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Original Research Article

A study of bone marrow angiogenesis and its correlation with serum vascular endothelial growth factor levels in acute leukaemia

Neha Yadav[1], Sant P. Kataria[2]*, Sanjay Kumar[2], Oshin Vashishth[3], Sudhir Atri[4], Mahak[2], Sunita Singh[2]

[1]Department of Pathology, Rewari, Haryana, India
[2]Department of Pathology, [4]Department of Medicine, Pt. B. D. Sharma PGIMS, Rohtak, Haryana, India
[3]St. Martinus Faculty of Medicine, Willemstad, Curacao

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***Correspondence:**
Dr. Sant P. Kataria,
E-mail: santprakashkataria8@gmail.com

ABSTRACT

Background: Angiogenesis, which is the development of new capillaries from existing blood vessels, occurs in both the developing embryo and postnatal life. The growth of solid tumours requires the development of micro vessels; therefore, tumour expansion correlates with the extent of angiogenesis.
Methods: A prospective study was conducted on bone marrow trephine biopsies of 40 new cases of acute leukaemia diagnosed on complete blood count, bone marrow aspiration examination, and flow cytometry, including 20 control cases. Micro sections were stained with immuno-histochemical stains using monoclonal antibodies to CD31, CD34 and vWF. Micro vessel density was analysed at a 400× using automated image analyser by two investigators independently. Data were calculated, tabulated and statistically analysed using statistical package for the social sciences (SPSS) statistical program version 18.
Results: A total of 1522 micro vessels were analysed using CD31 including 802 in acute myeloid leukaemia (AML), 512 in acute lymphocytic leukemia (ALL) and 208 in the control group. The bone marrow microvessel density (MVD) in acute myeloid leukaemia using CD31, CD34 and Von Willebrand factor (VWF) was 70.83±20.76, 66.48±18.99 and 60.32±18.75 respectively while MVD in acute lymphoblastic leukaemia was 62.74±21.09, 70.58±22.46 and 51.22±21.13 respectively. The study revealed significant difference between AML, ALL and normal bone marrow cases by using CD31, CD34 and vWF antibody. Serum vascular endothelial growth factor (VEGF) concentration in AML, ALL and control group was 163.74±119.03, 168.23±154.22 and 43.45±9.14 respectively.
Conclusions: Higher micro vessel density was observed in acute leukaemia. Present findings suggested the potential significance of characteristics of micro vessel density as potential prognostic marker as well as its application in improved selection of patients for anti-angiogenic and other treatments.

Keywords: Angiogenesis, CD31, CD34, Leukaemia, Serum VEGF, vWF

INTRODUCTION

Angiogenesis, which is the development of new capillaries from existing blood vessels, occurs in both the developing embryo and postnatal life. The growth of solid tumors requires the development of micro vessels, therefore tumor expansion correlates with the extent of angiogenesis.[1] Micro vessels not only provide nutrients and oxygen but also remove catabolic substances, while endothelial cells produce growth factors for tumor cells in a paracrine fashion.[2] Without adequate vascularization, tumors larger than 1 mm^2 may undergo necrosis and cannot grow beyond a critical size or metastasize to

another organ. Similarly, without an efficient blood supply, it is difficult to deliver anti-cancer drugs to all regions of a tumor in effective quantities.[3]

Leukemia is a group of malignant disorders of the haemopoietic tissue characteristically associated with increased number and clonal proliferation of leucocytes in the blood. Leukemias are progressive and fatal conditions resulting in death most often from hemorrhage or infections. The course may vary from a few days or week to many years depending on the type.[4]

Leukemia accounts for 0.15–0.6% of the total medical admission in many general hospitals in India. Males are affected more frequently than females. Male to female ratio in acute leukemia is 3:2. Frequency of leukemia seen in India of acute myeloid leukaemia (AML) is 20–25% and acute lymphocytic leukemia (ALL) is 15–25%. The Annual incidence rates of AML and ALL are 5.6 and 30.9 per million population respectively.[5] Acute lymphoblastic leukemia is primarily a disease of children and young adult, whereas AML occurs primarily in adults. ALL shows a peak incidence in the age group of 1-5 years. Each year, approximately 10,000 children of age group 0-21 years develop AML. Current epidemiological studies predict about 6.4-6.7 AML cases per million in the USA, while in China it is about 11 cases per million and in India 3.5 cases per million. The mechanisms underlying these differences have not yet been determined.[6]

