Predictive value of the proliferation marker Ki-67 in patients with acute leukemia undergoing hematopoietic stem cell transplantation

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Introduction Hematopoietic stem cell transplantation (HSCT) is increasingly used in cases of acute leukemia. Ki-67 protein is an excellent marker of proliferation in many solid tumors and hematological malignancies.

Aim The aim was to evaluate the utility of Ki-67 as a prognostic marker before HSCT.

Patients and methods The study included 40 adult Egyptian patients with acute leukemia undergoing HSCT. Plasma Ki-67 levels were measured by enzyme-linked immunosorbent assay before transplant and after (at the date of engraftment or day 30 if engraftment failure occurred).

Results The median levels and (range) of plasma Ki-67 before and after transplant were 1.85±5.48 and 1.19±3.22 ng/ml, respectively. We found higher plasma Ki-67 levels (before and after transplant were correlated with more delayed engraftment (r=0.425,P=0.007 before transplant; r= 0.361 P=0.024 after transplant), and more prolonged neutropenic fever duration (r=0.377, P=0.017 before transplant; r=0.387, P=0.014 after transplant). Patients having acute graft-versus-host disease (GVHD) during the first month of HSCT also had higher levels of plasma Ki-67 before and after HSCT compared with patients who did not develop acute GVHD, with median (range) of 15.85 (0.20-27.40) ng/ml vs 0.30 (0.10-1.70) ng/ml (P=0.029) before transplant and 6.15 (3.90-18.90) ng/ml vs 0.20 (0.10-2.80) ng/ml (P= 0.001) after transplant. Plasma Ki-67 levels of greater than or equal to 6.4 ng/ml before transplantation (75% sensitivity and 100% specificity) and greater than or equal to 3.35 ng/ml after transplantation (100% sensitivity and specificity) are predictive values for detection of occurrence of acute GVHD.

Conclusion High levels of plasma Ki-67 in patients with acute leukemia undergoing HSCT may be a predictor of delayed engraftment, prolonged neutropenic fever and occurrence of acute GVHD.

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Introduction

Acute leukemia is a fatal disease. The therapy for acute leukemia, both acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL), is separated into two phases: the induction phase and the postremission phase to eradicate all blasts from the bone marrow and peripheral blood then to prevent relapse [1].

Hematopoietic stem cell transplantation (HSCT) has been advocated as an effective consolidation therapy in patients with AML especially those who relapse after standard therapy and patients with AML or ALL with poor prognostic factors [1]. Prognosis of HSCT varies widely dependent upon age, disease type, transplantation type, human leukocyte antigen-matched status, and conditioning regimen [2].

Ki-67 is a large nuclear nonhistone protein, encoded by the gene localized at 10q25. It is expressed at the end of G1, S, and G2 to reach maximal expression in mitosis. It is not expressed in G0, which means its expression is an absolute requirement for proliferation [3]. The fact that Ki-67 protein is present during all active phases of the cell cycle (G1, S, G2, and mitosis) and is absent in the resting cells (G0) makes it an excellent marker for proliferation [4].

Although the functional significance of Ki-67 remains to be fully elucidated, it has valuable prognostic utility in assessing many solid tumors and also some hematological malignancies [5] including malignant lymphoma [3] and leukemia [6].

Monoclonal and polyclonal anti-Ki-67 antibodies were developed to detect the proliferative compartment in tissue sections. Yet, detection in plasma would be advantageous as this would circumvent the need for costly and invasive biopsy procedures, and already

quantification of Ki-67 levels has become an important aspect in monitoring the progress of human neoplasms, notably prostate and breast carcinomas.

The aim of this study was to evaluate the plasma level of Ki-67 in patients with acute leukemia undergoing HSCT as a potential prognostic marker in this group of patients.

