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ORIGINAL ARTICLE

Assessment of CD34 using immunohistochemistry in bone marrow biopsies of acute myeloid leukemia patients.

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Abstract

Background: Acute myeloid leukemia (AML) is a heterogeneous hematopoietic tissue neoplasm. Response assessment is evaluated by undergoing bone marrow examination on day 28 from initiation of therapy. **Patients and methods:** 60 newly diagnosed AML cases were. Blast percentage obtained in bone marrow biopsy using CD34 immunohistochemistry on day 28 after induction chemotherapy compared with that obtained in bone marrow aspirate smears and flow cytometry. In addition, counting of immature CD34 positive clusters and evaluation of bone marrow microvessel density (MVD) and relating them to prognosis were performed.
Results: Blast percentage in bone marrow aspiration smears and CD34 immunohistochemistry was discordant in 21.7% with a statistically significant difference. While, discordance between flow cytometry and CD34 immunohistochemistry was 13.3 % with no statistically significant difference. upstaging by CD 34 immunohistochemistry had a statistically significant shorter OS, DFS and EFS. In addition, immature CD34 clusters found to have a significant effect on remission rate and MVD suggested to be important in predicting relapse.
Conclusion: Assessment of response to chemptherapy using CD34 immunohistochemistry provides more accurate method for counting blast cells with subsequent reinduction therapy decision. In addition, immature clusters and MVD could be considered as prognostic factors of AML patients

Keywords: CD34, Acute myeloid leukemia, Immunohistochemistry, Bone marrow biopsy, Survival.

INTRODUCTION

Acute myeloid leukemia (AML) is a complex hematopoietic cellular neoplasm characterized by clonal expansion of immature myeloid cells in the bone marrow, and peripheral blood with uncontrolled proliferation and impaired differentiation program of the affected cells. Although 50–75% of patients with AML have an excellant response to chemotherapy, relapse represents the major cause of treatment failure [1].

Early assessment of response to treatment after induction chemotherapy regarding actual blast count is one of the major tools to assess achievement of complete remission and prediction of survival outcomes of treated AML patients [2,3]. In the updated WHO guidelines [4] cytomorphology is still the gold standard method for counting of blast cells, despite the inaccuracy in manual counting. In addition, some studies has established the limitation for the assessment of bone marrow blasts morphologically from smears after induction chemotherapy and reported that flow cytometry (FCM) and immunohistochemistry (IHC) techniques can improve the sensitivity of blast cells counting as they are more reproducible than cytomorphological assessment on day 14 [5,6]. Bone marrow biopsy provides better information as regard cellular localization and bone marrow microenvironment [7], Orazi highlighted the importance of a bone marrow trephine biopsy at diagnosis and on follow up of AML cases for a better assessment of blast cell count and creation of a baseline picture for later comparisons and exclusion of a minimal residual disease [8]. CD34 is a cluster of differentiation described as a cell surface glycoprotein [9]. It acts as a cell adhesion molecule to other cells and mediate attachment of bone marrow stem cells to the stromal cells or extracellular matrix [10]. Microvessel density (MVD), determined by using CD34 on bone marrow biopsy, is a measure of angiogenesis state and is increased in AML patients in comparison with healthy individuals [11]. Angiogenesis can predict AML

aggressiveness and the patient outcome [12]. In our study, we assessed blast count on day 28 after initial induction chemotherapy by using anti CD34 antibody for immunohistochemistry on bone marrow biopsies and compared it with the blast count detected by FCM or cytomorphology on bone marrow aspiration smears. We also studied the effect of the discordance between IHC and cytomorphology on treatment response and survival outcomes of AML patients. Secondarily, we evaluated bone marrow MVD and immature CD34 positive clusters and relating them to prognosis.

PATIENTS AND METHODS

This is a prospective study that carried out in department of Clinical Pathology, Faculty of Human Medicine, Zagazig University, during the period from August 2016 to November 2019. A total of 60 newly diagnosed AML patients that were CD34 positive by FCM, were included in the study. They were 39 males & 21 females and their ages ranged from 18 to 60 years.

Samples: Peripheral blood (PB) and bone marrow (BM) samples were collected from all patients at the time of presentation before therapy was initiated. Venous blood samples were aseptically withdrawn from each patient. One ml of the sample was delivered into a sterile container containing EDTA for complete blood count (CBC) examination. 1.5 ml of the BM were aspired from each patient in EDTA vacutainer tube for immunophenotyping. Also, leishman stained BM smears were prepared and examined. Another Bone marrow aspirates with immunophenotyping for CD34 and bone marrow trephine biopsies with CD34 IHC were obtained and evaluated in all cases on Day 28.

