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 vactutainer tubes containing Na2EDTA (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 PETHHEMA 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), $\sim$5/$\sim$7 or complex karyotype. All demographic features are shown in Table 1.

| 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     | –             | 20/40     |
| studies (favorable/unfavorable)        |               |           |

**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
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
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
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-DR\(^+\) patients was significantly more than its expression in HLA-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 targeting 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].

![Fig. 5 Kaplan–Meier curves according to high or low BTLA fold expression](image-url)
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. AE contributed to methodology, data analysis, and wrote the first draft of the manuscript. NSE contributed to methodology, data analysis, and wrote the first draft of the manuscript. SMR 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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