Original Article

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Morphometric Approach to Angiogenesis In Acute Leukemia: An Attempt to Bring Objectivity

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Abstract

Background:Angiogenesis is central to growth and development of all tissues, whether they are healthy or diseased. Angiogenesis has a key role in progression of tumor and in its metastasis and invasion. The role of angiogenesis in solid tumors is well established through various studies, but the same needs to be studied more extensively with respect to hematological malignancies. This could aid in prognostication and has therapeutic implications. The aim of the present study was to evaluate bone marrow microvessels in cases of acute leukemias by morphometry, for the purpose of providing objectivity, and study the angiogenic activity in these cases.

Methods:In this study, all retrospective and prospective cases of acute leukemias diagnosed on bone marrow biopsies over a period of two years, from 2018 to 2020, were included. A total of 46 cases and 27 controls were studied. Hematoxylin and Eosin (H&E) and CD34 stained bone marrow biopsies were reviewed. For quantification of microvessel density (MVD), three "hotspots" (areas with maximum number of microvessels) on bone marrow biopsy were identified by scanning at x100 power. Then, at x400 magnification, the field was set to cover the maximum number of microvessels within the hotspot, and microvessels were counted in one field in each of the three hotspots. MVD was reported as the mean of the three hotspots expressed as the number of microvessels per mm^2. The mean values of the measurements in the three fields were used for statistical analysis.

Result: The cases of acute leukemia were found to have significantly higher MVD as compared to the control group. Present study also found significant correlation of MVD with marrow fibrosis and blast percentage in peripheral blood. However, the mean MVD did not show any significant correlation with the bone marrow cellularity. A significant reduction in MVD was found post therapy in the remission cases, while MVD was still high in case with relapse.

Conclusion: There is a definite role of angiogenesis in pathophysiology of hematological malignancies. Assessment of bone marrow MVD by morphometry can help in lending objectivity to angiogenesis, which can aid in prognostication and therapeutics in cases of acute leukemias.

Keywords: *Leukemia, angiogenesis, microvessel density, morphometry*

Introduction

Angiogenesis is central to growth and development of all tissues, whether they are healthy or diseased. Angiogenesis has a key role in progression of tumor and in its metastasis and invasion. It is a highly regulated process which is mediated by angiogenic and anti-angiogenic factors.[1] For the tumor to grow, an essential requirement is nutrients and oxygen, which is provided by the new blood vessels that are formed by angiogenesis. So, it is evident that angiogenesis, is fundamental to tumor growth and spread, without which the tumor cannot grow beyond few millimeters. This being the reason for cancer cells to promote angiogenesis very early in the formation of a tumor.[1,2]

The role of angiogenesis in solid tumors is well established through various studies, but the same needs to be studied more extensively with respect to hematological malignancies, especially in different types of leukemias in adults as well as pediatric population as they form a major bulk of hematological malignancies. This could aid in prognostication and also has therapeutic implications.[3–6]

Various studies have been published till now, where angiogenic activity was calculated after immunostaining.

But this method has its own drawbacks of being subjective in selection of area for counting the microvessels. Standardization of the method of quantification of microvessels has still not been achieved, which affects the objectivity in interpretation and center to center variation arises.[7–9]

Only few studies have calculated angiogenic activity by morphometry, even fewer have compared multiple peripheral blood and bone marrow parameters with microvessel density to find correlations of the same.[7,10,11] The present study has focused on calculating angiogenic activity through morphometry and comparison with various factors was done to find relevant correlations.

The aim of the present study was to evaluate bone marrow microvessels in cases of acute leukemias, in the patients who were diagnosed on BM biopsy and study the angiogenic activity in these cases. This was done following CD34 immunostaining on these biopsies. The study is intended to find an association between MVD and tumor burden, and the change in MVD post induction therapy. The present study found increased bone marrow microvessel density in patients of acute leukemia, which points towards a possibility of the role of angiogenesis in pathophysiology of the disease, which would help in prognostication as well as therapeutics.

Material and methods

Ethics and consent- Institutional ethics committee clearance was obtained prior to proceeding with the study. Informed consent was obtained from all the subjects for review of tissue blocks and for study of immunohistochemistry (IHC) on such samples.

