Original Article

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Haematology Section

Does CD34 Staining Reflect the Angiogenic Process in the Bone Marrow? An Analysis of a Series of Chronic Myeloid Leukemia Patients

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ABSTRACT

Backgorund: Angiogenesis is associated with growth, dissemination and metastasis of tumours. Measurement of Microvascular Density (MVD) is a quantitative method of assessment of angiogenesis and would give a proportional co relate of the angiogenic process in tumours. The aim of this study is to measure the MVD by using CD34 staining in various phases of Chronic Myeloid Leukemia (CML) and type of CML (Granulocytic/Granulocytic Megakaryocytic) (G/GM) and to co-relate micro vascular densities with the grade of fibrosis.

Materials and Methods: Bone marrow biopsy specimens of 30 CML patients and 20 non-CML (controls) cases that required bone marrow biopsy were subjected to CD34 staining and H&E staining. The mean MVD in CD34 slides was assessed by selecting hot spots and MVD was measured in these fields in high power (40 x magnification) and the mean MVD was calculated by taking the average of four hot spots per field. Grade of fibrosis and phase of CML, type (G/GM) were assessed in H&E slides. The controls were matched with respect to age and gender.

Results: Among 30 patients with CML, 21 were in chronic phase, five in accelerated and four in blast crisis. A normal distribution was obtained for MVD of both CML cases and controls using tests for normality. Comparison of mean MVD between CML and controls by student *t*-test showed a significant increase in MVD of CML cases (p = 0.00026). However, no significant difference in MVD between the three phases viz, Chronic, accelerated and blast crisis phase (p = 0.302) was obtained by using one way ANOVA. Comparison of Grade of fibrosis with MVD using independent *t*-test showed no significant difference in MVD between low (Grade1&2) and high grade (Grade 3&4) (p = 0.805). No significant difference in MVD was obtained between G and GM types of CML using independent t-test (p =0.381).

Conclusion: The study shows that there is a significant increase in MVD in CML cases than controls but no significant difference in MVD could be demonstrated between different phases of CML, histological types of CML and grades of fibrosis in CML.

Keywords: Angiogenesis, CD34 (Cluster of differentiation), MVD (Microvascular Density) Chronic Myeloid Leukemia (CML)

INTRODUCTION

Angiogenesis is the formation of new blood vessels from an existing vasculature [1]. Angiogenesis plays an important role in several physiological and pathological processes. In addition to its physiological role in processes such as placentation, embryogenesis and ovulation, angiogenesis has been associated with the growth, dissemination and metastasis of various tumours. Formation of new blood vessels involve extracellular matrix proteins, activation, proliferation and migration of endothelial cells in a multistep process.Since cytokines like Vascular Endothelial Growth Factor (VEGF), Basic Fibroblast Growth Factor (bFGF), Tumour Necrosis Factor-Alpha (TNF), Hepatocyte Growth Factor (HGF) have been ascribed angiogenic functions and increased levels of these have been co-related with poor prognosis of tumours [2-4], it is logical to adopt maneuvers to measure the ongoing angiogenic process. Angiogenesis by quantitative and qualitative methods can be assessed on tissue sections. Microvascular Density (MVD) being one quantitative method demonstrated by immunohistological markers viz VEGF, CD34, CD31, CD105 and factor VIII related antigen gives a proportional co-relate of the angiogenic process in the bone marrow.

Studies on spectrum of angiogenesis in Bone marrow of children with Acute Lymphocytic Leukemia (ALL) showed significantly increased MVD as well as high urinary levels of bFGF [5]. Studies on acute, chronic leukemias and Myelodysplastic syndromes showed significant increase in vascularity (MVD) in all leukemias with the exception of CLL when compared to controls [6]. Haematological malignancies, have traditionally been regarded as “liquid tumours” because of the terminology and reflection of the disease process in the peripheral blood. Now there is evidence for the concept that these hematological malignancies may depend on vascular supply in the bone marrow like solid tumours in other tissues [7]. The data from studies also suggested that vascularity and angiogenic factors are increased in leukemias and MDS and may play a major role in the leukemogenic process. Hence this study is taken up to study angiogenesis in CML which would be instructive in assessing prognosis and treatment plan.