Treatment plan: Patients were treated by an induction 3+7 regimen, consisting of continuous infusion of cytarabine (100 mg/m^2) daily for 7 consecutive days combined with 3 days of doxorubicin (30 mg/m^2). Patients having poor performance status were treated by 2+5 (cytarabine 100 mg/m^2) daily for 5 combined with 2 days of doxorubicin (25 mg/m^2) regimen of low dose cytarabine (10 mg/m^2/12 h) for 14 days. Patients who achieved complete remission received consolidation therapy which is composed of three to four courses of high-dose cytosine arabinoside (3 g/m^2 every 12 h on days 1, 3 and 5; total, 18 g/m^2)

Response to therapy and survival outcomes : Complete remission (CR) was characterized by morphologically normal marrow with less than 5% blasts, neutrophil count more than 1.5 x10^9/L, and platelet count more than 100 x10^9/L. Relapse was defined as more than 5% leukemic blasts in the BM aspirate or new extra medullary leukemia. Overall survival (OS) was measured from the time of diagnosis until the date of death regardless of the cause. Event free survival (EFS) was defined as the time from diagnosis to treatment failure, disease relapse or death by any causes. Disease-free survival (DFS) was estimated from the time of first CR to relapse or death in CR. Patients who didn't reach the endpoint of follow up as being lost or didn't express the event were considered as censored.

Patients' follow-up: Bone marrow aspiration was performed on day 28 after receiving induction chemotherapy to evaluate morphological remission. Patients were followed once every 3 months with clinical examination and complete blood cell counts. Marrow examination was done if there was any doubt of a relapse on clinical examination or blood smear. The patients were followed up for two year to evaluate OS, EFS, and DFS.

Methods: Participants enrolled in the study were subjected to the following: full history taking, clinical examination, complete blood count, bone marrow aspiration & examination, and immunophenotyping by flow cytometry using Becton Dickenson FacsCalibar device , San Diego, USA, to detect the following markers: MPO, CD13, CD33, HLADR, TDT, CD14, CD64, CD34, CD3, CD20 and CD22. Bone marrow aspiration, immunophenotyping for CD34 and Bone marrow biopsy with CD34 IHC were performed on day 28 and evaluated for the presence of blast cells. Also, MVD and immature CD34+ clusters were evaluated.

CD34 immunohistochemistry: The procedure was carried out manually according to the manufacturer's instructions: Antigen retrieval on slides done by putting them in citrate buffer (PH 6.6) for 15 minutes in a steamer at 95 C, then the slides were submerged in peroxidase blocking solution for 10 minutes and washed with buffer to remove excess solution. Primary antibody (Thermo Scientific CD34 Ab-1, Clone no. QBEnd/10, Catalogue no. MS-363-PO and, lot no. H02069) added to completely cover the tissue and incubated for 30 minutes at room temperature. The antibody binding was detected using poly HRP Conjugate (Reagent A) and incubated for 15 minutes, then rinsing with wash buffer was done. DAB substrate chromogen was added for detecting the reaction and prepared by addition of DAB Chromogen Solution (Reagent B1) to DAB Buffer Solution (Reagent B2) with mixing in a 1:1 ratio. Finally, counter staining with Mayer's hematoxylin for 5-10 seconds was performed.

Interpretation of results:

1-Determination of blast cells percentage on CD34 bone marrow biopsy sections was performed manually: CD34 positive cells were counted per 1000 nucleated cells using BX53 optical microscope imaging system (Oil immersion 100x objective, camera adaptor 0.5x), (CU30=3 megapixels, CellSense Ver.01.08) and calculated as percentage. The pattern of CD34 positivity is a granular brown cytoplasmic and membranous staining (Figure 1(A)). Positive control was performed by using endothelial cells as a positive internal control.

Morphological examination of Leishman-stained bone marrow smears was done, blast percentage per 500 WBCs were counted using 100x oil immersion lens objective (total magnification of 1000x) (Bx53 Olympus microscope, Japan) and compared with that obtained by CD34 immunohistochemistry.

2- Determination of CD34 positive immature clusters and their distance from endosteum in good and intermediate responder patients by CD34 IHC: Sections were observed randomly for 10 fields at x400 magnification (HPF) for the presence of CD34 positive doublets or clusters (3-5 immature precursors) away from the bone endosteum [14]. The distance of the clustered precursors from the bone endosteum was measured using BX53 optical microscope imaging system (CellSense Ver.01.08) (Using 20x and 40x objectives, camera adaptor 0.5x)(CU30=3megapixels) [13,14]. For detection of the precise distance between precursors and endosteum, computer image processing technology was Performed (CellSense Ver.01.08) (Figure 1 (B) & (C)).

3- Determination of MVD using CD34: The average MVD was counted in each patient in 10 consecutive microscopic fields at 20x objective (total magnification of 200x) and an average count of vessel density /HPF was calculated **[15].**

Ethical approval: The study was done according to The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans. The patients were informed about the nature and purpose of the study and informed written consent was taken from all the patients for the required investigations including bone marrow aspiration and biopsy. Patients were not exposed to any harm or risk and the patient's data was confidential. Also, approval of ethical committee in Faculty of Medicine was done.

Statistical analysis: Analysis of data was performed using SPSS computer program (version 20; SPSS Inc. Chicago, Illinois, USA). $\chi 2$-test and Mann–Whitney tests were used for statistical analysis. Kaplan-Meier method was used to estimate survival and the difference between groups was analyzed by the log rank test. Hazard ratio (HR) with its 95% confidence interval (CI) was used for risk estimation. The receiver operating characteristic curve (ROC) analysis was used to detect a best cut off value and pearson correlation was used for analysis of association between two quantitative continuous variables. A P value less than 0.05 was considered statistically significant, and $P<0.001$was highly significant.