Patients and controls- In this study, all retrospective and prospective cases of acute leukemias diagnosed on bone marrow biopsies over a period of two years, that is from 2018 to 2020, were included. We studied a total of 46 patients who fulfilled our criteria, and the bone marrow biopsies were adequate for evaluation. They were diagnosed as cases of acute leukemia on bone marrow aspirate and biopsy along with immunophenotyping. The cases included 32 cases of acute myeloid leukemia (AML) and 14 cases of acute lymphoblastic leukemia (ALL). The control group included 27 cases, which comprised the non-leukemic conditions like immune thrombocytopenia, pyrexia of unknown origin, cytopenia for investigation, lymphomas with no deposits in the bone marrow. The age group of patients ranged from 1 to 75 years.

Bone marrow specimens- Bone marrow trephine biopsy specimens with adequate material were included. Archival formalin fixed and paraffin embedded blocks were retrieved. The formalin fixed and paraffin-embedded tissue blocks from patients and controls were cut into sections of 3–4-micron thickness. The slides were stained with Hematoxylin and Eosin (H&E) stain and reviewed.

Immunohistochemistry- IHC was done using antibodies directed against CD34 to highlight the vascular endothelial cells. Streptavidin-biotin method was utilized to perform the staining. DAB was used as the chromogen and hematoxylin was used as the counterstain.

Evaluation and calculation of bone marrow microvessel density- The sections of bone marrow biopsies were stained with CD34 IHC, following which they were examined at 100x magnification to find “hot spots”, which is the area with highest vascularity. Three such hot spots were identified to count the average number of microvessels. The microvessels were counted at 400x magnification. Microvessel density (MVD) was expressed as the average of the total number of vessels per 0.17mm2 area. The microvessel caliber (MVC) (in microns) was also calculated. Criteria taken into consideration for counting as microvessel included counting the circles and ellipses. Two parallel lines and solid bars were taken as a single vessel.

Morphometric analysis was done using computerized digital photomicrograph system using Video test size 5.0 software.

Parameters correlated- The MVD and MVC were compared with peripheral blood parameters including hemoglobin, total leukocyte count, platelet count, blast count as well as bone marrow parameters including blast count, marrow fibrosis and cellularity.

Statistical analysis- The data was analyzed using Statistical Package for Social Sciences (SPSS version 25.0, IBM corporation, USA) for MS Windows. Kruskal Wallis test was used to compare MVD, MVC and Vessel count in all the groups with hemoglobin, TLC count, platelet count, blasts in peripheral blood and bone marrow and with myelofibrosis and bone marrow cellularity. Similarly, the MVD in new, relapse and remission cases were compared.

Mann-Whitney U test was used to compare the MVD for pair wise comparisons of each group with controls. Spearman’s correlation coefficient was used to find the correlation between marrow cellularity, myelofibrosis with MVD in all the groups. Similarly, correlation between peripheral blood and marrow blasts with MVD was established.

Result

A total of 46 cases of acute leukemia were included in the study, with 12 cases of ALL and 34 cases of AML. (Table-1). 27 controls were included in the study. The mean age of the patients was 32 years, ranging from 1 to 75 years. Male: female ratio was 1:1 with 23 males and 23 female patients. Mean age of controls was 41 years.

Mean MVD in acute leukemia patients was significantly higher in cases compared to controls (144.98/mm2 versus 91.28/mm²; p value<0.001) though there was no statistically significant difference in the MVD between the AML and the

ALL group (AML- 152/mm2 versus ALL- 125/mm2; p-value=0.255). (Table-1)

Table 1: Distribution of cases and controls with mean MVD

	Frequency (n) (%)	Mean MVD (per mm²) ±SD
AML	34 (74%)	152.0 ± 70.0
ALL	12 (26%)	125.1 ± 46.9
Acute Leukemia (Total)	46	144.98 ± 65.34
Controls	27	91.28± 36.22
p-value		Cases vs controls p<0.001
p-value		AML vs ALL p=0.255

The mean MVD increased significantly with increasing reticulin fibrosis (p-value= 0.011) and correlated well with blast percentage in peripheral blood (p-value=0.029). No significant correlation with marrow blasts or BM cellularity was seen. The study also correlated the mean MVD with the percentage of blasts in the BM aspirate, though this was not statistically significant. (Table-2)

