, CD33 and HLA-DR expression in AML patients are listed in Table 2.

BTLA expression levels in AML patients

As shown in Fig. 1, *BTLA* expression levels showed 155% up-regulation in AML patients (median: 1.77; 25th and 75th centiles-quartiles: 0.96–3.68) as compared to the healthy control group (median: 1.14; 25th and 75th centiles-quartiles: 0.70–1.15) (significant, $p = 0.024$). This up-regulated expression was independent of patients' age or gender.

BTLA expression levels regarding individual prognostic markers in AML patients

As for individual prognostic markers, the expression level of *BTLA* in $CD13^+$, $CD33^+$, HLA-DR^+ patients was significantly higher than in $CD13^-$, $CD33^-$, HLA-DR^- patients ($p = 0.003$, $p < 0.001$, $p = 0.001$, respectively) (Fig. 2A–C, respectively). On the other hand, *BTLA* expression levels were higher in AML patients with unfavorable prognosis (median: 1.9; 25th and 75th centiles-quartiles: 1.00–3.7) as compared to AML patients with favorable prognosis (median: 1.00; 25th and 75th

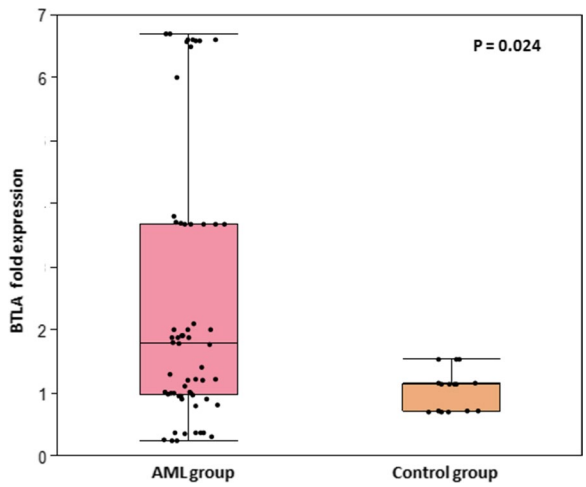

Fig. 1 *BTLA* fold expression in cases of AML and control group. Box represents the interquartile range. Line inside the box represents the median. Whiskers represent minimum and maximum values. Dots represent individual observations

Table 2 CD13, CD33 and HLA-DR expression in AML patients

	CD13 expression	CD33 expression	HLA-DR expression
AML patients	52 $CD13^+$/8 $CD13^-$	52 $CD33^+$/8 $CD33^-$	36 HLA-DR^+/24 HLA-DR^-