MATERIALS AND METHODS

We evaluated 30 Bone Marrow Biopsies, BMBs from consecutive patients diagnosed as CML, from the Hematology and Internal Medicine department. Also 20 control BMBs were taken which comprised of BMBs evaluated for solid tumours or lymphomas who did not have evidence of bone marrow involvement and were histologically within normal limits. The study design was a retrospective case control study and all 30 BMBs of CML patients in the period of 2007–09 were collected from the archives of the Pathology department. BMBs included in the study were all a part of the diagnostic workup of the patients. Informed consent was taken from all the patients as per hospital protocol. The preserved

paraffin blocks of the CML patients were subject to H&E staining and CD34 staining (Novacastra™; Lot 6023453). Unmasking of the antigen in formalin preserved slides was done using citrate buffer under pressure and CD 34 primary antibody was applied. Subsequently a secondary kit consisting of biotin, streptavidin and di-amino–benzidene was used and subsequently counterstained with hematoxylin.

Measurement of BM Mean Micro Vasculardensity (MVD)

All the Bone marrows were evaluated for cellularity by two observers using a light microscope independently. Screening for areas of increased angiogenesis i.e., HOT SPOTS was done with 10x power ocular lens. These areas which had increased CD34 staining were chosen randomly and MVD was measured in these fields in high power (40 x magnification) and the mean MVD was calculated by taking the average of four hot spots per slide. All the hot spots were marked and seen by both observers and a consensus was reached whenever there was a discrepancy in MVD assessment. Care was taken to identify ongoing angiogenic CD34 positive endothelial cells and not to count the blasts, formed blood vessels and other CD34 positive artefacts such as skin tissue in BM.

Grade of fibrosis in the cases were identified usingWergertReticulin stain and Masson trichromestained slides of the corresponding patients (WHO 2004). Also phase of CML (chronic, accelerated, blast crisis phase), type (G/GM) (WHO 2008) were assessed in H&E slides.

The CCGM-CML recommendation was followed for categorization of CML stages:

- **Chronic Phase (CP):** The presence of < 15 percent blasts, < 20 percent basophils and < 30 percent blasts plus promyelocytes in peripheral blood and marrow.
- **Accelerated Phase (AP):** The presence of at least 15 percent blasts in blood or bone marrow, at least 30 percent blasts plus promyelocytes in blood or bone marrow, and atleast 20 percent peripheral basophils or thrombocytopenia (platelets < 100 x 109/L).
- **Blast Crisis (BC):** The presence of at least 30 percent blasts in blood or bone marrow or extra medullary involvement.

The controls comprised of 20 patients whose BMBs were taken for assessing metastasis, staging of other malignancies. Their BMBs were found to be normal and also were matched with respect to age and gender.

RESULTS

Among 30 patients with CML, 21 were in chronic phase, 5 in accelerated and 4 in blast crisis phase. A normal distribution with minimal skewing [–0.155] was obtained using SPSS software for MVD of both CML cases and controls using tests for normality [Kolmogorov-Smirnov]. A two-sided significance level of 0.05 was used for all statistical tests. All statistical analyses were performed using SPSS version 21.

Comparison of Mean MVD between CML Cases and Controls

The mean MVD of the cases of CML was 15.21 as compared to normal control of 8.77 (p=0.000265) by using unpaired t-test [Table/Fig-1].

Mean MVD in Blast crisis was significantly higher, 20.76 when compared with all other CML cases 14.35 (p = 0.001).

Comparison of means using One way ANOVA considering each phase independently showed no significant difference in MVD between Chronic, accelerated and Blast crisis phase (p = 0.302).

Grade of Fibrosis vs MVD in CML

Grade of fibrosis was assessed in H & E stained slides and Grades I and II were assigned to Low grade group and Grades III and IV were assigned to High grade group. Among the CML cases, 13 of them had Low grade fibrosis and 17 of them had high grade fibrosis [Table/Fig-1].

