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Original Article

Degree of fibrosis and its association with angiogenesis in the myelofibrotic bone marrow

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ABSTRACT

Background: Primary and secondary myelofibrosis has become a global burden due to its increased mortality and morbidity. Angiogenesis is a significant driving force in the development of fibrogenesis in the bone marrow, which leads to myelofibrosis. The microvascular density (MVD) with immunomarker CD34 can be used to assess the degree of angiogenesis. The objective of this study was to examine the association between degree of myelofibrosis and angiogenesis in hematological malignancies.

Methods: Forty-six trephine biopsy specimens of various hematological malignancies with myelofibrosis were studied at the Department of Pathology of Bangabandhu Sheikh Mujib Medical University. Extent of myelofibrosis in each case was assessed by examining the reticulin and Masson's trichrome stained sections using a semiquantitative grading system of bone marrow fibrosis (MF) within a scale of MF-0 to MF-3. Angiogenesis was measured by counting MVD in the 'hotspots' after immunostaining with CD34 antibody.

Results: The trephine biopsy cases were grouped into early fibrotic (MF-1) and advanced fibrotic (MF-2,3) consisting of 16 (34.8%) and 30 (65.2%) patients, respectively. Angiogenesis was estimated as mean MVD count which revealed 16.7 ± 5.4 and 32.0 ± 11.5 in these groups, respectively. Significant difference of mean MVD values (P<0.001) between the early and advanced fibrotic groups revealed the association of angiogenesis and degree of myelofibrosis.

Conclusion: MVD may be used to measure angiogenesis in myelofibrotic marrow along with other clinical and laboratory indices as a marker of disease activity in hematological malignancies, thus aiding disease prognosis.

Keywords: myelofibrosis, angiogenesis, microvascular density, anti-angiogenic drug, CD34

INTRODUCTION

Myelofibrosis is a cytokine mediated process of the bone marrow stroma which is associated with many different types of reactive conditions including autoimmune and granulomatous disease and a variety of neoplastic disorders.[1] The condition is of major concern for compromising quality of life from debilitating disease-related constitutional symptoms, progressive bone marrow failure and extramedullary hematopoiesis resulting in progressive splenomegaly or hepatosplenomegaly.[2]

Myelofibrosis may occur in clonal myeloproliferative disease with characteristic proliferation of megakaryocytic and granulocytic components in the bone marrow, which is associated with reactive

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HIGHLIGHTS

1. **Angiogenesis is necessary not only for the survival of malignant progenitor cells but also gradual fibrogenic replacement of bone marrow in hematological malignancies which can be assessed easily by estimation of CD34 positive microvascular density (MVD).**
2. **Degree of myelofibrosis and MVD both can predict prognosis of the disease and response to therapeutic agents.**
3. **Evaluation of degree of myelofibrosis and angiogenesis can be practiced in trephine biopsies of all hematological malignancies.**

deposition of bone marrow connective tissue progressively from early to overt fibrotic stage culminating in ineffective hematopoiesis and extramedullary hematopoiesis. This condition is known as chronic idiopathic myelofibrosis (CIMF) or primary myelofibrosis (PMF). It is associated with JAK2/CALR/MPL mutation.[3-4] Bone marrow fibrosis associated with other neoplastic and non-neoplastic hematological and non-hematological disorders is called secondary myelofibrosis. Among hematologic malignancies, several myeloid neoplasms, including MPNs other than PMF, myelodysplastic/ myeloproliferative neoplasms (MDS/MPN), myelodysplastic syndromes (MDS), lymphoma, leukaemia etc. are associated with secondary myelofibrosis.[5-6]

Several conditions are associated with increase in reticulin as well as collagen fibrous content in the bone marrow. 'Reticulin fibrosis' denotes an increase in reticulin fibres. Deposition of collagen fibres of any quantity constituting the dominant pattern of fibrosis in bone marrow is implied with the term 'collagen fibrosis'. In bone marrow biopsies, stromal structural fibres are detected by reticulin and Masson's trichrome stains.[6] Some patients with collagen fibrosis may also present with new bone formation in the marrow space, the condition is known as 'osteosclerosis'.[7] "European consensus on bone marrow fibrosis (MF) grading, 2005" allows quantitative and qualitative evaluation of bone marrow fibrosis ranging from MF-0 to MF-3 based on reticulin fibrosis, collagen fibrosis and osteosclerosis, which was adopted by the latest WHO classification system for tumours of the hematopoietic and lymphoid tissues.[4,8]