Patients and methods

Patients

The current study included 40 adult Egyptian patients with acute leukemia who were in remission and were prepared and underwent allogeneic HSCT during the period from July 2013 to June 2016 at either Ain Shams University hospitals, Cairo, Egypt (20%) or Nasser Institute Bone Marrow Transplant units (80%), Cairo, Egypt. The study protocol was approved by the scientific ethical committee of Faculty of Medicine, Ain Shams University and Nasser institute. An informed consent was obtained from each patient before study entry in accordance with the Declaration of Helsinki.

Criteria for diagnosis of acute leukemia were based on standard morphology, cytochemistry, immunophenotyping, and cytogenetic studies of leukemic cells [7,8].

Inclusion criteria

The following were the inclusion criteria:

(1) Age more than 18 years old.
(2) Diagnosis of acute leukemia [myeloid, lymphoblastic or mixed phenotype acute leukemia (MPAL)] undergoing allogeneic HSCT for the first time.

Exclusion criteria

The following were the exclusion criteria:

(1) Children with acute leukemia.
(2) Patients with relapsed leukemia after previous HSCT.

Methods

All patients were subjected to diagnostic laboratory investigations, human leukocyte antigen matching, and clinical follow-up.

Two peripheral blood samples on EDTA were withdrawn for detection of circulating plasma Ki-67 level. The first sample was withdrawn before stem cell transplantation and the second sample was taken at the date of engraftment or at day 30 if engraftment failure occurred.

All patients received peripheral blood collected stem cells from matched related donors. Human leukocytic antigen typing was done for each patient and his/her relatives by both serology and molecular techniques. 6/6 match was observed in 21 (52%) cases, and 8/8 matching was observed in 19 (47.5%) patients.

All patients were then followed up for 1 month after day 0 of HSCT procedure for determining the day of engraftment as defined as an absolute neutrophil count greater than 500/mm for 3 consecutive days or a single absolute neutrophil count greater than 1500/mm, and platelet engraftment is defined as having a platelet count of greater than 20 000/mm and not supported by a platelet transfusion in the previous 7 days [9].

Circulating plasma Ki-67 levels were measured using Enzyme-linked Immuno-sorbent Assay (ELISA) kit for Ki-67 protein (Ki-67P) (Uscn Life Science Inc., Wuhan, China) catalog no. E92047Hu 96 according to the manufacturer's instructions.

Statistical methods

Data were coded and entered using the statistical package SPSS (statistical package for the social science; SPSS Inc., Chicago, Illinois, USA) version 22. Data were summarized using mean and SD for quantitative parametric data, as median and range in non parametric quantitative data and using frequency (count) and relative frequency (percentage) for categorical data.

Comparisons between quantitative variables were done using the nonparametric Mann–Whitney tests, and for categorical data, the χ^2-test was performed. Correlations between quantitative variables were done using Spearman's correlation coefficient. *P* values less than 0.05 were considered as statistically significant.

A receiver operating characteristic curve was constructed to establish clinically relevant cut-off values for studied parameter with calculation of sensitivity, specificity and prognostic accuracy.

Results

Demographic data

The study included 26 (65%) males and 14 (35%) females. Their age ranged between 20 and 52 years with a median age of 30 years.

Among our patient cohort, the initial diagnoses were 26 (65%) AML, 12 (30%) ALL and 2 (5%) MPAL.

Regarding patients with AML, there were 16 males and 10 females. Their age ranged between 20 and 52 years old, with mean age of 29.76±8.36 years.

Regarding patients with ALL, there were nine males and three females. Their age range between 23 and 49 years old, with mean age of 33.8±10.3 years old.
As for patients with MPAL, there were one male and one female.

Subtypes of acute leukemia cases and their cytogenetic and molecular analysis are shown in Table 1.

Outcomes of allogeneic hematopoietic stem cell transplantation in the study population

Day of engraftment

A total of 38 patients had developed engraftment, with mean engraftment time of 18.36±3.74 days, and two patients had graft failure.