Patient group	No.	MVD	p-value
CML Cases	30	15.21	0.00026
Chronic phase	21	14.5	(Vs controls)
Accelerated phase	5	13.73	0.302
Blast crisis	4	20.76	
Controls	20	8.77	
Grade of fibrosis			
Low (I & II)	13	14.96	0.805
High (III & IV)	17	15.40	
Chronic Phase			
CML-G	12	12.74	0.148
CML-GM	9	17.11	

[Table/Fig-1]: Assessment of MVD in CML patients
Key: CML- Chronic Myeloid Leukemia; MVD – Micro vascular density

CML cases	No.	MVD	p-value
Blast crisis	4	20.76	0.001
All other cases	26	14.35	
Blast crisis with high grade fibrosis	3	21.35	0.02
Low grade fibrosis	13	14.96	

[Table/Fig-2]: MVD in blast crisis phase of CML

Three among four patients in blast crisis had high grade fibrosis in the bone marrow and had a mean MVD of 21.35 significantly higher than the group with low grade fibrosis 14.9 (p = 0.02) [Table/Fig-2]. However no significant difference in MVD was found in the accelerated and chronic phase with respect to grade of fibrosis [Table/Fig-3,4].

The mean MVD in the group with low grade fibrosis was 14.9 as opposed to a mean MVD of 15.4 in the high grade group. However comparison of grade of fibrosis with MVD using independent t-test showed no significant difference in MVD between low and high grade of fibrosis with CML (p = 0.805) [Table/Fig-1].

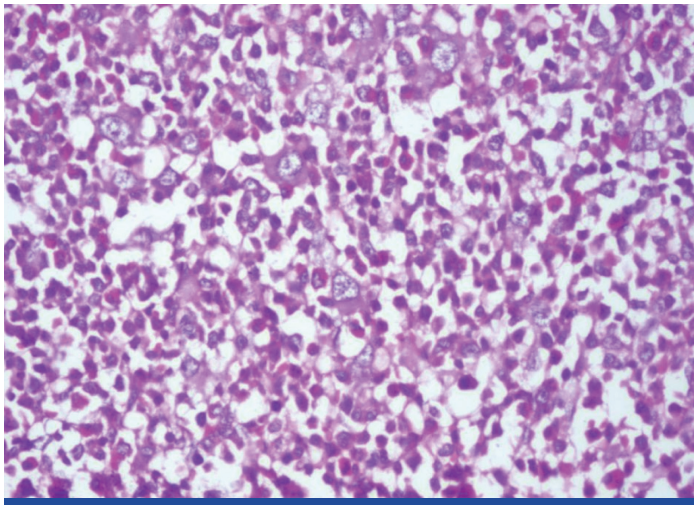
G/GMVsMVD: The 21 cases of CML in chronic phase were morphologically classified as Granulocytic (CML-G) and granulocytic megakaryocytic (CML-GM). Twelve cases were CML-G and the remaining 9 were CML-GM. The difference in mean MVD of both these groups was however not found to be statistically significant [Table/Fig-1].

DISCUSSION

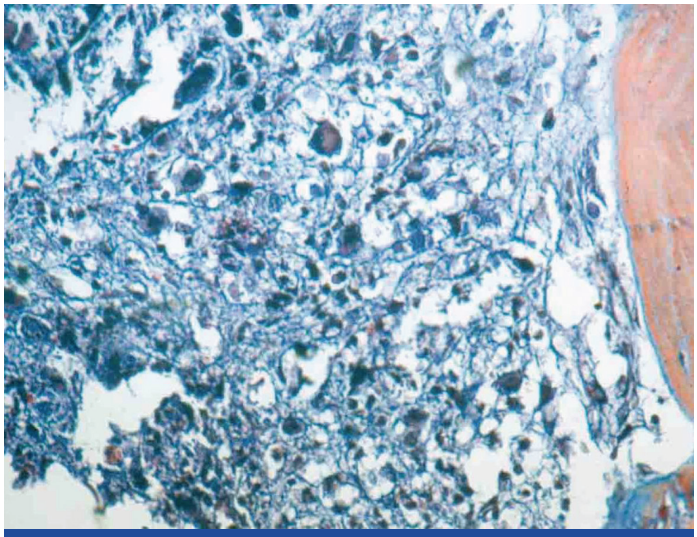
The normal bone marrow vascular bed consists of complex anastomosing network of sinusoids. The close interdependence between the bone marrow vessels and the hematopoietic cells has long been appreciated. Recent interest in bone marrow vascularity has brought to light the production of hematopoietic growth factors by endothelial cells [8,9]. These capillaries and sinusoids are functionally dormant and non-patent in a normal marrow which accounts for sparse MVD in a normal marrow.