The histological quantification of human tumour angiogenesis was introduced by Weidner et al in 1991.[7] He and his colleagues used immunohistochemically techniques to highlight tumour blood vessels with antibodies to factor VIII related antigen. Since then, various other markers have been employed including CD31, CD34 and CD105.[8] Highlighting the vessel wall made it possible to numerically count micro vessels, announcing the emerging of a new parameter that might be used in the characterization of solid tumours. Among the various methods to assess angiogenesis, micro vessel density (MVD) has been studied extensively in various tumors.[9] He introduced for the first time the term intra tumoral MVD. The reason why these parameters should be studied is to yield important information on the relationship to other clinicopathological tumour characteristics and help testing of antiangiogenic therapies.[10]

The angiogenic activity of haemopoietic neoplasms (so called liquid tumors) has not yet been demonstrated. It is not known whether leukemic cells, which usually grow in absence of visible connective tissue stroma and circulate in the blood and body fluids; are also dependent on neovascularization to proliferate and expand. [11] Various studies have shown that patients with hematological malignancies have increased bone marrow MVD and increased levels of angiogenic factors in serum levels; hence supporting the notion that bone marrow angiogenesis plays a role in pathogenesis and progression of this cancer.[12-14]

Aims and objectives

Aims and objectives of the study were: to evaluate angiogenesis by using immunohistochemically markers (CD31, CD34 and vWF) on bone marrow trephine biopsies and measuring micro vessel density by computer assisted quantitative image analysis in acute leukaemia; and tcorrelate angiogenesis with serum VEGF levels in acute leukaemia.

METHODS

A prospective study was conducted in the department of pathology in collaboration with the department of medicine over a period of two and half years i.e. from June 2014 to December 2016 in Pt. B. D. Sharma, PGIMS, Rohtak, Haryana. The study included bone marrow biopsies of 40 new cases of acute leukaemia diagnosed on complete blood count, peripheral blood smear, bone marrow aspiration examination, and flow cytometry. Informed consent was taken for bone marrow trephine biopsy from each patient. The design of the study was approved by the institutional ethical committee. Cases of acute leukaemia were classified according to FAB (2008) classification. Twenty cases of bone marrow trephine biopsies received for non-leukemic conditions like for staging of neoplasm or pyrexia of unknown origin were taken as control. These 60 cases were divided into three groups. Group I included 23 cases of acute myeloid leukaemia (AML), group II included 17 cases of acute lymphoblastic leukaemia (ALL) and group III or the control group included 20 cases of normal bone marrow. Cases of inadequate bone marrow trephine biopsies were excluded from the study. Representative paraffin-embedded tissue sections of all the cases were stained with routine haematoxylin and eosin along with immuno-histochemical stains with monoclonal antibodies to CD31, CD34 and vWF at 1:50 dilution (Dako, polyclonal). “Hotspots,” that is, areas of maximal MVD, were identified. In two such hotspots, vessel number and MVD were analysed at 400× using an automated image analyser by two investigators independently. The mean value of both investigators was considered as the MVD in each case. Sections of normal bone marrow biopsy were used as positive controls for anti CD31, anti CD34 and anti vWF antibodies. Negative controls were prepared by replacing the antibody by PBS or non-immune serum.

The quantitative morphometric studies were done by image analysis. Images provided by a charged device video camera coupled with Olympus BX51 microscope at a magnification 400x were stored on a host computer based on Pentium 4 processor with operating system Microsoft Windows Vista/ XP through a digital frame grabber and processing was done by image analysis software Image Pro Plus version 6.3.