Angiogenesis is a major driving force in numerous types of solid as well as hematological malignancies by providing a vascular support for delivering oxygen and nutrients for the growth of tumours. It is the process of development of new blood vessels from pre-existing vasculature induced by the production and release of angiogenic factors from tumour cells.[9] Estimation of the extent of tumour vascularity, as measured by pathological microvascular density (MVD), is necessary to predict aggressiveness of a tumour. The endothelial cell or clusters of endothelial cells, clearly separated from adjacent microvessels, neoplastic cells and other connective tissue elements, is considered as a single countable microvessel.[10-11] Angiogenesis is also assessed semiquantitatively by estimation of microvessel grade.[12] Several hematological malignant neoplasms with increased MVD are associated with unfavorable prognosis as revealed by previous studies, which potentiates measurement of angiogenesis in the investigation of a useful therapeutic strategy.[13-14]

Angiogenesis is a complex multistage process involved in the development of myelofibrosis. The increase in collagen type IV formation in myelofibrosis is thought to be generated by the pronounced endothelial cell proliferation in the course of neovascularization.[15] Mitogenic cytokines for endothelial cells, such as PDGF and b-FGF etc. are derived from clonal proliferating population of hematopoietic stem cells and/or its progeny in bone marrow microenvironment which appear to be necessary for fibrogenesis along with angiogenesis to take place.[2,9,13] CD34 immunomarker is a highly specific pan-endothelial marker for staining of small and large vessels with equal intensity.[16-17] CD34 expression is predominantly found on the luminal membrane of cellular process but also may be seen on the abluminal membrane of cells found at the tips of vascular sprouts.[18] Assessment of mean MVD and microvessel grade is done using CD34 for quantification of microvasculature.[12,19]

Analysis of angiogenesis in myelofibrotic bone marrow may provide further prognostic significance. It may lead to the direction of potent therapeutic anti-

angiogenic drugs. We, therefore, designed the study to find out any possible association between degree of marrow fibrosis and CD34 marker aided angiogenesis, based on evaluation of 46 trephine biopsy specimens of myelofibrosis, irrespective of their primary or secondary origin in hematological malignancies.

METHODS

Study design

Between March 2020 and February 2022, a total of 46 patients of hematological malignancies who underwent trephine biopsy and histopathologically diagnosed with myelofibrosis were enrolled in this analytical cross-sectional study. This study was conducted at the Department of Pathology of Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka. After approval by the Institutional Review Board of BSMMU, the study patients were identified according to sample selection criteria. Cases of hematological malignancies were included in this study which revealed myelofibrosis in H&E stain and/or, bone marrow fibrosis with at least grade MF-1 in routinely provided reticulin and Masson's trichrome stains.

The sample size was determined by the number of cases reported during the study period. Clinical and laboratory information was obtained at diagnosis and recorded systematically in a prepared proforma. Myelofibrosis secondary to chemotherapy or radiotherapy, myelofibrosis associated with non-malignant conditions, bone marrow specimen showing extensive crush artifact or inadequacy (< 1cm in length), and patients unwilling to give informed consent were excluded from the study.

The study population comprised cases with primary myelofibrosis (n=20), chronic myeloid leukaemia (n=15), myeloproliferative neoplasm associated myelofibrosis (MPN-MF) (n=3), plasma cell dyscrasia (n=3), polycythaemia vera (n=1), essential thrombocythaemia (n=1), myelodysplastic syndrome with fibrosis (MDS-F) (n=1), Hodgkin lymphoma (n=1) and acute leukaemia (n=1).

For the purpose of this study, all the H&E and special stained slides were collected and reviewed. Paraffin blocks of selected cases were taken for subsequent sectioning and immunohistochemical staining with CD34 antibody.