RESULTS

Regarding laboratory characteristics of the patients, statistically significantly higher BM blasts, CD34 by FCM on D1 and immature cell clusters were detected in CD34 IHC high expression group than CD34 IHC low expression group. While, no statistically significant differences were detected between both groups as regards age, sex, WBCs count, hemoglobin, platelets and MVD or clusters distance. CD34 expression demonstrated non-significant heterogeneity among FAB subtypes of AML between both groups (P=0.12) (Table S1 and S2).

Comparison of blast percentage by bone marrow aspiration and the percentage of CD34-positive blast cells by IHC: When we compared both techniques, concordance was detected in 47 (78.3 %) of the cases. While both techniques were discordant in 13 (21.7 %) of the cases. IHC revealed a higher blast percentage than bone marrow aspiration in 12 out of 13 cases, and only one case had a higher blast percentage by aspiration technique (Table 1). A statistically significant difference was detected between both techniques (P= 0.001) (Table S3).

Comparison of percentage of CD34 positive blast cells by FCM and IHC techniques: By comparing them, both techniques were concordant in 52 (86.7%) of the cases. While discordance was detected in 8 (13.4%) of the cases. IHC revealed a higher blast percentage than FCM in 5 cases out of 8 and 3 cases had a higher blast percentage by FCM (Table 2). No statistically significant difference was detected between both techniques (P=0.193) (Table S4).

Prognostic impact of higher blast percentage by CD34 IHC on bone marrow biopsy in comparison to bone marrow smears at D28 after induction: We studied the effect of higher blast percentage by IHC in predicting treatment outcome of AML patients. We assessed the CR rate of the 43 patients who were described as good responder with blast count ≤5% morphologically and we divided them into 2 groups, one group described as good responder by both

cytomorphology and IHC. While the second group was upstaged by IHC with blast count >5%. CR rate was not statistically significantly different in both groups (P=0.06). While relapse rate was statistically significantly higher in IHC upstaged group (P=0.02). Regarding survival rates, including OS, EFS and, DFS, we applied Kaplan meier survival analysis to evaluate them. Higher blast percentage by IHC was associated with a statistically significant shorter OS, EFS and, DFS (P= 0.002, 0.001, and 0.014; respectively) (Table 3 and Figure 2).

Effect of number of immature CD34 positive clusters and micro vessel density on CR and relapse rates:

A higher number of immature CD34 positive clusters had a statistically significant effect on CR achievement (p=0.001). While, this was not significantly associated with higher relapse rate (p=0.06). While, their distance from endosteum neither affect CR or relapse rate. In contrast, regarding micro vessel density, a higher MVD value didn't have a statistically significant effect on CR rate (p=0.38). While, it was significantly associated with higher relapse rate (p=0.03) (Table 4 & 5). By applying the receiver operating characteristic curve for determination of the optimal cut off value of MVD for predicting relapse we used was ≥6.95/HPF with sensitivity 80%, specificity 50%, PPV 53.3, NPV 77.8, and accuracy 62.5% (Table S5 and Figure S1). Also, by applying spearman correlation, micro vessel density showed a strong positive correlation with D28 CD34 IHC but did not reach a significant level (r=0.666,P=0.093)(Figure S2).

Multivariate analysis using Cox regression model (HR) for survival analysis was done. Multivariate modeling including D28 CD34 IHC, D28 CD34 FCM, number of immature clusters, their distance from endosteum, MVD, BM blasts, FAB, and cytogenetic risk was designed. CD34 by IHC or FCM, and MVD were independent prognostic factor which significantly affects OS, EFS and DFS in the AML group. While, immature clusters was significantly affect OS and EFS only (Table S6).

Table 1: Comparison of blast percentage by cytomorphology and CD34 IHC on bone marrow biopsy (D-28):

BM blasts %	Percentage of CD34 positive cells by IHC			Total
	≤5 %	>5 <20%	≥ 20%	
≤5 %	32 (53.3%)	11 (18.3%)	--	43 (71.6)
5-20 %	1 (1.7%)	8 (13.3%)	1 (1.7%)	10 (16.7)
≥ 20 %	--	--	7 (11.7%)	7 (11.7)
Total	33	19	8	60

Table 2: Comparison of percentage of CD34 positive blasts by FCM and IHC (D-28):

Percentage of CD34 positive cells by FCM	Percentage of CD34 positive cells by IHC			Total
	≤5 %	>5 <20%	≥ 20%	
≤5 %	31 (51.7%)	--	--	31 (51.7)
5-20 %	2 (3.3%)	18 (30%)	5 (8.3%)	25 (41.6)
≥ 20 %	--	1 (1.7%)	3 (5%)	4 (6.7)
Total	33	19	8	60

Table 3: Comparison of the group with ≤5% blasts on bone marrow aspiration smears as well as CD34 IHC to the group with ≤5% blasts on bone marrow aspiration and >5% blasts on CD34 IHC regarding remission, relapse and survival rates:

	≤5 % by both techniques (no.= 32)	≤5 % by BMA and >5 % by IHC technique (no.= 11)	P value
CR # [no. (%)]	30/32 (93.8 %)	8/11 (72.7 %)	**0.06**
Relapse # [no. (%)]	2/30 (6.7%)	3/8 (37.5%)	**0.02**
2 years OS Mean (95% CI) (Months) Percent probability HR	15(10-20.4) 81.3 % 2.2 (20.19 - 23.9) 36.4 % 12.8 (6.7-18.9) 36.4 %		**0.002**

	≤5 % by both techniques (no.= 32)	≤5 % by BMA and >5 % by IHC technique (no.= 11)	P value
2 years EFS Mean (95% CI) (Months)Percent probability HR	22.9 (21.2 - 24.6)	81.3 %	**0.001**
2 years DFS Mean (95% CI) (Months) Percent probability HR	22.16 (19.9 -24.5) 15.3 (8.8-21.7)	% 50 %86.7	**0.014**

P<0.05, significant; P<0.001, highly significant; #, χ2-test.

Table 4: Effect of immature CD34 + clusters and micro vessel density on remission status:

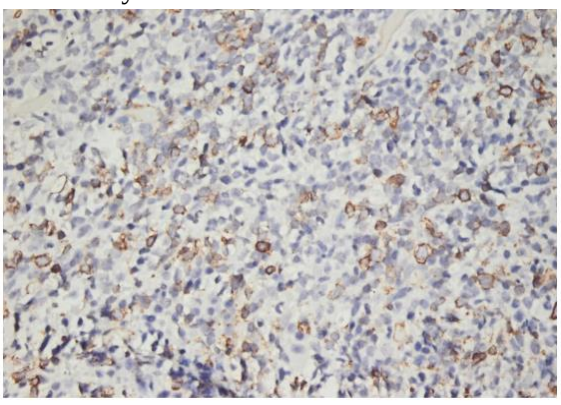
	CR (no.= 38)	No CR (no.= 5)	P
No. of immature CD34 +clusters * Median (range)	0 (0-7)	8 (0-12)	**0.001**
Distance * Median (range)	599.42 (420.52-880.57)	527.76 (316.99-799.21)	**0.07**
Micro vessel density * Median (range)	10 (1.4-61)	18.8 (1.9-40)	**0.38**

*, Mann Whitney test

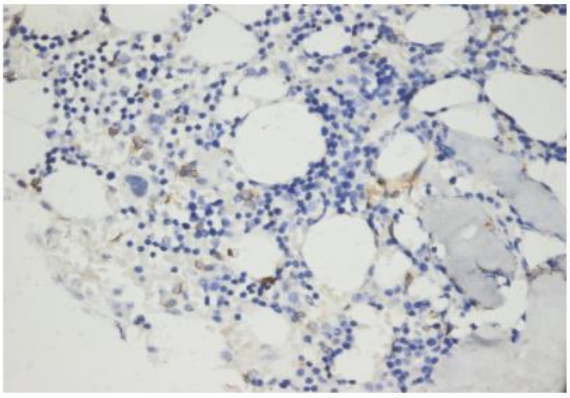
Table 5: Effect of immature CD34 + clusters and microvessel density on relapse rate

	Relapse (no.= 5)	No relapse (no.= 33)	P
No. of immature CD34 +clusters * Median (range)	5 (0-6)	0 (0-7)	**0.06**
Distance * Median (range)	690.32 (505.1-880.57)	612.77(420.52-790.51)	**0.09**
Micro vessel density * Median (range)	20 (10.6-61)	8.5 (1.4-43)	**0.03**

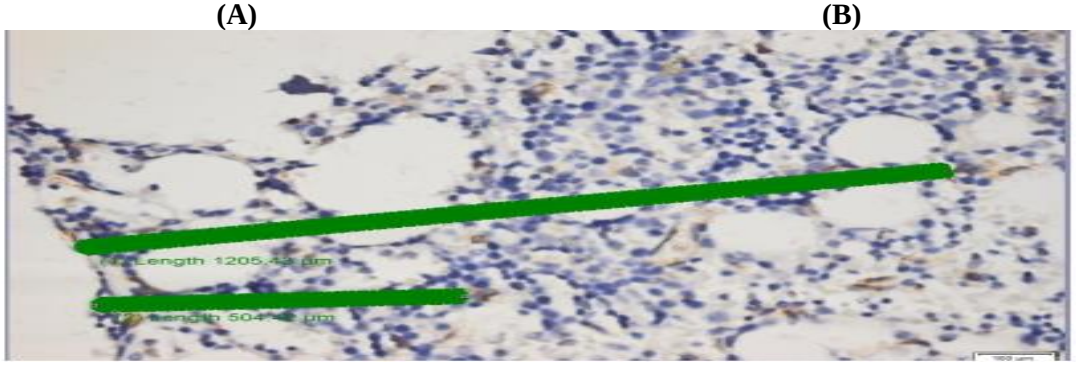
*, Mann Whitney test



(A)



(B)



(C)

Figure 1: (A) Trephine biopsy (CD34 IHC) showing AML M4 with 45% CD34 +ve immature cells (x40). (B) Trephine biopsy (CD34 IHC) showing AML M1 with a cluster of immature cells (x40). (C) Trephine biopsy (CD34 IHC) of AML M1 showing one doublet of CD34 positive immature cells at 1205.43µ and another doublet at distance of 504.42µ from the trabecular bone (x40).