MVD is considered a very important prognostic factor in Myelodysplastic syndromes and in assessing anti angiogenic therapy [10]. Though some studies on CML and AML demonstrate the increase in MVD, studies on other Chronic Myeloproliferative disorders like Polycythemia Vera, where angiogenesis was considered to be significantly higher than normal BM did not demonstrate significant increase in MVD assessed using CD34 staining [11]. These findings too question the reliability of CD34 staining to determine BM angiogenesis.

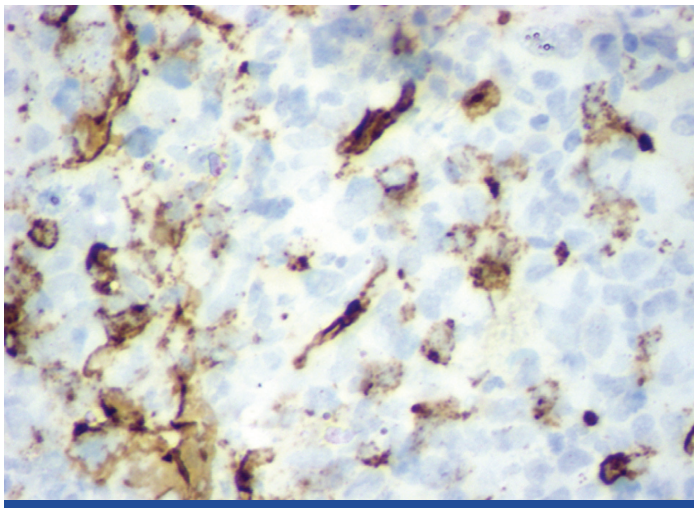
[Table/Fig-5,6] show cases of CML with high angiogenetic activity as demonstrated by a CD34 stain while [Table/Fig-7] depicts low angiogenesis.



[Table/Fig-3]: Chronic Myeloid leukemia H & E x 400



[Table/Fig-4]: A case of CML showing high grade(grade 3) fibrosis (WHO). (Masson trichrome x 100)

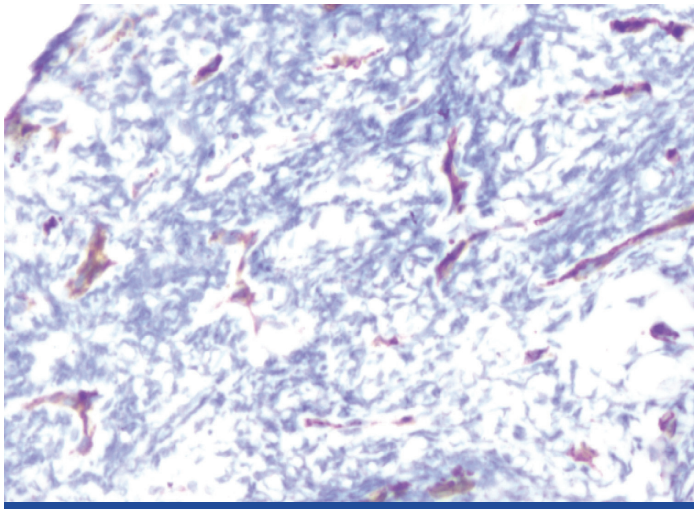


[Table/Fig-5]: Increased angiogenesis in a case of CML demonstrated by CD34 stain (CD34 x 400)

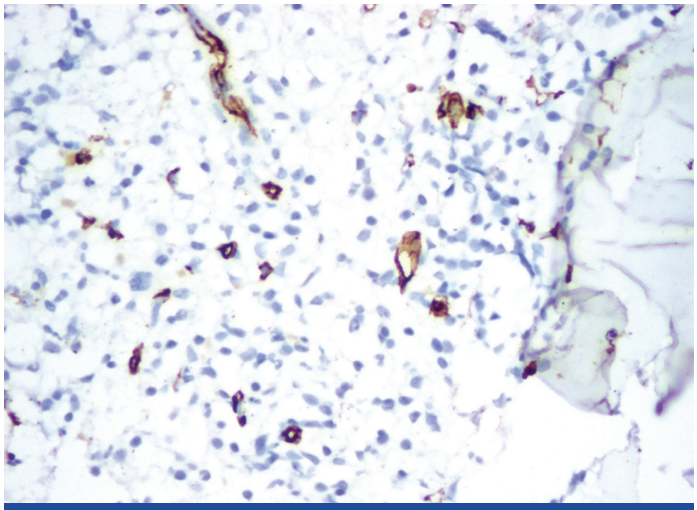
Why Choose CD34?