Re-evaluation of bone marrow sections

H&E stained sections with at least three well preserved marrow spaces were studied for cellularity, status of granulopoiesis, erythropoiesis and megakaryopoiesis, presence of dysplasia and other hematological elements. Reticulin and Masson's trichrome stained sections were reviewed for grading of bone marrow according to pattern of reticulin fibrosis (MF-1 to MF-3), collagen fibrosis (grade: 0-3) and osteosclerosis (grade: 0-3) as followed by Kvasnicka et al.[20] Total study population was divided into two groups after evaluation of bone marrow fibrosis. Those cases with degree of fibrosis MF-1 belonged to the early fibrotic group, whereas MF-2 and MF-3 cases constituted advanced fibrotic group.

Semiquantitative bone MF grading system based on "European consensus, 2005"[20]

- MF-0: Scattered linear reticulin with no intersections corresponding to normal bone marrow
- MF-1: Loose network of reticulin with many intersections, especially in perivascular areas
- MF-2: Diffuse and dense increase in reticulin with extensive intersections, occasionally with only focal bundles of thick fibres mostly consistent with collagen and /or focal osteosclerosis
- MF-3: Diffuse and dense increase in reticulin with extensive intersections with coarse bundles of thick fibres mostly consistent with collagen with significant osteosclerosis

Semiquantitative grading of collagen deposition[20]

- Grade 0: Perivascular collagen only (normal)
- Grade 1: Focal paratrabecular or/and central collagen deposition without connecting meshwork
- Grading 2: Paratrabecular or/and central collagen deposition with focally connecting meshwork or generalized paratrabecular apposition of collagen
- Grading 3: Diffuse (complete) connecting meshwork of collagen

Semiquantitative grading of osteosclerosis [20]

- Grade 0: Regular bone trabeculae (distinct paratrabecular borders)
- Grade 1: Focal budding, hooks, spikes or paratrabecular apposition of new bone
- Grade 2: Diffuse paratrabecular new bone formation with thickening of trabeculae,

occasionally with focal interconnection

- Grade 3: Extensive interconnecting meshwork of new bone with overall effacement of marrow spaces

Immunohistochemical analysis

For evaluation of microvessels, CD34 antibody was selected as a formalin resistant endothelial cell marker having the property of highest average immunolabeling intensity for endothelial cells in tumour neovascularisation with comparison to CD31 and FVIII-RA.[17,19,21] Four µm-thick sections of formalin-fixed, paraffin-embedded tissues were mounted on poly-L-lysine coated slides for immunohistochemistry. The sections were deparaffinized in xylene and rehydrated in a descending ethanol series. Sections were incubated for 5 minutes in 3% hydrogen peroxide to block endogenous tissue peroxidase. The sections were incubated with a monoclonal antibody CD34 in appropriate dilution. Appropriate positive control was used in trephine biopsy of normal bone marrow for each batch of slides.

Microvessels count

Angiogenesis was assessed by estimation of MVD in early and advanced fibrotic groups as followed by Pereira et al.[22] At first, the stained section was screened at low power (10x) to identify the hot spot (highest area of microvessel concentration). Blood vessels were then counted under x40 magnification. Those brown colored endothelial cells positive with CD34 which may be single without lumen or form cluster of endothelial cells with a lumen were considered as a microvessel.[11] Vessels with muscular wall, vessels in the periosteum and open sinusoids were not counted. Areas occupied by fat were also excluded from measurement. The presence of blood cells or fibrin without any detectable endothelial cells was considered to be insufficient to define a microvessel. MVD of three hot spots were counted and calculated for the mean of MVD. The value was recorded as mean (standard deviation) of MVD/ HPF per slide. The analyses were performed independently by two observers (SA and BPD) to avoid observer bias.

Statistical analysis

The statistical analysis of all the recorded data was carried out using the Statistical Package for Social Sciences version 24.0 for Windows (SPSS Inc., Chicago, Illinois, USA). Continuous variables were expressed as mean and standard deviation, and categorical variables were expressed as absolute frequency and percentage, which were presented in the tables. Chi-squared test was done to compare the features of age related cellularity and status of granulopoiesis and megakaryopoiesis between two groups. Student's t-test was done to analyze the association between degree of fibrosis and MVD. The correlation between the degree of fibrosis and MVD was also analyzed using the Pearson correlation. $P \leq 0.05$ was considered statistically significant.