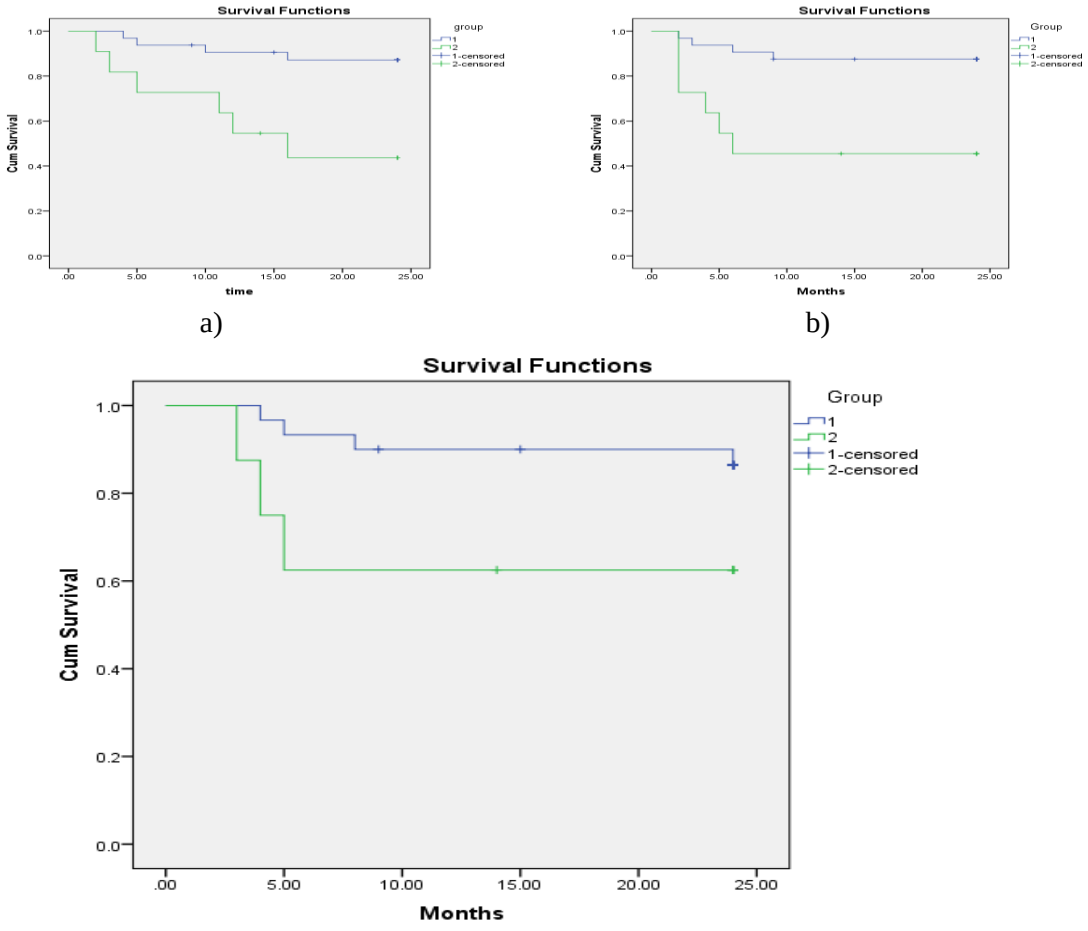


Figure 2: Kaplan–Meier curve shows probability of (a) Overall survival, (b) Event-free survival, and (c) Disease-free survival for the group with <5% blasts by cytomorphology and and CD34 IHC and CD34 IHC upstaged group.

DISCUSSION

Assessment of bone marrow blast percentage after initial induction chemotherapy is very important in the determination of survival outcomes of AML patients. Here, in this study, we assessed the possible prognostic impact of blast count on day 28 after initial induction chemotherapy on the outcome of AML patients. We assessed that either morphologically or immunologically by using CD34 IHC and FCM for the detection of CD34 positive myeloid blast cells. Also, other studies reported the value of early blast clearance after induction chemotherapy to determine prognosis individually of AML patients [2,16]. In the past, IHC was not recommended because of the inconsistent results, but with improved antigen retrieval methods and staining techniques, this concept had been changed and IHC on bone marrow biopsies became a major diagnostic and prognostic tool of AML patients [17]. In our study, we compared the blast count detected by using CD34 IHC on bone marrow biopsies on day 28 after induction chemotherapy with that detected using leishman stained bone marrow smears and flow cytometry. Patients were divided into 3 groups according to the blast count detected morphologically or immunologically using anti CD34 Ab as follows: ≤5% (good responder), >5 <20% (intermediate responders) and ≥ 20% (poor responders). The distribution of the patients according to the blast count on bone marrow smears and CD34+ blast cells by IHC on bone marrow biopsies represents 47 out of 60 patients lies within the same blast range, CD34 IHC revealed a higher blast percentage in 12 patients. Immature cell clusters were detected in 10 out of 12 patients by IHC technique. This may be highlighted the importance of immature clusters for early response assessment of AML patients. Also, 3 patients out of 12 had more than grade 2 bone marrow fibrosis. Bone marrow fibrosis together with unequal blast distribution may contribute to the lower blast percentage by aspiration technique and this can add a value for