Current understanding of immunohistochemistry indicates that staining reaction is very reliable with CD34, although few and dispersed progenitor cells are co-stained, they are very easily distinguishable from endothelial cells by morphology. One of the problems with CD34 in blast crisis is the co-staining of blasts [11], although they could be differentiated from endothelial cells on careful observation.

Other studies too show that estimation of MVD by means of CD34 staining appear to be the most reliable method although none of the tested molecule qualifies as a specific marker for leukemia associated vessels in the bone marrow. Another commonly used marker to assess MVD is vWf which shows a consistently higher MVD than CD34 staining. This difference raised the question of whether vWf staining identified more vessels that characterise the Disease. One confounding factor, which might account for a higher MVD by vWf was a high positivity for mega karyocytes. Also, platelets localized near endothelial cells could also be mistaken for vWf staining endothelial cells. In order to preclude these issues, we chose to use CD34 staining in our study [11].



[Table/Fig-6]: Increased angiogenesis in a case of CML demonstrated by CD34 stain (CD34 x 400). Increased fibrosis in the marrow is also noted



[Table/Fig-7]: Low angiogenesis in a case of CML demonstrated by CD34 stain (CD34 x 400)

Why Hot Spots?

Haematologic neoplasms have a considerable amount of heterogeneity in contrast to solid tumours. Hot spot counting neutralizes the possibility of heterogeneous distribution within the neoplastic tissue. Also areas of intense neo-vascularization reflect the close and functionally important interaction between neoplastic cells and ensuing proliferation of endothelium associated with tumour growth and spread.

Some important conclusions from the results of the study were:

1. There is an increase in angiogenic process in CML patients when compared with controls, which is demonstrated by the CD34 stain.
2. The MVD in blast crisis was significantly higher when compared with the MVD in chronic and accelerated phases taken together.
3. Interestingly, there was no statistical difference in MVD between chronic and accelerated phases of CML and the mean MVD

was actually lower in the accelerated phase. This could be due to the relatively low number of cases in accelerated phase and needs to be elucidated further.

4. Grade of fibrosis is another parameter that is considered pathophysiologically related to the angiogenic process in the marrow. Most patients in blast crisis phase of CML had high grade fibrosis with significantly higher MVD than the low grade fibrosis group. Although the mean MVD was higher with higher grade of fibrosis (15.4 vs 14.9), there was no significant difference in MVD w.r.t. grade of fibrosis. One plausible explanation in this study could be the substantial presence of chronic phase patients in higher grade of fibrosis and relatively fewer cases in blast crisis phase.
5. In contrast to the Blast crisis patients, patients in the accelerated phase (3/5) had high grade fibrosis and the rest, low grade fibrosis without a significant difference in mean MVD between them.

These conclusions derived from the results affirm that there is certainly greater angiogenic process in the blast crisis phase of CML. However it raises the natural question – is not the angiogenic process different between the accelerated and chronic phase? This question stems from the fact that there was not any difference in MVD between the chronic and accelerated phase and no significant increase in MVD w.r.t. the grade of fibrosis.

Although our current understanding of Myeloproliferative disorders show that MVD assessment is an important prognostic factor, determination of vascularity has to include pathophysiologic aspects of perfusion and therefore quantification of MVD has to be improved by parameters that characterise blood flow with greater precision such as microvessel area (Luminal distension), shape (form factor), tortuosity and branching [12]. Microvessel number and branch points determined by confocal microscopy in studies also demonstrate increased branching from straight blood vessels in CML patients [7]. Studies on CML patients also show that BM angiogenesis plays an important role in the pathophysiology and prognosis of the disease. Also significant decrease in MVD through VEGF, CD34 has been demonstrated after interferon, imatinib and hydroxyl urea therapy. The 3-dimensional reconstruction of bone marrow also confirm these findings in CML patients [13].

In conclusion even though increased angiogenesis has been long recognised in various hematological malignancies, the molecular basis underlying this angiogenic switch in CML remains poorly understood. In addition to the usually applied quantification techniques, more elaborate morphometrical methods are required to assess tumour vascularity and angiogenesis in order to obtain a better insight of vascular architecture of the bone marrow in these patients.

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