Complications of hematopoietic stem cell transplantation

The most common observed adverse effects were mucositis (vomiting and diarrhea). Vomiting was seen in all patients with mean duration of 7.5±3 days. Diarrhea was also seen in all patients with a mean duration of 4.15±2.2 days. Neutropenic fever was observed in all patients with a mean duration of 5.35±2.13 days. Symptoms of acute graft-versus-host disease (GVHD) were seen in four (10%) patients; three cases of them were diagnosed as relapsed AML and the fourth case as MPAL. Graft failure was seen in two (5%) cases, both were diagnosed as relapsed AML. Other complications included viral infection Cytomegalovirus (CMV) in seven cases and bacterial infections in nine cases, hematuria in two cases, impaired kidney function tests in four cases, hepatitis in one case and jaundice in one case.

Table 1 Patients' leukemia subtypes and cytogenetic analysis

Patients	*n* (%)
Diagnosis	
ALL	12 (30)
AML	26 (65)
MPAL	2 (5)
ALL subtypes	
B-cell ALL	6 (15)
T-cell ALL	6 (15)
AML subtypes	
M6	1 (2.5)
M5	2 (5)
M4	4 (10)
M3	4 (10)
M2	8 (20)
M1	7 (17.5)
Philadelphia chromosome analysis in B-ALL	
Philadelphia chromosome positive	4 (66.7)
Philadelphia chromosome negative	2 (33.3)
Cytogenetic and molecular analysis for AML	
t (15;17)	4 (10.0)
inv. 16	2 (5)
FLT3 mutation.	2 (5)
del (11q)	1 (2.5)
Complex cytogenetic abnormalities	1 (2.5)
Normal karyotype	16 (40)

This table shows the distribution of subtypes of acute leukemia and their cytogenetic and molecular abnormalities. ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; MPAL, mixed phenotype acute leukemia.

Comparative studies

There were no significant differences between both sexes regarding day of engraftment or occurrence of HSCT complications (including acute GVHD, neutropenic fever, infections, vomiting, diarrhea, and graft failure).

Age was found to be negatively correlated with the day of engraftment, with a highly significant *P* value of 0.001, r=-0.571. Moreover, age was negatively correlated with the severity and duration of mucositis, with a significant *P* value of 0.003, r=-0.459. Acute GVHD was seen more in younger patients with mean age of 21.75±2.87 years and less at elder age group of patients, with mean age of 31.53 ±8.97 years, with significant *P* value of 0.020.

There was a significant difference between the three diagnoses groups regarding day of engraftment (18.7 ±3.2 days for patients with ALL, 17.6±3.5 days for patients with AML, and 25±1.4 days for patients with MPAL; *P* value was significant at 0.049.

Incidence of infections was also highest in MPAL cases (100%), compared with ALL cases (66.7%) and AML cases (19.2%). The *P* value was significant at 0.003.

There were no significant differences between the three diagnoses groups regarding occurrence of acute GVHD, vomiting, diarrhea, and graft failure.

Circulating plasma Ki-67 levels (before and after hematopoietic stem cell transplantation) and its relation to leukemia subtypes and outcomes of allogeneic hematopoietic stem cell transplantation

Plasma Ki-67 levels

The median (range) of plasma Ki-67 before transplant was 0.30 ng/ml, (range: 0.1–27.4 ng/ml), whereas the median level after transplantation (at the date of engraftment or day 30 if engraftment failure occurred) was 0.25 ng/ml (range: 0.1–18.9 ng/ml).

Correlation studies

For the 38 patients who developed engraftment during the observation time, there was a positive correlation between plasma Ki-67 level before and after HSCT and early engraftment (the day of engraftment) (r=0.425 and 0.361, respectively), with a significant P value (P=0.007 and 0.024, respectively). This means that the higher plasma Ki-67 level, the more delayed the engraftment.

Moreover, there was a positive correlation between plasma level of Ki-67 before and after HSCT and duration of neutropenic fever (r=0.377 and 0.387, respectively), with significant P value (P=0.017 and 0.014, respectively). Thus, the higher the plasma Ki-67 level, the more prolonged the neutropenic fever duration.