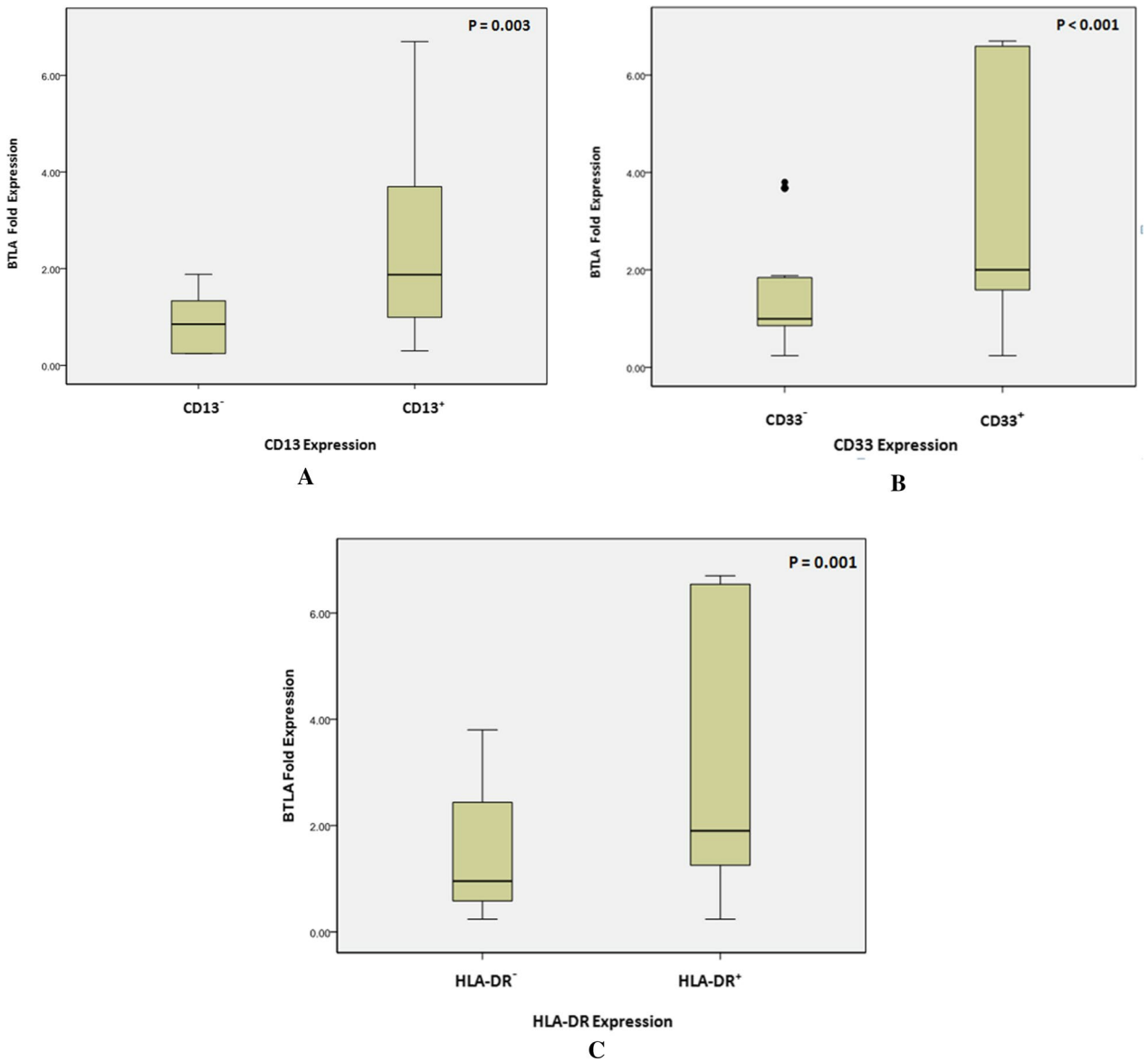

Fig. 2 *BTLA* fold expression with respect to **A** CD13 expression, **B** CD33 expression, **C** HLA-DR expression. Box represents the interquartile range. Line inside the box represents the median. Whiskers represent minimum and maximum values

centiles-quartiles: 0.60–2.00) but this increase was not statistically significant ($p = 0.06$).

BTLA expression levels and response to chemotherapy in AML patients

To assess the correlation between *BTLA* expression levels and response to chemotherapy, the patients' responsiveness to induction chemotherapy was studied. By the end of the induction phase, 20% (12 patients) of the study group deceased; this may be attributed to respiratory tract infections, septicemia and/or hemorrhage. All the deceased patients have shown high *BTLA* expression levels. On the other hand, the survivors (80% of the cohort, 48 patients) were further classified into 36 (75%) responders and 12 (20%) non-responders. *BTLA* fold expression was found to be significantly up-regulated in non-responsive patients as compared to those responsive to chemotherapy ($p = 0.022$, Fig. 3).