RESULTS

The mean (standard deviation) age of the participants was 44.5 (16.0) years. Thirty-one patients in this study were male (67.4%) and only 15 cases (32.6%) were female. All the patients had constitutional symptoms. Thirty-three (71.7%) patients presented with splenomegaly or hepatosplenomegaly. A total of 34 (73.9%) cases had history of blood transfusion. Complete blood count findings of 46 patients are shown in **TABLE 1**.

According to the findings of special stains, degree of myelofibrosis including pattern of reticulin and collagen fibrosis and osteosclerosis were evaluated **(TABLE 1 and 2)**. Age related cellularity, status of granulopoiesis and megakaryopoiesis were evaluated from the H and E preparations **(FIGURE 1- a and b)**. All the patients had demonstrable deposition of reticulin fibres using reticulin stain as followed by Kvasnicka et al.[20] **(FIGURE 1– c and d)**. Collagen fibrosis and osteosclerosis were present in 23 (50%) and 15 (32.6%) patients, respectively within proposed grade

TABLE 1 Complete blood count findings and distribution of patients according to pattern of marrow fibrosis (n=46)

Variables	Results
Complete blood count, mean (standard deviation)	
Haemoglobin (g/dl)	8.4 (2.1)
White blood cells x10^9/L	13.6 (11.3)
Platelets x10^9/L	271.9 (234.7)
Pattern of fibrosis, Number (%)	
Reticulin fibrosis	46 (100.0)
Collagen fibrosis	23 (50.0)
Osteosclerosis	15 (32.6)

TABLE 2 Distribution of patients according to degree of myelofibrosis (reticulin fibrosis, collagen fibrosis and osteosclerosis) (n=46)

Variables	Advanced fibrotic (n=30)	Early fibrotic (n=16)
	n (%)	n (%)
Bone MF grade		
Grade 0	0	0
Grade 1	0	16 (100.0)
Grade 2	21 (70.0)	0
Grade 3	9 (30.0)	0
Mean (SD*) of bone MF grade	2.3 (0.5)	1.0 (0.0)
Collagen fibrosis grade		
Grade 0	7 (23.3)	16 (100.0)
Grade 1	12 (40.0)	0
Grade 2	9 (30.0)	0
Grade 3	2 (6.7)	0
Mean (SD*) of collagen fibrosis grade	1.2 (0.9)	0
Osteosclerosis		
Grade 0	15 (50.0)	16 (100.0)
Grade 1	8 (26.7)	0
Grade 2	7 (23.3)	0
Grade 3	0	0
Mean (SD*) of osteosclerosis grade	0.7 (0.8)	0

*SD indicates standard deviation

category of '1-3'. The former is best demonstrated with Masson's trichrome stain.[20] The mean (standard deviation) values of bone MF, collagen fibrosis and osteosclerosis grades were 2.3 (0.5), 1.2 (0.9) and 0.7 (0.8), respectively. Finally, sixteen (34.8%) patients were classified in early fibrotic group (MF-1) and 30 (65.2%) patients in advanced fibrotic group (MF-2 to MF-3).

TABLE 3 Morphological findings of bone marrow and microvascular density of patients (n=46)

Variables	Advanced fibrotic (n=30)	Early fibrotic (n=16)	*P*
	n (%)	n (%)	
Age related cellularity			
Increased	13 (43.3)	14 (87.5)	
Decreased	17 (56.7)	0	<0.001
Normal	0	2 (12.5)	
Granulopoiesis			
Increased	9 (30.0)	13 (81.3)	
Decreased	15 (50.0)	0	0.001
Active	6 (20.0)	3 (18.8)	
Megakaryopoiesis			
Increased	20 (66.7)	16 (100.0)	0.009
Decreased	10 (33.3)	0	
Mean (SD*) of microvascular density	32.0 (11.5)	16.7 (5.4)	<0.001

*SD indicates standard deviation

All of these parameters were found significantly diminished with advancement of bone marrow fibrosis (P<0.001). Mean MVD is calculated in two myelofibrotic groups using CD34 **(FIGURE 1- e and f)**. Student's t-test demonstrated that patients with advanced myelofibrosis has significantly higher MVD values than those with early stage of fibrosis (P<0.001). **TABLE 3** illustrates the morphological findings of bone marrow and difference of MVD of patients.