Comparative studies

Among the study population, lowest plasma level of Ki-67 before HSCT was seen in patients with ALL, with median (range) value of 0.20 (0.10-0.50) ng/ml compared with 0.40 (0.10-20.60) ng/ml in patients with AML and 14.0 (0.60-27.40) ng/ml for patients with MPAL. P value was significant at 0.011.

However, plasma Ki-67 after HSCT did not differ significantly between different diagnoses (P=0.143). Median (range) plasma Ki-67 level in patients with ALL was 0.20 (0.10-2.80) ng/ml compared with 0.25 (0.10-6.60) ng/ml in patients with AML and 9.60 (0.30-18.90) ng/ml for patients with MPAL.

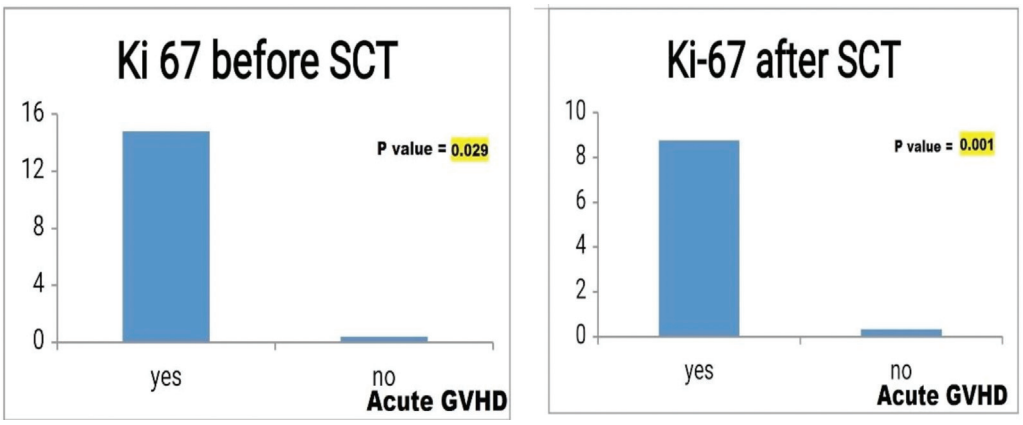
Patients who had acute GVHD during the first month of HSCT had higher plasma level of Ki-67 before and after HSCT, with median (range) value of 15.85 (0.20-27.40) and 6.15 (3.90-18.90) ng/ml, respectively, compared with patients who did not develop acute GVHD, with median (range) value of 0.30 (0.10-1.70) and 0.20 (0.10-2.80) ng/ml, respectively; the P value was significant (P=0.029 before transplant and P=0.001 after transplant; Fig. 1).

Patients who developed graft failure had higher plasma Ki-67 level after HSCT compared with patients who did not develop this complication (median (range) value of 5.25 (3.90-6.60) ng/ml compared with 0.20 (0.10-18.90) ng/ml), with a significant P value (0.026). However, their plasma Ki-67 levels before HSCT did not show statistically significant difference, with P value of 0.53. (median (range) value of 5.65 (0.20-11.10) ng/ml compared with 0.30 (0.10-27.40) ng/ml).

Receiver operating curve

Plasma Ki-67 levels of greater than or equal to 6.4 ng/ml before transplantation (75% sensitivity and 100% specificity) and greater than or equal to 3.35 ng/ml after transplantation (100% sensitivity and specificity) were proposed for detection of occurrence of acute GVHD (Table 2).

Figure 1



Plasma Ki-67 level before and after hematopoietic stem cell transplantation (HSCT) in relation to incidence of acute graft-versus-host disease (GVHD). This figure shows that patients who had acute GVHD during the first month of HSCT had higher level of Ki-67 before and after HSCT, with median (range) of 15.85 (0.20-27.40) and 6.15(3.90-18.90) ng/ml, respectively, compared with patients who did not develop acute GVHD, with median (range) values 0.30 (0.10-1.70) and 0.20 (0.10-2.80) ng/ml, respectively; the P value was significant (0.029 and 0.001, respectively).