Micro vessel (MV) was defined as any highlighted endothelial cell or endothelial cell cluster clearly separate from adjacent micro vessels, tumor cells and other connective tissue elements. Vessel lumens were not necessary for a structure to be defined as a micro vessel. MVD was assessed by light microscopy in representative areas of sections with highest numbers of capillaries and small venules (neovascular ''hot spots'') according to the method that was first described by Weidner et al.[7] The micro sections stained with CD31, CD34 and vWF were scanned first at low magnification (100x), and after the most intense areas of neovascularization (hot spot) were identified in sections, micro vessel (MV) counts were done on a minimum of two fields at a 400X. Computer assisted image analysis was performed on all cells, tissues and vessels expressing antibody staining, avoiding confounding background and including all positive staining vessels, whereas contaminating areas were excluded using the specific computer function. Data was collected as the number of micro vessels in a 400X field. All the cases were evaluated by two different observers. Mean of two microscopic fields was taken and micro vessel density per mm^2 was calculated. Serum VEGF was estimated in patients and control samples stored at -80^0 C using the VEGF ELISA kit by Peninsula Inc. USA following the manufacturer instructions. All the data was tabulated in excel sheet from which mean and median was calculated in all categories of acute leukemia.

Statistical analysis

Data were calculated, tabulated and statistically analyzed using statistical package for social studies (SPSS) statistical program version 18. The values entered were mean of morphometric parameters. In all tests, p values below 0.05 were regarded as significant. For comparison between immunohistochemically staining i.e., CD31, CD34 and vWF between AML, ALL and control group analysis of variance (ANOVA) test was applied. To compare the number of MVs and MVD between acute myeloid leukemia and acute lymphoblastic leukemia t-test was applied. For finding correlation between MVD and serum VEGF concentration Spearman correlation coefficient test was applied.

RESULTS

Present study consisted of three groups which included 23 (38.33%) cases of acute myeloid leukemia in group I, 17 (28.33%) cases of ALL in group II and 20 (33.33%) cases of normal bone marrow as control in group III. Maximum number of cases i.e., 15 each belonged to AML-M1 and M2 and ALL-L2 comprising 37.5% of all total cases. Four cases were of AML-M3 (10%) and 3 cases of AML-M4 (7.5%) respectively, whereas 2 cases (5%) were of ALL-L1 type followed by a single case of bi-phenotypic leukemia. However, in control group maximum number of cases (n=8, 40%) were of normal hematopoiesis followed by 5 (25%) cases of megaloblastic erythroid hyperplasia, 3 (15%) cases of normoblastic erythroid hyperplasia and 2 (10%) cases each of dimorphic erythroid hyperplasia and non-specific myeloid reaction respectively. Male to female ratio was 1.07:1.00 in AML cases and 1.12:1.00 in ALL cases, while age ranged from 15-95 years in AML and 5-84 years in ALL cases.

The total number of micro vessels evaluated using different antibodies in the study were 1522 with CD 31, 1534 with CD34 and 1164 with vWF. A total of 802 numbers of micro vessels were identified in AML using CD31 antibody followed by 512 in ALL and 208 in the control group. On using CD34 as the endothelial marker the number of micro vessels counted were 734 in AML, 576 in ALL and 224 in the control group. Numbers of micro vessels counted were 666 using vWF in AML, 418 in ALL and 80 in the control group (Table 1).

The mean MVD was 70.83±20.76 using CD31, 66.48±18.99 per mm^2 using CD34 and 60.32±18.75 per mm^2 with vWF in cases of AML, whereas the mean MVD was 62.74±21.07 per mm^2 using CD31, 70.58±22.46 per mm^2 using CD34 and 51.22±21.13 per mm^2 using vWF in cases of ALL (Figures 1-5). The mean MVD was 21.65±11.69 per mm^2 using CD31, 23.33±9.31 per mm^2 using CD34 and 8.33±3.31 per mm^2 using vWF in control group (Table 1). ANOVA test applied showed a significant difference in AML, ALL and control group with a p value (<0.001).

Serum VEGF concentration in cases of AML was 163.74±119.03 pg/ml, 168.23±154.22 pg/ml in cases of ALL and 43.45±9.14 pg/ml in cases of the control group. A significant increase in serum VEGF concentration was noted in three cases i.e., 350, 400 and 600 respectively, which were diagnosed as CML in blast crisis of lymphoid origin (Table 2). Differences in serum VEGF concentration between AML, ALL and control group were significant on applying ANOVA test with a p value 0.01.

Table 1: Comparison of total number of blood vessels and mean MVD at 400x using various antibodies in leukemia cases and control group.