The correlation co-efficient was evaluated between degree of bone marrow fibrosis and CD34+ MVD. A significant positive correlation was found between MVD and myelofibrosis grade by Pearson correlation test (correlation co-efficient, r=0.75 and P<0.001) **(FIGURE 2)**.

DISCUSSION

Due to frequent association of myelofibrosis with dry tap, it is diagnostically important to obtain an adequate trephine biopsy from fibrotic bone marrow. Though study of bone marrow morphology is the basis for diagnosis, collaboration of clinical features, laboratory parameters and additional molecular testing becomes necessary in severe myelofibrosis with distorted histomorphologic features.[23-25] Most diseases with increased bone marrow fibrosis are associated with abnormalities of the number and/ or function of megakaryocytes and platelets and significant elevation in circulating pro-inflammatory cytokines. TGF-β, PDGF, FGF, cytokines and other growth factors from megakaryocytes and platelets, monocytes, even mast cells, appear to be necessary for fibrogenesis to take place. TGF-β is a pleiotropic cytokine that potently stimulate fibroblasts to produce extracellular matrix (ECM). In altered bone marrow microenvironment, ECM induces growth of new capillaries. Increased expression of basic fibroblast growth factor (b-FGF) and PDGF by megakaryocytes and endothelial cells is associated with both fibroblasts growth and higher degree of angiogenesis. These steps act reciprocally to promote proliferation of malignant progenitor cells and tumour progression.[2,6,13,26] Recent study hypothesized the role of pericytes in the secretion of fibrosis stimulating cytokines. These cells can differentiate into fibroblasts as well as endothelial cells.[27]

In the present study, most of the patients (70%) were present in MF-2 category corresponding to advanced fibrotic stage of myelofibrosis and only two patients