Table 2 Predictive Performance of Ki-67 for acute GVHD occurrence

Test Result Variables (s)	Area under curve	P Value	95% Confidence Interval		Cutoff value	Sensitivity (%)	Specificity (%)
			Lower Bound	Upper Bound			
Ki 67 before SCT	.833	.030	.545	1.122	6.4	75	100
Ki-67 after SCT	1.000	0.001	1.000	1.000	3.35	100	100

Discussion

Allogeneic HSCT is the treatment of choice for treatment of patients with high-risk features of acute leukemia or those who relapsed after standard treatment. Compared with autologous transplantation or consolidation chemotherapy, allogeneic HSCT has a higher potential for complications, with particular difficulty arising from regimen-related toxicity, infection and GVHD, but offers the therapeutic potential of graft-versus-leukemia (GVL) effect [7]. However, these transplant-related morbidity and mortality complications are considerably decreasing now owing to updates in selecting donors, conditioning regimens, early diagnosis and treatment of life-threatening conditions [10].

Ki-67 is a nuclear antigen, and its expression is of prognostic importance in a variety of cancers. It is an excellent operational marker to determine the growth fraction of a given cell population. Numerous studies have been performed to examine the usefulness of this marker in various types of malignant neoplasms.

The aim of this work is to measure the plasma level of the proliferation marker Ki-67 in patients with acute leukemia who undergo HSCT and correlate it with other clinical and laboratory data and to assess its clinical and prognostic value in such patients.

The current study included 40 adult patients with acute leukemia who underwent allogeneic HSCT. The most observed adverse effects in our study were vomiting, diarrhea and neutropenic fever, which were seen in all patients. This is in accordance to the incidence in literature at 82%, with a median duration of 5–6 days [11]. Symptoms of acute GVHD were seen in four (10%) patients compared with incidence in literature (35–50%). [12] This large difference may be because of shorter follow-up duration, as we only followed up patients for the first 30 days after HSCT; however, acute GVHD may occur during the first 100 days after HSCT. Graft failure was seen in two (5%) cases; this was in accordance with the literature incidence (5.4%) [13].

When we studied the relation between recipient ages with the outcome of HSCT, we found that age was significantly negatively correlated with the day of engraftment. These results are in accordance with Gonçalves *et al.*, [2] who studied the factors that influence the success of cell transplantation, including the relation between patients' age and the day of engraftment. They found that neutrophil engraftment was earlier in patient aged greater than 50 year old compared with patient less than 29 year old, with median time of engraftment of 11 days and 19 days, respectively.

Acute GVHD was seen more in younger patients, with mean age of 21.75±2.87 years, and lesser in older age group of patients, with mean age of 31.53±8.97 years. Similar to our findings, Weisdorf *et al.* [14] analyzed the factors associated with acute GVHD following allogeneic bone marrow transplantation and found in univariate analysis that patient or donor age less than or equal 18 years was significantly associated with increased GVHD risks versus patients older than 18 years, without incremental risk in older adults.

We found that there was a significant difference between the 3 diagnoses groups regarding the day of engraftment, being earlier in ALL. However, there were no significant differences between the three diagnoses groups regarding acute GVHD, vomiting, diarrhea and graft failure. These results were in accordance with other studies [2–16].

To our knowledge, this is the first study to explore the prognostic significance of proliferation marker in transplantation setting. However, the prognostic importance of Ki-67 proliferation marker has been studied in various types of solid and hematologic malignancies. In breast cancer, Inwald *et al.* [17] have categorized Ki-67 tissue expression into five categories and found that Ki-67 was an independent prognostic parameter for both disease-free survival (Ki-67 >45%; P=0.001) as well as for overall survival (P=0.002), independent of common clinical and histopathological factors.