IHC over bone marrow aspiration technique. While a higher blast percentage by bone marrow aspiration was detected in only one patient. A statistically significant difference was detected between both methods. A close result reported by Jain et al. [18], who assessed the blast count early after induction chemotherapy on day 14. This study reported 81 out of 100 patients (81%) that were within the same range by both techniques. While discordance was detected in 19 cases with a higher percentage detected by IHC than cytomorphology. A diluted bone marrow aspirate sample was obtained in 15 out of 19 cases and only one case showed sheets of CD34+ blast cells. A statistically significant difference was reported between both methods. So, CD34 IHC on bone marrow biopsies on day 14 improved the detection of any residual blast cells in bone marrow when compared with the cytomorphological assessment of bone marrow aspirate for follow up of treated AML patients[19]. In agreement with our results, other studies reported that the count of CD34 + blast cells by IHC in patients with MDS was slightly higher than the blast percentage that counted morphologically in bone marrow smears, which subsequently resulted in change of MDS classification [20-22]. While, in contrast to our results, other studies reported high concordance for positive and negative results for blast cells detected by IHC when compared to bone marrow smears [23,24]. Differences in blast count between IHC and cytomorphology may be attributed to the presence of CD34 immature clusters and the uneven distribution of CD34 blast cells, both of which are easily detected by IHC on bone marrow biopsies [22]. In our study, the patient's distribution according to CD34+ blast cells by FCM and IHC represented 52 out of 60 patients were within the same blast range. IHC was higher in 5 out of 60 patients. This is likely to be explained by unequal distribution of blast cells in bone marrow and the presence of immature CD34+ clusters that could be easily detected by IHC [22]. Also, IHC may be more accurate than FCM or cytomorphology technique as bone marrow aspirate is sometimes diluted on day 28 due to the hypoplastic effect of chemotherapy on bone marrow. So, in such samples, FCM is usually difficult to detect the actual blast count. While IHC using CD34 on bone marrow biopsies would be easily performed for more accurate evaluation of real marrow cellularity and CD34+ blast cells count [18], and subsequently, this can prevent a repeated aspiration procedure [29]. Only three cases had higher percentages by FCM than IHC technique with similar results for blast percentages in bone marrow smears, this may be attributed to weak antigen expression on blast cells [19], no statistically significant difference was detected between both methods. In agreement to our results, other studies reported a high concordance between FCM and IHC for CD34+ blast cells for both positive and negative results [23-27]. These findings may indicate that bone marrow biopsies detect higher blast percentages in some cases that may be more difficult to be detected by bone marrow smears or FCM technique [28]. In addition, other studies reported that CD34 in AML and MDS patients increase diagnostic accuracy [21]. Assessment of bone marrow on day 28 has an important role in predicting the remission rates of the patients. We evaluated the CR rate in 43 patients who were described as good responders on day 28 with ≤5% blast cells detected morphologically by bone marrow aspiration technique. These patients were subsequently divided into 2 groups. One group (32 patients) with blast cells ≤5% by both bone marrow aspiration and IHC techniques. The other group (11 patients) with blast count ≤5% by bone marrow aspiration technique and >5% by CD34 IHC on bone marrow biopsies. CR rate in the first group was achieved in 30 out of 32 patients (93.8 %). So, only 2 patients didn't achieve CR or showed CBC recovery, failure of CR achievement was persistent after reinduction for these 2 patients. While, in the second group, CR was not achieved after initial induction chemotherapy, but with reinduction, CR was achieved in 8 out of 11 patients (72.75%). A higher CR rate was reported in the first group, but this was not statistically significant (P=0.06). Actually, cytomorphology can't be completely replaced with either FCM or IHC but if FCM and IHC revealed a higher blast percentage after initial induction. So, this must be taken into consideration for proper decision making of reinduction. In agreement to our results, Jain et al. [18] reported higher CR rate in their first group but also with no statistically significant difference between both groups regarding CR rate, which was 77.6% in the first group and 66.7% in the second group (P=0.506). So, persistent disease after initial induction detected by IHC may affect the remission rate. CD34 IHC is better than morphological assessment of blast count and thus, it can be used as a major tool for picking up a group of patients who may get a treatment benefit with reinduction of chemotherapy. Regarding survival rates, the patient group who was upstaged by IHC over bone marrow smears, was associated with poor survival rates in AML patients. 2 years OS, EFS and DFS were statistically significantly shorter in