Table 2: Correlation of mean MVD with various blood and bone marrow parameters

Correlation with mean MVD	Correlation co-efficient (ρ)	p-value
Cellularity	0.230	0.125
Reticulin fibrosis	0.370	0.011
BMA Blasts	0.222	0.138
PBS Blasts	0.322	0.029

The acute leukemia group was further sub-classified into new cases, remission cases, and relapse cases in both AML (Figure-1) and ALL groups (Figure-2). (Table-3) MVD was compared amongst these groups to find out the change in MVD in post therapy cases. It was found that the MVD in relapse group was significantly higher than the remission group, while the MVD in new cases though higher than the remission group, was not statistically significant. There was a significant reduction in the MVD following remission, thus suggesting a role of anti-angiogenic therapy in achieving remission in hematological malignancies. (Table-3).

Table 3: Change in MVD in new versus relapse and remission cases

Cases	Frequency (n)(%)	Mean MVD (per mm²)±SD	p-value
New	24 (52%)	152.4 ± 72.28	New vs Remission = 0.106
Remission	17 (37%)	118.34 ± 45.67	New vs Relapse = 0.083
Relapse	5 (11%)	200 ± 50.12	Relapse vs Remission = 0.005

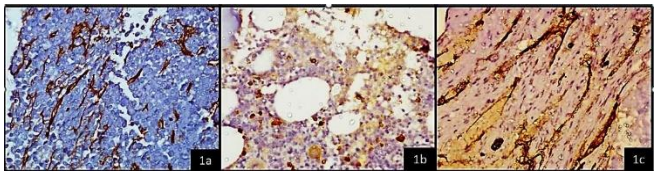

Figure 1: (CD34 IHC; x400) Vascularity in cases of AML (a) new (b) remission (c) relapse marrow

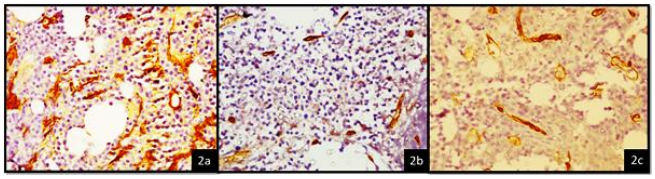

Figure 2: (CD34 IHC; x400) Vascularity in cases of ALL (a) new (b) remission (c) relapse marrow.

Discussion

Angiogenesis has a key role in progression of a tumor and in its metastasis and invasion. It is a process controlled by delicate balance between angiogenic and anti-angiogenic factors.[2,10,12,13]

An essential requirement for the tumor to grow, invade and metastasize is nutrients and oxygen, which is provided to it by the new blood vessels that are formed by angiogenesis. So, it is evident that angiogenesis, is fundamental to leukemogenesis.[2,9,10,14]

The pro-angiogenic cytokines are expressed by the progenitor cells, endothelium, and stroma of BM. These cytokines affect the entire BM microenvironment by various stimulations. Thus, leading to high MVD and VEGF mRNA level.[9,14]

Based on the above data, a hypothesis was postulated that the MVD would be higher in newly diagnosed cases of hematological malignancies who have not yet been treated, as well as in the cases where there has been relapse of the disease or the patient did not attain remission. While MVD is expected to be comparatively lower in post treatment cases who have achieved a state of remission.

In the present study, MVD and its various parameters were studied in cases of acute leukemia and controls with the help of morphometric analysis of the hotspots with maximum number of microvessels. MVD is used as a surrogate estimate of the metabolic burden represented by tumor cells.

A major limitation of this study was that the sample size was small. Also, there was limited follow-up data for survival analysis, so the final outcome could not be assessed. The selection of the area of microvessel enumeration and vessel evaluation has an element of subjectivity, though there is no interobserver variability as the microvessel evaluation was done by single observer.

There are a huge number of studies that have shown increase in angiogenesis and its mediators in cases of acute leukemias.[7,15–18,20] The present study analyses angiogenesis and compares it with various parameters in peripheral blood and bone marrow in cases acute leukemias and controls. In addition to this, the alteration in these parameters following therapy in cases of ALL, AML were also studied. The MVD among cases of ALL and AML were also compared with each other. The present study includes pediatric as well as adult patients, as compared to previous studies where mostly the pediatric population has been studied.