Cases	Number of vessels and type of antibodies					
	Number CD31 of vessels		Number CD34 of vessels		Number vWF of vessels	
AML	802	70.83±20.76	734	66.48±18.99	666	60.32±18.75
ALL	512	62.74±21.07	546	70.58±22.46	418	51.22±21.13
Control group	208	21.65±11.19	224	23.33±9.31	80	8.33±3.31
P value	<0.001		<0.001		<0.001	

Table 2: Comparison of serum VEGF concentration in various categories of acute leukemia cases and control group.

Cases	Mean serum VEGF concentration (pg/ml)
Acute myeloid leukemia	163.74±119.03
Acute lymphoblastic leukemia	168.23±154.22
Control group	43.45±9.14

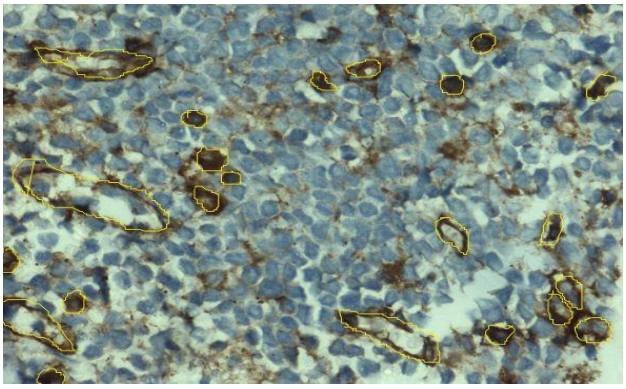

Figure 1: Micro vessels in acute myeloid leukaemia (CD31; 400×).

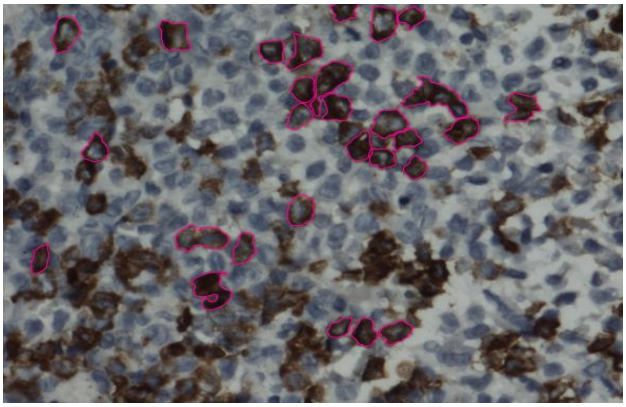

Figure 2: Micro vessels in acute myeloid leukaemia (CD34; 400×).

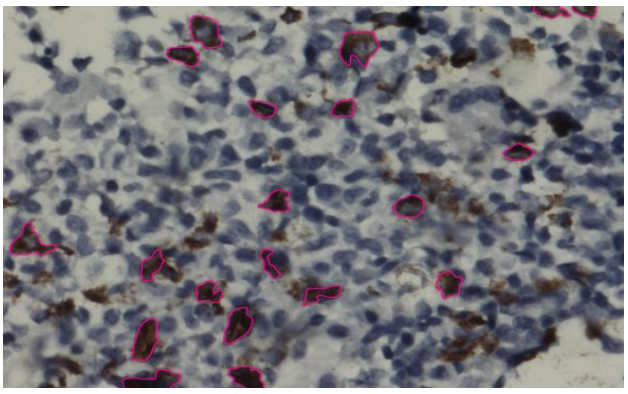

Figure 3: Micro vessels in acute myeloid leukaemia (vWF; 400X).

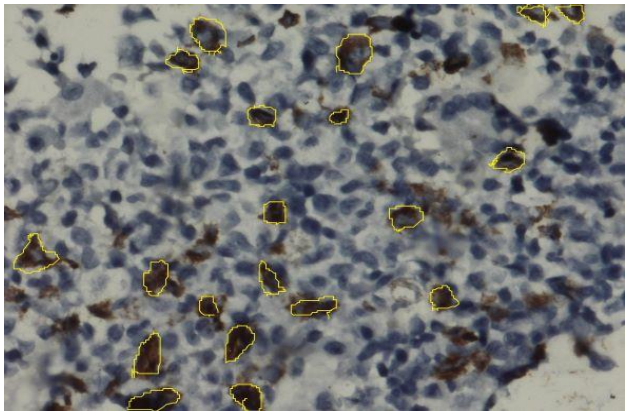

Figure 4: Micro vessels in acute lymphoblastic leukaemia (CD31; 400X).