BTLA expression levels concerning AML patients' survival

Regarding patients' survival, *BTLA* fold expression was found to be significantly negatively correlated with

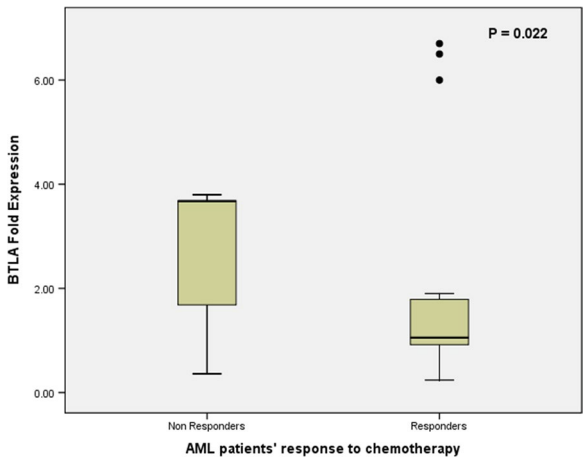

Fig. 3 *BTLA* fold expression with respect to AML patients' response to chemotherapy. Box represents the interquartile range. Line inside the box represents the median. Whiskers represent minimum and maximum values. Dots represent individual observations

patients' overall survival ($r = -0.538$, $p < 0.01$, Fig. 4). Additionally, Kaplan–Meier survival analysis revealed that AML patients with high *BTLA* expression showed significantly worse survival than those with low *BTLA* expression ($p = 0.001$, Fig. 5). Finally, multivariate analysis was performed using the Cox Proportional Hazards Model adjusted for age and gender. The test revealed significant relation between decreased survival and high *BTLA* expression (Cox proportional hazards = 1.282, 95% CI = 1.121–1.465, $p < 0.001$).

Discussion

Cancer evolution is currently documented as an outcome of developing crosstalk between different tumor cells and the surrounding stroma. While normally stroma is not permissive for cancer development, malignant cells have the ability to transform it. This may include their capability of changing the proportions of effector to regulatory T-cells, as well as altering co-stimulatory and co-inhibitory molecule expression, resulting in immune suppression, tumor evolution and immune evasion [18]. Understanding this immune environment in AML and manipulating coherent approaches to target its immune biology are an area of deep constant research in AML [19].

BTLA has been considered a novel co-inhibitory receptor, similar in structure and function to *CTLA-4* and *PD-1*, and present on most lymphocytes [18]. The present study demonstrated the significant up-regulation of *BTLA* expression in AML patients versus the control group. Previous data reported AML blasts to be involved in immune suppression and in creating suppressive microenvironments [3]. Accordingly, immune response is inhibited; leading to immune escape by cancer cells and AML progression. Moreover, *BTLA* is linked with T-cell differentiation; previous studies concerning dysfunctional T-cells in cancers illustrated that exhausted T-cells demonstrate up-regulated expression in inhibitory receptors (IRs) and lost ability to eradicate tumor cells. However, the crucial procedure of T-cell exhaustion in cancer is still unclear [7].

Lately, the up-regulation of *BTLA* expression has been linked to tumor progression, with the worst