In non-Hodgkin lymphoma, Broyde *et al.* [15] found that Ki-67 proliferation index (PI) was correlated with clinical course and outcome and suggested that the mean Ki-67 PI differs by type of lymphoma. A cut-off value of 45% can help differentiate indolent from aggressive disease. In diffuse large B-cell lymphoma, a cut-off value of 70% can distinguish patients with a good and bad prognosis when combined with other prognostic factors.

In 2010, Bruey *et al.* [18] described an electrochemical-based enzyme immunoassay which detects Ki-67 in plasma. This was the first report of an assay that monitors circulating levels of this protein. Detection in plasma would be advantageous, as this would circumvent the need for costly and invasive biopsy procedures, and the quantification of plasma Ki-67 levels has already become an important aspect in

monitoring the progress of human neoplasms. Additionally high degree of proliferation and elevated levels of Ki-67 have been reported in patients with ALL, Burkett's lymphoma and non-Hodgkin's lymphoma [5].

Bruey *et al.* [18] have studied the circulating level of Ki-67 in patients with ALL compared with healthy controls. They found that significant higher levels of plasma Ki-67 in patients with newly diagnosed ALL than in healthy control. Moreover, elevated plasma Ki-67 was associated with significantly shorter survival in patients with ALL. They also suggested that Ki-67 can be detected in circulation and has potential for use as a biomarker for predicting clinical behavior in ALL.

In our study, we found a significant positive correlation between plasma Ki-67 level before and after transplantation and the day of engraftment; the higher the Ki-67 level, the more delayed the engraftment.

There was a statistically significant prognostic value for plasma Ki-67 level to detect the incidence of acute GVHD, with 75% sensitivity and 100% specificity before transplantation at cut-off value of greater than or equal to 6.4 ng/ml, whereas at cut-off value of greater than or equal to 3.6 ng/ml after transplantation had 100% sensitivity and specificity.

Patients who developed graft failure had also significantly higher Ki-67 level after HSCT compared with patients who had engraftment, but its level before HSCT was not significantly higher in such patients.

We think that higher plasma Ki-67 level before transplant may be related to residual leukemic cells as it reflects PI of malignant cells, and this may explain poorer outcome for patients with higher Ki-67 before transplant, with higher incidence of transplant-related complications and graft failure.

On the contrary, Ki-67 after transplantation may correlate with proliferation of normal engrafting cells during period of engraftment that return to normal level after complete engraftment. This explains higher level for graft failure may be either owing to continuous proliferation of residual recipient cells that lead to delayed engraftment or even graft failure or owing to excessive proliferation of donor cells, which in turn may explain higher incidence of acute GVHD during early post-transplant period.

We think further investigation with correlation with chimerism may explain if this excessive proliferation is owing to the patient's own clone of HSCs or donor-related HSCs.

We also studied the relation between circulating Ki-67 level in plasma and different diagnoses of acute leukemias among the forty patients included in the study. There was a significant difference regarding Ki-67 level before HSCT and different diagnoses, with lowest level in patients with ALL then AML and highest level seen in MPAL. However, there was no significant difference regarding Ki-67 level after HSCT. This explains that before HSCT procedure, the proliferation marker is affected by the type of leukemia, which may be owing to minimal residual cells before transplantation, and after successive engraftment, there is no more relation between the leukemic subtype and the proliferation marker level as these cases became almost cured.

Conclusion

There was a statistically significant positive correlation between plasma Ki-67 level before and after transplantation and the day of engraftment and duration of neutropenic fever. We also found a statistically significant positive correlation between circulating Ki-67 level and incidence of acute GVHD, where a cut-off value of greater than or equal to 6.4 ng/ml) before HSCT had 75% sensitivity and 100% specificity and a cut-off value of greater than or equal to 3.6 ng/ml after HSCT had 100% sensitivity and 100% specificity for the detection of occurrence of acute GVHD.

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Conflicts of interest

There are no conflicts of interest.

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