this patient group when compared with the good responder group (P= 0.02, 0.001, and 0.014; respectively). This finding was also reported by Saft et al. [22] who collect OS data for all MDS patients and reported that higher blast percentages in bone marrow histology in comparison to cytomorphology is associated with shorter 2 years OS than the good responder group as it was 10% and 55% for both groups; respectively (P<0.0001). Also, kern et al. [2] used cut off 10% for blast percentage on day 16 and found a statistically significant effect of higher blast count by IHC on RFS (P=0.0049) and OS (P=0.0068) and reported that this count represents an assessment of in-vivo sensitivity to chemotherapy. Regarding the effect of number of immature CD34 + clusters, their distance, and MVD on CR and relapse rates, we found that the number of immature clusters had a significant effect on CR rate with no significant effect on relapse rate. While their distance from endosteum didn't affect CR or relapse rates. Also, other studies reported that immature CD34+ cluster is a poor prognostic factor that affects CR and survival rates including OS, DFS and RFS in AML patients [27,30]. Our result differs from that of Yu et al. [14] who suggested that clustered precursors are associated with leukemic relapse. They stated that clustered precursors in relapsed patients were significantly higher than those in non-relapsed patients (P = 0.0075). These discrepancies in the results may be due to the different size of the studied cases. Therefore, the power of our study may be increased by inclusion of a larger number of AML patients. On the other hand, MVD had a significant effect on the relapse rate without a significant effect on CR rate. Jothilingam et al. [31] assessed MVD in a control group and stated that MVD was significantly higher in leukemia population when compared to controls (P< 0.001). Also, Kuzu et al. [32], stated that higher MVD was associated with poor prognosis and short overall survival in patients with AML. Furthermore, evaluation of MVD at presentation will be helpful in confirmation of its prognostic value [32]. In addition, our study showed that MVD was a strong predictor of relapse in AML patients. ROC curve analysis showed the best cut off value of MVD in prediction of relapse is ≥6.95 / HPF with sensitivity of 80% and specificity of 50%. This result encourages us to support the concept of assessing MVD on post induction bone marrows and using it as a predictor of disease prognosis. In the present study, there was a strong positive correlation between CD34 IHC and MVD assessed at D28 post induction chemotherapy. This result coincides with results reported by Padro et al. [33], who stated that patients with at least 5% residual leukemic blast cells at day 16 of induction had higher micro vessel count than patients without blast infiltration of the bone marrow and differ significantly from micro vessel count at time of complete remission. This may reflect angiogenic effect of residual blast cells on bone marrow endothelial cells. Several cytokines are produced by blast cells and induce migration and proliferation of endothelial cells e.g vascular endothelial growth factor [34]. So, evaluation of both immature clusters and MVD should be taken into consideration when performing CD34 IHC on bone marrow biopsies after initial induction to add a prognostic value for AML patients. Recently, a study is currently being performed to establish the standards for the accurate counting of CD34 + blast cells in bone marrow by members of European bone marrow working group [35].

CONCLUSION

Cytomorphology can't provide accurate counting of blast cells in bone marrow in 100% of AML patients and CD34 IHC on bone marrow biopsies can assist blast cell count after induction, especially those with fibrosis or hypocellular bone marrow, in which blast count is often underestimated. Also, IHC allows identification and counting of CD34 + immature cell clusters, which are found to have a prognostic impact on CR rate and allows measurement of MVD which is a predictor of relapse. So, we recommend the combination of the three methods for accurate counting of bone marrow blasts, and this can provide a prognostic value.

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Table S1: Demographic and laboratory characteristics at day 28 CD34 expression groups by IHC analysis

Characteristics	CD34 IHC low expression group (≤5%) at D-28 (no.=33)	CD34 IHC high expression group (>5%) at D-28 (no.=27)	P value
Sex (no.): # Male Female	 20 13	 19 8	**0.81**
Age (years): * Median Range	 47 (18-58)	 50 (25-60)	**0.32**
WBCs: ($x10^9$/L) * Median Range	 16 (1.9-120)	 7.3 (1.1-64)	**0.127**
Platelet: ($x10^9$/L) * Median Range	 65 (10-120)	 50 (12-299)	**0.749**
Hemoglobin (gm/dl) * Median Range	 7.8 (5.7-9.9)	 8 (6-10.1)	**0.422**
BM blasts (%): * Median Range	 45 (21-91)	 73 (42-97)	**0.005**
AML FAB subtypes: # M1 M2 M4 M5a M5b M7	 1 9 13 6 3 1	 2 7 11 4 2 1	**0.12**
CD34 expression by flow cytometry in Day 1: * Median Range	 34.3 (26.8-81.9)	 66.7 (40.6-89.6)	**0.001**

WBCs, white blood cells; BM, bone marrow; P<0.05, significant; P<0.001, highly significant; *, Mann Whitney test; #, χ2-test.

Table S2: Micro vessel density, immature CD34+ clusters and their distance at day 28 CD34 expression groups by IHC analysis

Characteristics	CD34 IHC low expression group (≤5%) at D-28 (no.=33)	CD34 IHC high expression group (>5% <20%) at D-28 (no.=19)	P value
Micro vessel density/HPF: * Median Range	 8.5 (2-43)	 10 (1.4-61)	**0.** **694**
Immature CD34 + clusters: (no.) * Median Range	 6 (0-8)	 7 (0-12)	 **0.001**
Distance: * Median Range	 729.59 (578.61-880.57)	 472.81 (316.99-628.63)	**0.19**

P<0.05, significant; P<0.001, highly significant; *, Mann Whitney test; #, χ2-test.