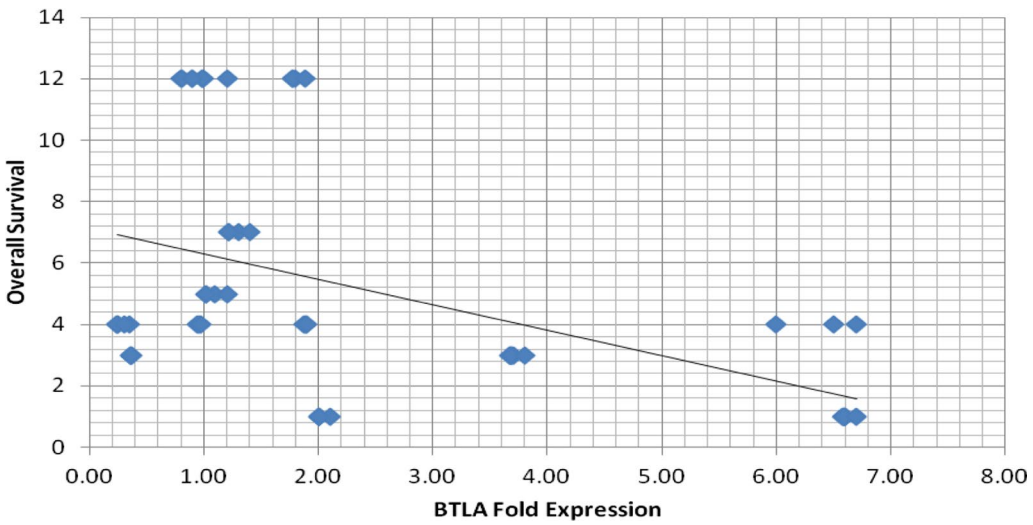

Fig. 4 Correlation of *BTLA* fold expression with overall survival in AML patients ($n = 60$). Each individual value is represented by a symbol (■). r = Spearman's rho

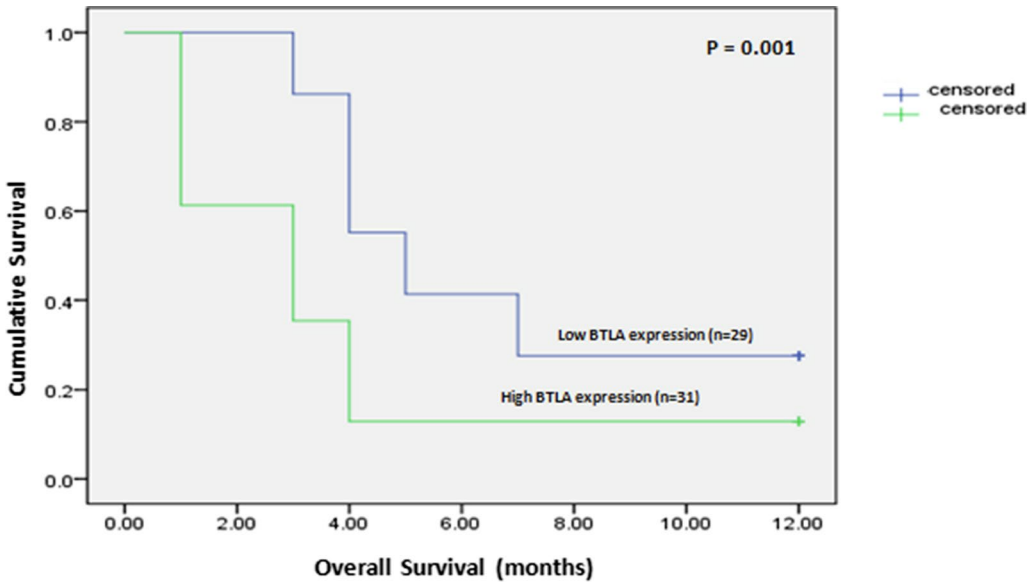

Fig. 5 Kaplan–Meier curves according to high or low *BTLA* fold expression

prognosis reported for human melanoma [20], chronic and small lymphocytic leukemias [21], colorectal cancer patients [22], as well as gastric adenocarcinoma [23]. Those results come in accordance with the findings of this study, as the up-regulated *BTLA* expression was correlated with a decrease in the overall survival of AML patients, suggesting its potential use as a prognostic marker. Moreover, the significant up-regulation of *BTLA* expression in chemotherapy non-responder patients in our study as compared to responders may indicate that high *BTLA* expression may be related to resistance to chemotherapy. Similarly, other immune checkpoints have also demonstrated prognostic significance in AML patients such as T-cell immunoglobulin and mucin domain 3 (TIM-3) [24]. Additionally, the study of Chen et al. demonstrated that increased immune checkpoints co-expression of PD-1/CTLA-4, PD-L2/CTLA-4 or PD-1/LAG-3 correlated with poor OS in AML patients [25].