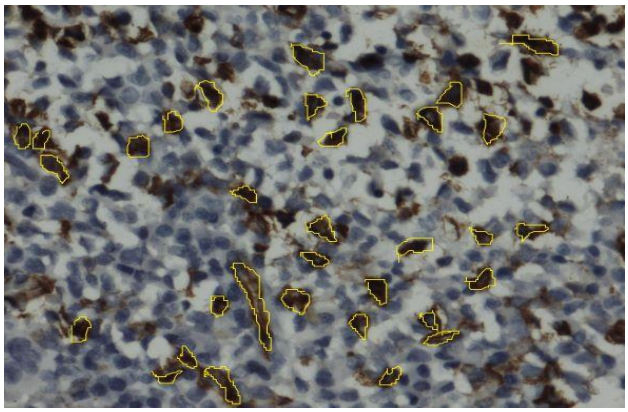

Figure 5: Micro vessels in acute lymphoblastic leukaemia (CD34; 400X).

DISCUSSION

Acute leukemia comprises a heterogeneous group of conditions that differ in etiology, pathogenesis, molecular mechanisms and prognosis. The heterogeneity is reduced if cases of acute leukemia are divided into AML, ALL and mixed phenotype acute leukemia (MPAL); even then, however, considerable heterogeneity remains within each of the groups.[15] During the past few years, it has been proposed that angiogenesis may play a role in not only solid tumors, but also in hematological malignancies. Highlighting the vessel wall using various endothelial markers made it possible to numerically count micro vessels, announcing the emergence of a new parameter that might be used in the characterization of haematolymphoid malignancies and other solid tumors. Immuno-histochemical staining for pan endothelial markers clearly highlights the vascular endothelium. Of the various methods to assess angiogenesis, micro vessel density has been studied extensively in various tumors.[16]

The reason why these parameters should be studied is to yield important information on the relationship to other clinico-pathological tumor characteristics and help testing of anti-angiogenic therapies.[1]

Comparison of micro vessel density in acute myeloid leukemia and acute lymphoblastic leukemia

Angiogenesis is important for the development of a malignant phenotype and numerous studies have demonstrated that the vascular density of a tumor directly correlates with metastasis and patient outcome. Marked increase in micro vessel counts in the bone marrow of patients with AML is related to reactivation of dormant bone marrow sinusoids.[17]

The differences between the number of vessels/mm^2 in AML and normal marrows were highly significant ($p<0.0001$ for vWF staining) as studied by Hussong et al who also demonstrated a positive correlation between the percentage of marrow blasts and vessel score.[18] We observed a significantly higher number of bone marrow micro vessels count in AML and ALL as compared to the control group.

Padro et al demonstrated significantly increased bone marrow micro vessel density in newly diagnosed AML patients compared with control on thrombomodulin as well as vWF staining and observed that micro vessel count lowered significantly on achieving complete remission as compared to initial diagnosis and showed morphology similar to those in the controls.[11]

MVD values examined by Kuzu et al showed a significant increase in cellularity and MVD with both CD31 and CD34 markers in AML as compared to the control group.[15] P value for CD31 was 0.004 and for CD34 was <0.001 which suggested higher sensitivity of CD34 marker. It was also revealed that decrease in MVD and angiogenic factors with therapy may support the relationship between leukemic cells and endothelial growth in AML in relevance to the prognosis.

We observed an increase in micro vessel density using CD31, CD34 and vWF antibody in acute myeloid leukemia cases as compared to control, which was statistically significant ($p<0.001$). Increased MVD was observed using CD31 and CD34 as compared to vWF in cases of AML (Table 3). This may be due to frequently observable strong staining of myeloid leukemia blasts by anti-CD31 and anti-CD34 antibodies. Thus, our study was comparable with other studies mentioned above as all were showing increased micro vessel density in leukemia patients comparable to control.