In various studies, the BM of patients of AML has shown increased angiogenesis and neoangiogenic processes. Also, the MVD is increased with presence of hot spots, branching microvessels with variable morphologies.[11,19,20]

Hussong et al found significantly increased MVD in the BM of AML patients ($P<0.001$), suggesting a role of angiogenesis in AML. They also correlated the cellularity of BM in AML patients with the controls as well with the MVD, though they did not find any significant correlation. The present study also found similar results, with absence of correlation between marrow cellularity and MVD, signifying that it was unlikely that the increased angiogenesis was due to hypercellularity.

In a study for cases of ALL by Perez-Atayde et al, they demonstrated the increased number of blood vessels in bone marrow of children with ALL, as compared to the normal counterparts, the present study also shared similar results. This is indicating that leukemia could be angiogenesis dependent and suggesting a role of anti-angiogenic therapy in treatment of leukemia. The study by Perez-Atayde was a pilot study after which the degree of angiogenesis was measured using MVD in the BM biopsies of acute and chronic leukemia cases and was found to be raised. In the present study, the angiogenesis was measured as MVD. This was done with the help of computer assisted morphometry. They found no significant decrease in the MVD in children with acute leukemia following achievement of remission. Whereas, in the present study, the mean MVD in relapse cases was found to be significantly higher than the cases who achieved remission, though a significant decrease was not found in remission cases compared with the new cases.[13]

A study was done by Jothilingam et al, where the cases of acute leukemia were found to have increased MVD. They also studied proliferation index and compared it to the controls, which was not done in the present study. They found the MVD to be significantly higher in patients compared to the controls as was found in the present study.[1]

A study by Aguayo et al analyzed angiogenesis between AML and ALL cases. They did not find any significant difference in the MVD of these two leukemias. The present study, possibly the only study, which in addition to MVD, also compared the marrow blasts, cellularity, reticulin fibrosis among the AML and ALL cases, and found out there was no significant difference between the two.[20]

Studies have tried to show the relation between the marrow cellularity and angiogenesis. A mechanical link was established, saying that increased marrow cellularity leads to increased demand supply relationship which in turn leads to the increased angiogenesis, as was studied by Kalmanti et al.[7] The present study compared angiogenesis with the cellularity as well as the BM reticulin fibrosis and found that the MVD in acute leukemia cases significantly correlated with reticulin fibrosis, however, it did not significantly correlate with the cellularity, so a direct relationship could not be established. Thus, highlighting that increased angiogenesis was not due to hypercellularity in the BM. Rather, the secretion of angiogenic factors by leukemic cells are a more likely cause of increased angiogenesis.

The present study found a significant correlation of MVD with reticulin fibrosis and peripheral blood blasts, though the marrow blasts did not significantly correlate significantly with the MVD, suggesting angiogenesis to be independent of the bone marrow blasts.

The cases of AML and ALL in the present study who went into remission, showed a clear-cut decline in the MVD post-induction as compared to the pre-treatment MVD and the control values, indicating role of anti-angiogenic therapy to achieve remission in hematological malignancies.

Conclusion

To summarize, the present study has demonstrated the bone marrow microvessel density to be significantly higher in cases of acute leukemia as compared to control group in pediatric as well as adult population, suggesting a role of angiogenesis in hematological malignancies. The study also found a significant reduction of MVD following remission, stressing on the therapeutic implication of anti-angiogenic therapy to achieve remission in hematological malignancies.

Present study found a significant correlation of MVD with marrow fibrosis in acute leukemia cases and increase in MVD in new and relapse cases when compared to the cases in remission, illustrating the point that the angiogenesis gets intensified due to neoplastic proliferation, while there a reduction in MVD with remission due to decrease in tumor load.

Absence of correlation between marrow cellularity and MVD in the present study, suggests that its unlikely that the increase in angiogenesis is due to hypercellularity and points to a more complex relation between the two.

To conclude, the study shows a definite role of angiogenesis in pathophysiology of hematological malignancies. Though,

further larger studies are required to establish exact mechanism and interaction of angiogenesis and BM cellular components and microenvironment. Inhibitors of angiogenesis can serve as a potential therapeutic strategy alone or in combination with the established chemotherapy regimens.

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Competing Interests: The authors declare no conflict of interest.

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