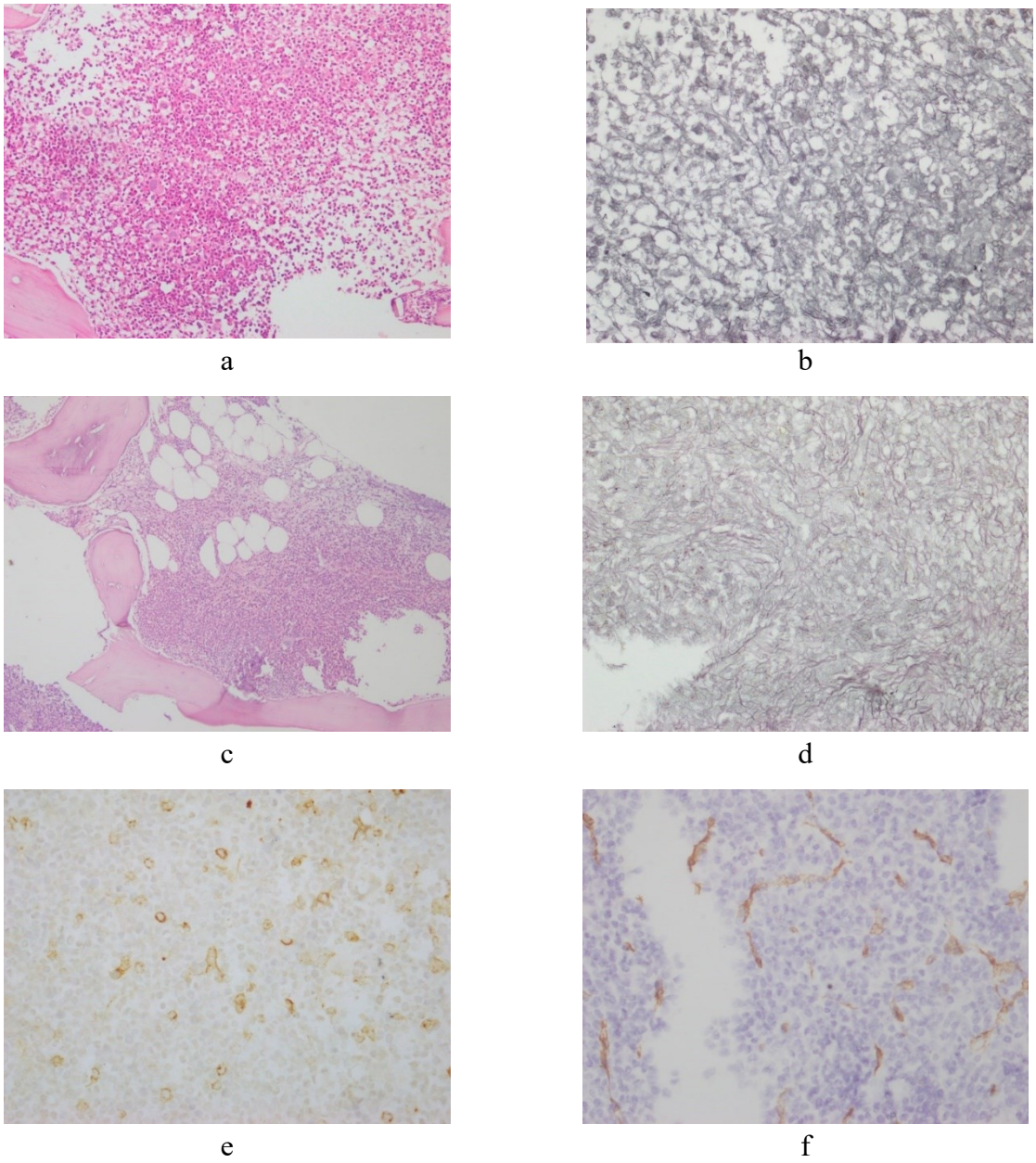

FIGURE 1 Photomicrograph showing cases of (a) chronic myeloid leukaemia, H&E stain (200x) with early fibrotic marrow revealed, (b) primary myelofibrosis, H&E stain (200x) with advanced fibrotic marrow revealed, (c) MF-1 with reticulin stain (400x), (d) MF-2 with reticulin stain (400x), (e-f) microvessels in above cases, respectively highlighted with CD34 immunostain (400x)

had diffusely deposited collagen fibres corresponding to grade-3 collagen fibrosis. Reticulin fibrosis is often reversible after therapeutic intervention, while collagen fibrosis is less likely to be so. The later signifies more severe concurrent disease conditions of the marrow.[6] Higher degree of bone marrow fibrosis predicts poor prognosis in terms of survival.[8] Age related cellularity was found significantly diminished with advancement of fibrosis. Determination of cellularity and fibrosis is important to assess therapeutic efficacy and to predict disease progression.[1,20]

We demonstrated here that advanced fibrotic bone marrow cases with hematological malignancies are associated with an increased mean MVD compared to

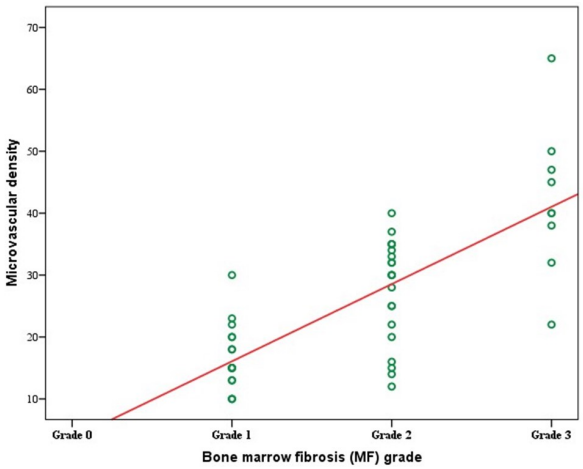

Figure 2 Correlation of degree of bone MF with microvascular density (n=46)