Interestingly, *BTLA* expression level showed a significant increase in $CD13^+$ in comparison with $CD13^-$ patients and in $CD33^+$ as compared to $CD33^-$ patients. CD13 expression is generally regarded as a bad prognostic sign, when it is not detected better outcomes are expected [26], while CD33 is more expressed on AML blasts as compared to healthy donor myeloid progenitors, which makes it an attractive nominee for targeted AML therapy [27]. Furthermore, *BTLA* expression in $HLA\text{-}DR^+$ patients was significantly more than its expression in $HLA\text{-}DR^-$ patients, indicating low chances of complete remission [26]. Accordingly, *BTLA* can be possibly suggested as an indicator of prognosis and survival in AML patients.

The fact that allogeneic hematopoietic cell transplantation is effective in AML treatment suggests that AML may be immune-responsive and indicates that novel immune-therapies such as immune checkpoint inhibitors might be able to provide a significant disease control [20]. The purpose of AML immunotherapy is enhancing the immune cells' capacity to eliminate leukemic cells. However, chemotherapy provokes several alterations within immune effector cells and might hinder T-cells functionality, thus the optimal timing of immunotherapy with chemotherapy should be taken into consideration [3].

The study of Chen et al. evaluated the prospective of BTLA as targets for ovarian cancer therapy preclinically. Obvious *BTLA* expression was prognostic for poor survival. Moreover, inhibition of *BTLA* combined with chemotherapy was found to encourage immune activation and produce influential antitumor effects in an animal model. Therefore, the combination of chemotherapy and anti-*BTLA* Ab might show clinical potential [28], but still future studies regarding *BTLA* targetting as a novel therapeutic strategy in AML are necessary. Several monoclonal antibodies targeting the *CTLA-4, PD-1* and *PD-L1* immune checkpoints on T-cells are now approved for clinical use in several solid tumors and hematological malignancies [29]. Some different immunotherapies are now under evaluation in AML such as PD1 inhibitors nivolumab and pembrolizumab. Early promising signals demonstrated in AML suggest a prospective future role for these targeted checkpoint therapies [19]. Moreover,

dual blockade treatments were proposed to have a superior effect [30]. Nevertheless, resistance to treatment necessitates the development of unorthodox approaches.

Finally, the up-regulated *BTLA* expression demonstrated in the present study and its negative impact on prognosis and survival may be a basis for designing future immune-therapies. The use of multiple combinations targeting immune checkpoints may provide superiority in cancer therapy. Accordingly, it is proposed that our findings concerning the prognostic value of *BTLA* expression in AML would be valuable in the upcoming studies to develop this novel approach.

Conclusions

As a final conclusion, these results demonstrate that *BTLA* gene expression may be considered as a promising significant determinant of AML patients' prognosis and survival. Additional studies are necessary for evaluating the significance of *BTLA* inhibitors in designing future immunotherapies.

Abbreviations

BTLA: B and T lymphocyte attenuator; AML: Acute myeloid leukemia; *CTLA-4*: Cytotoxic T lymphocyte antigen-4; *LAG-3*: Lymphocyte activation gene-3; *PD-1*: Programmed cell death protein-1; *TIM-3*: T-cell immunoglobulin and mucin domain 3; HLA-DR: Human leukocyte antigen—DR isotype.

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Authors' contributions

SMR contributed to the study conception, design, methodology, investigation, data analysis and wrote the first draft of the manuscript. NSE contributed to methodology, investigation, validation and resources. NAN interpreted the patient data regarding the hematological disease and contributed to methodology, validation and resources. AE contributed to methodology, data analysis, validation and resources. AMK contributed to the study conception, design, methodology, investigation and data analysis. All authors read and approved the final manuscript.

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Availability of data and materials

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethical Committee of Research, Faculty of Medicine, Ain Shams University (FMASU M D 355/2018) and was conducted in accordance with the Declaration of Helsinki. Written consents were obtained from all controls and patients.

Consent for publication

Written consents were obtained from all controls and patients.

Competing interests

The authors declare that they have no competing interests.

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