A significantly increased bone marrow micro vessel density was observed in children with ALL compared to cases of bone marrow control ($p<0.0001$) by Atayde et al, which also persisted at the time of complete remission.[19]

Higher MVD and proliferative index (PI) was observed by Jothilingam et al in the entire leukemia population when compared to controls ($p<0.001$) and further found a significant drop in MVD following remission.[20] ALL had a significantly higher MVD compared to AML ($p=0.041$). To support higher angiogenesis, ALL was more fastidious in its requirement for supporting stromal environment. The higher vascularity could also mean higher drug delivery and hence better chance at remission.

We also observed significantly increased micro vessel density using CD31, CD34 and vWF antibody in acute lymphoblastic leukemia cases as compared to control, which was statistically significant ($p<0.001$). Out of the above mentioned two markers, CD34 revealed higher sensitivity in ALL as compared to CD31 and vWF. As we did not have any information regarding patients follow up, we could not determine the relationship between micro vessel density and remission status. Our study showed lower values of micro vessel density in the control group by using vWF as compared to leukemia cases. This was because of low levels of vWF expression and dormant marrow sinusoids in normal people. Our results were comparable with studies performed by Padro et al, Hussong et al and Atayde et al.[11,18,19]

Comparison of serum VEGF concentration in AML, ALL and control group

VEGF is regarded as the most important pro-angiogenic factor which is crucial for vasculogenesis and angiogenesis and plays a key role in the development and progression of leukemia. Previous studies have indicated that increased levels of serum VEGF and bone marrow micro vessel density (BM-MVD) were observed in patients with various types of hematological malignancies and were associated with the severity of disease in patient with leukemia.[15,19]

We have studied levels of serum VEGF concentration in cases of AML, ALL and in cases of control group; which were found significantly higher in leukemic patients compared with control group with significant p value <0.01. Increased serum VEGF level was observed in cases of AML as compared to ALL which was seen in the majority of studies performed by various authors.[12,16,21] However, in our study levels of serum VEGF were slightly higher in ALL as compared to AML. This might be due to inclusion of cases of CML in blast crisis of lymphoid origin as these cases showed very high levels of serum VEGF (Table 4).

The prognostic significance of neo-vascularization has been demonstrated for solid tumors, but few reports are available for hematological malignancies. Won et al found significantly higher peripheral blood mononuclear cell VEGF expression in recurrent ALL compared to newly diagnosed ALL and that VEGF levels in newly diagnosed ALL showed prognostic value and hence VEGF at diagnosis may play an important role in progression of childhood ALL.[22]

A significantly lower value of serum VEGF was reported by Kalra et al in patients of ALL as compared to controls.[23] Authors hypothesized that low serum VEGF levels in

children with ALL may reflect a higher VEGFR expression on leukemia cells that would bind serum VEGF, thus decreasing unbound serum VEGF proportionately to tumor burden via ligand receptor interaction.

Correlation between VEGF and MVDs in cases of AML

It has been well studied by Sorady et al that both micro vessel density in bone marrow biopsies and serum VEGF levels in patients with hematological malignancies significantly exceeded those of the controls.[12] They also found a significant positive correlation between the vessel count and VEGF levels in patients with multiple myeloma and non-Hodgkin's lymphoma.

Padro et al showed that decreased levels of BM-MVD and serum VEGF were detected in patients with acute myeloid leukemia subsequent to remission.[11]

Furthermore, Kuzu et al described that an increased levels of BM-MVD were associated with poor prognosis in patients with AML.[15]

Song et al observed that significant increase levels of BM-VEGF and BM-MVD in AML patients suggested that VEGF associated vasculogenesis and angiogenesis might support the proliferation of malignant progenitor cells and may be associated with the pathogenesis of AML.[21] In addition, VEGF may be a target for the design of novel therapies for AML.

Table 3: Comparison of micro vessel density in AML and ALL in various studies.

Studies	Endothelial marker	MVD in AML	MVD in ALL	MVD in control group
Padro et al[11]	vWF	25.5	-	13.2
Kuzu et al[15]	CD31	28.9±16.2	-	-
	CD34	41.3±26.1	-	-
Hussong et al[18]	vWF	8.6±3	-	4.9±2.2
Atayde et al[19]	vWF	-	56.75	8.10
Jothillingam et al[20]	CD34	-	49.81	13.5
Our study	CD31	70.83±20.76	62.74±21.09	21.65±11.19
	CD34	66.48±18.99	70.58±22.46	23.33±9.31
	vWF	60.32±18.75	51.22±21.13	8.33±3.31

Table: 4: Comparison of serum VEGF concentration in AML, ALL and control group.