the bone marrow of early fibrosis. The present data were consistent with previous observation in other populations and suggest pathophysiological association of angiogenesis and evolution of advanced myelofibrosis. A case control study conducted by Panteli et al.[14] revealed MVD values with CD34 ranged from 1 to 15 (median value-8) in controls with 27 healthy subjects without bone marrow fibrosis. That study confirmed a significantly higher degree of angiogenesis in myelofibrosis with myeloid metaplasia (MMM) compared with controls. Ponce et al.[12] demonstrated higher MVD values with TGF-β1 immunostain in patients with fibrotic and prefibrotic primary myelofibrosis than those with essential thrombocythaemia and the controls and a strong correlations between MVD with degree of fibrosis (P<0.001). A correlation was present between latent TGF-β1 immunoexpression in megakaryocytes and angiogenesis which promotes the development of bone marrow fibrosis.

According to the study performed by Ni et al.,[13] the higher degree of angiogenesis was observed to be associated with increased expression of basic fibroblast growth factor (b-FGF) in megakaryocytes and endothelial cells in the bone marrow of primary myelofibrosis. However, they did not find any significant difference of MVD between the pre-fibrotic and overt fibrotic groups (P=0.25). In another study, Ponzoni et al.[10] found significant difference of mean MVD between those groups using CD34 (P<0.001). Mesa et al.[19] found out positive correlation of angiogenesis with increases in marrow cellularity and megakaryocyte clumping (P=0.01) in a study on 114 patients with MMM. Prominent megakaryocytic VEGF expression produces secretion of angiogenic cytokines as evidenced by higher serum levels of VEGF in most patients with MMM.[9,19,28] Mesa et al.[19] also revealed that increased angiogenesis, advanced age and increased percentage of circulating blasts were significant risk factors for overall survival.

The main purposes of application of therapeutic regimen in hematological malignancies are to impede their progression and leukaemic transformation and to lessen myelofibrosis associated complications. Presence of higher level of microvessels density has proved its association with the pathogenesis of leukaemia.[29] Chemotherapeutic drugs exert their anti-angiogenic effects possibly by impairing the growth and function of microenvironmental cells and promoting apoptosis of endothelial cells.[30] Recent study revealed significant reduction of bone marrow MVD in completely recovered patients from acute myeloid leukaemia following chemotherapy.[31] Progression of myelofibrosis may be altered by probable changes in angiogenic cytokine release by these drugs.[32] In a phase II trial of an angiogenesis modulator, Lenaliomide have been shown significant reduction of bone marrow fibrosis. Janus kinase (JAK) inhibitors have minor impact on reduction of bone marrow fibrosis even after many years of treatment.[33]

The lack of evaluation of angiogenesis in separate groups of primary and secondary myelofibrosis has limited the study to analyze the comparison between two entities, which was not possible due to short number of cases. Though CD34 is most widely used pan-endothelial marker, it cannot differentiate between tumour associated newly formed vessels and pre-existing large vessels. The problem might be overcome with concurrent use of monoclonal CD105 antibody which preferentially reacts with blood vessels undergoing angiogenesis.[17]

Conclusion

Myelofibrosis, primary or secondary to any hematological malignancies, is well established by preceding angiogenesis which is reflected by a rise of MVD in accordance with degree of fibrosis. Thus, it can

be considered diagnostic research as it helps predict prognosis of the disease and monitor treatment progress. Diagnostic accuracy will be tested in near future. Anti-angiogenic drugs may be considered to be taken under clinical trial along with other specific therapy in the treatment of such "liquid tumours". For the assessment of prognostic relevance of angiogenesis, further study is required to be validated in larger cohort of patients.

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Author Contributions

- Conception and design: SA
- Data acquisition, analysis, and interpretation: SA, AKMNK, BPD, MMR, PR and SJ
- Manuscript drafting and revising it critically: SA, AKMNK, BPD, MMR, PR, RK, SSUM, UTNE and SJ
- Approval of the final version of manuscript: AKMNK
- Guarantor accuracy and integrity of the work: SA

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Conflict of Interest

The authors have no conflict of interest to disclose.

Ethical approval

Ethical approval was obtained from the Institutional Review Board of BSMMU (memo no. BSMMU/2021/1698) on 03 March 2021.

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