Table S3: Comparison of blast percentage by cytomorphology and CD34 IHC on bone marrow biopsy (D-28)

BM blasts %	Percentage of CD34 positive cells by IHC		P #
	≤5 %	>5%	
≤5 % >5 %	32 (53.3%) 1 (1.7%)	11 (18.3%) 16 (26.7%)	0.001

#, χ2-test

Table S4: Comparison of percentage of CD34 positive blasts by FCM and IHC (D-28)

Percentage of CD34 positive cells by FCM	Percentage of CD34 positive cells by IHC		P #
	≤5 %	>5%	
≤5 % >5 %	31 (51.7%) 2 (3.3%)	-- 27 (45%)	0.193

#, χ2-test

Table S5: Performance of micro vessel density in prediction of relapse in patients with AML.

Cut off	AUC	Sensitivity	Specificity	PPV	NPV	Accuracy	P
6.95	0.743	80	50	53.3	77.8	62.5	**0.046***

Table S6: Multivariate analysis of prognostic factors for OS, EFS and DFS

Variants	OS		EFS		DFS	
	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
CD34 IHC D28	2.2 (1.3-3.7)	**0.002**	1.9 (1.2-3.1)	**0.03**	2.1 (1.1-3)	**0.003**
CD34 by FCM D28	2 (1.2-2.6)	**0.03**	2.2 (1.2-3.1)	**0.001**	1.8 (1.4-2.6)	**0.04**
Immature CD34+ clusters	1.9 (1.7-2.2)	**0.02**	2 (1.4-2.6)	**0.002**	1.4 (0.8-1.7)	**0.08**
Distance	1.2 (0.6-1.4)	0.3	1 (0.7-1.3)	0.4	0.9 (0.8-1.2)	**0.4**
Microvessel density	2 (1.2-2.7)	**0.01**	1.8 (1.5-2.4)	**0.03**	1.9 (1.2-2.5)	**0.02**
FAB	1.2 (1-1.5)	0.3	1 (0.8-1.7)	0.1	1.5 (1-1.9)	**0.2**
Favorable cytogenetic	0.8 (0.6- 1.3)	0.4	0.9 (0.5-1)	0.3	0.7 (0.4-1.1)	**0.2**
Adverse cytogenetic	1.1 (0.8-1.5)	0.6	1.2 (0.6-1.4)	0.4	0.9 (0.7-1.3)	**0.5**
BM blasts	1.3 (1.1-1.9)	0.2	1.4 (1.1-1.7)	0.1	1.1 (0.9-1.5)	**0.4**

P<0.05, significant; P<0.001, highly significan

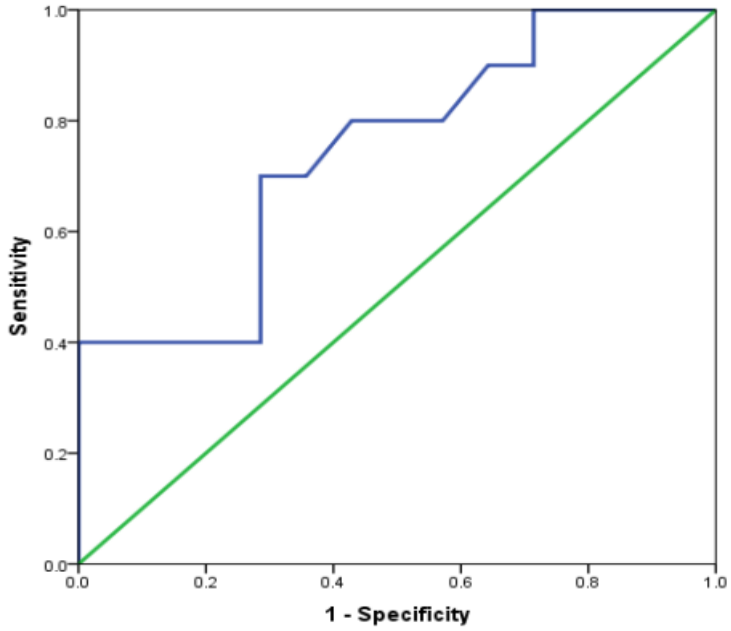

Figure S1: ROC curve showing the performance of vessel density in prediction of relapse in patients with AML.

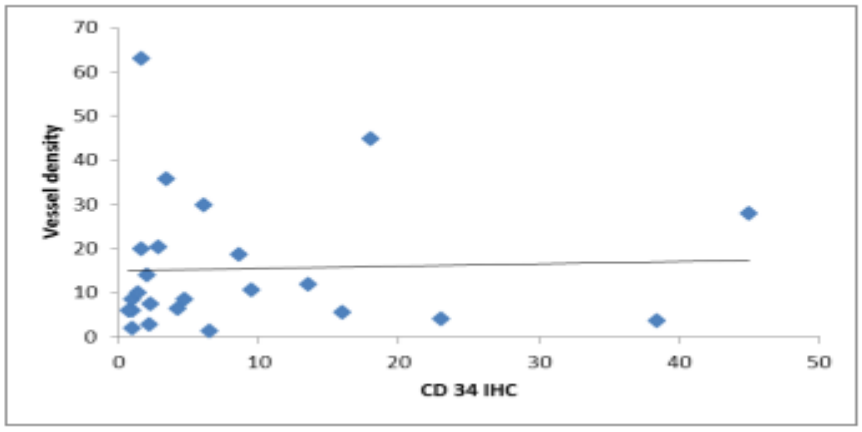

Figure S2: Scatter dot graph showing strong positive correlation between CD34 IHC and vessel density**.**

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