Various studies	Serum VEGF concentration (pg/ml)		
	AML	**ALL**	**Control group**
Sorady et al[12]	607.5±196.9	480.6±109.4	139.8±74.74
Erdem et al[16]	110.1±120.9	87.6±76.9	69.9±24.4
Song et al[21]	74.97±29.04	-	41.74±10.03
Won et al[22]	-	216.6±79.9	36.8±12.1
Kalra et al[23]	-	17.0	42.6
Chand et al[24]	78.75±33.83	163.64±95.81	-
Our Study	163.74±119.03	168.23±154.22	45.95±9.14

Correlation between VEGF and MVDs in total cases of acute leukemias

Our study showed a good correlation between VEGF and CD34 and VEGF and vWF with a significant p value of 0.004 and 0.018 respectively in overall leukemia cases (AML and ALL), whereas CD31 showed a poor correlation with VEGF.

All leukemia cases showed a significantly higher micro vessel density as well as serum VEGF levels as compared to the control group.

However, an inverse relationship was found between VEGF and micro vessel density in the control group (Table 5).

Table 5: Correlation between VEGF and MVDs in total cases of acute leukemia (AML+ALL).

Correlation	Coefficient value (r)	P value
VEGF with CD31	0.257	0.109
VEGF with CD34	0.443	0.004
VEGF with vWF	0.372	0.018

Comparison of CD31, CD34 and vWF as a marker of angiogenesis

Atayde et al observed that micro vessel density by using CD31, CD34 and vWF were similar.[19] De Bont et al similarly counted the number of vessels stained with CD34 and vWF and found no significant difference between the two.[14] Jothilingam et al also revealed no

significant difference between CD34 and vWF.[20] Our study also showed no significant difference between AML and ALL cases by using CD31 and CD34 antibody with a non-significant p value i.e., 0.234 and 0.536 respectively. On using vWF, it showed lesser number of vessels counted and lesser MVD comparable to other two antibodies used. This may be due to higher focal background staining frequently observed with the vWF antibody (probably because of plasma vWF) explaining the lower micro vessel counts found in bone marrow sections stained with this endothelial marker.

Previous studies of normal bone marrow have demonstrated a close association between the bone marrow vessels and the islands of developing blood cells, suggesting an important interdependence between the bone marrow hematopoietic function and its vascular bed.[19] The potential implication of studies on angiogenesis in hematological malignancies lies in its therapeutic application. One of the most critical regulators of angiogenesis is VEGF which causes endothelial cell proliferation. It is also involved in the "angiogenic loop" responsible for autocrine and paracrine tumor growth and survival.[26]

Our study identified positive correlation between micro vessel density analyzed and serum VEGF concentration indicating that both were significantly increased in leukemia cases as compared to control group.

In the present study, we could not determine the relationship between micro vessel density and survival due to non-availability of any information regarding patients follow up. But our study revealed that there was statistically significant difference in total micro vessel density between different groups i.e., AML, ALL and control group (p value <0.001) with a pattern of increasing angiogenesis in acute leukemia. Thus, it was suggested that micro vessel density could be used as a measure of angiogenic activity and aggressiveness in leukemia cases.

CONCLUSION

The present study concluded that malignant cell proliferation, angiogenesis and VEGF expression are linked in acute leukemia. Higher micro vessel density was observed in acute leukemia and plays a role in the pathogenesis and progression of leukemia. Recently published studies with different endothelial markers have shown parallel results demonstrating an increase in micro vessel density in leukemia patients. These findings further suggested the potential significance of characteristics of micro vessel density as potential prognostic markers as well as its application in improved selection of patients for anti-angiogenic and other treatments. In the present era of newer anti-angiogenic therapies, by demonstrating increased angiogenesis in the bone marrow of leukemia patients and blood or urine levels of angiogenic factors, we lend support to previous studies suggesting that angiogenesis may play a role in the pathophysiology of hematopoietic malignancies and thus raise the possibility of using antiangiogenic therapy as a novel therapeutic strategy.

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