Evaluation of Angiogenesis in Idiopathic Myelofibrosis with CD31 and CD34 by Light Microscope and Computer-Aided Image Analysis System.

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Abstract

Background: Myelofibrosis with myeloid metaplasia (MMM) is a clonal stem cell disease with varying degrees of fibrosis in the marrow.

Vasculogenesis is the formation of new blood vessels from pre-existing vessels during adult life; it plays a critical role in neoplastic development, progression and metastasization and has been shown to be an adverse prognostic factor in many solid tumors.

It is becoming increasingly evident that angiogenesis plays a key role in the pathophysiology of hematologic malignancies by estimating bone marrow microvessel density and by measuring circulating angiogenic factors. MMM is probably the disease with the more pronounced angiogenesis among Chronic Myeloproliferative Disorders.

Objective:

1. Evaluation of angiogenesis in Idiopathic Myelofibrosis (IMF) via immunohistochemical staining for CD31 and CD34 and quantifying them by Computerized Image Analysis System and Light Microscope.

2. To investigate whether angiogenesis can be considered as a marker for disease activity.

Methods: This cross sectional study was conducted on 31 formalin fixed paraffin embedded blocks of Idiopathic Myelofibrosis cases along with 10 age matched control cases having no abnormal bone marrow pathology, from January 2006 to June 2006. The sections from bone marrow biopsies were processed routinely and stained with Hematoxylin and Eosin (H&E) and with immunohistochemical stain for CD31 and CD34 markers.

The bone marrow Microvessel Density of IMF and control case was assessed by the means of Computer Aided Image Analysis System and by visual count using light microscope.

Results: This study revealed that there was a significant increase in microvessel density in IMF cases, using both the computerized method and the visual count by light microscope, with both CD31 & CD34 compared to control group ($P < 0.05$).

Also this study showed that the increase in angiogenesis was positively correlated with the Dupreiz score prognostic system and the age of the patients.

On the other hand; no significant correlation was found between angiogenesis and the following parameters: sex of the patient, Hb value, WBCs count, peripheral blood platelets count.

Conclusion: This study has showed that angiogenesis was an integral component of the bone marrow stromal reaction in MMM and it was closely related to many prognostic parameters; thus bone marrow angiogenesis can be used as a tool to assess the disease activity.

Key word: Idiopathic myelofibrosis, angiogenesis, Computeriz image analysis.

IRAQI J MED SCI, 2010; VOL.8 (3):25-33

Introduction

Idiopathic Myelofibrosis (IMF) or as also called Myelofibrosis with Myeloid Metaplasia (MMM) is one of the Philadelphia-negative chronic myeloid disorders and is stem cell disorder characterized by clonal megakaryocytic hyperplasia, a leukoerythroblastic peripheral blood smear, extramedullary hematopoiesis, and secondary bone marrow fibrosis[(1)].

Angiogenesis, or the formation of new blood vessels, is an important

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Received: 5th October 2009, Accepted: 21st April 2010.

process in health and disease and may be integral to solid tumor growth and metastasis.[2,3] The concentration of these vessels has been shown to be increased in various hematologic disorders, and it may provide useful prognostic informations [(2- 6)].

Indeed with the use of immunohistochemistry, various antibody markers for endothelial cells have been used to identify intratumoral vessels, the most commonly used markers are CD31 and CD34 [(7, 8)].

Quantification and analysis of the degree of intratumoral angiogenesis is mostly done by estimating the microvessel density (MVD) which is maximal number of blood vessels per unit area of section[(7, 8)].

The recent introduction of morphometric analysis using fully automatic Computerized Image Analysis (CIA) has offered objectivity, increase precision compared with direct visual appraisal and makes statistical analysis easier, as it is possible now to measure MVD and the intercapillary distance (ICD) [(9)].

Subjects, Materials, and Methods

This cross sectional study was conducted on 31 formalin fixed paraffin embedded blocks of Idiopathic Myelofibrosis cases along with 10 age matched control cases having normal bone marrow.

The cases were collected from the archives of the histopathology laboratory in Al Kadhimiya teaching hospital; Al Yarmook Center for Blood Disorders; the Teaching Laboratory of Medical City Hospital, and from private laboratories from January 2006 to June 2006.

The selection criteria of Idiopathic myelofibrosis cases was based on the biopsies reports which was examined by a specialized haematologist, and other diagnostic criteria of IMF; which include bone marrow fibrosis, atypical megakaryocytic hyperplasia, and peripheral blood leukoerythroblastosis, in addition to clinical & physical findings. Whereas the bone marrow biopsies of control cases showed no bone marrow abnormalities and no excess in fibrous tissue.

From each specimen, 3 sections of 5 µm thicknesses were taken, the first was stained with Hematoxyline and Eosin stain and the other two were stained immunohistochemically with CD31 and CD34 antibodies.

Both patients and controls sections were examined for bone marrow microvessel density at x100, x200, and x400 magnification, and five hot spot areas (areas of highest neovascularization) in each CD31 and CD34 stained sections were selected and the mean number of microvessels (MVD) was measured,and the same field was examined by light microscope (LM) and by computerized image analysis system (CIA)and was compared with that of normal reactive bone marrow specimens.

For performing visual count by LM, each of the study slide was first scanned at 100× magnification, and 5 hot spot areas were defined and the number of blood vessels were determined at 400× magnification, and that number was divided by the tissue area of that field (tissue area using Olympus microscope at 400× is 0.1885 mm^2) yielding microvessel visual count/area (MVC/Area) parameter which is comparable to the MVD estimated by CIA system and this was used for statistical analysis [(10)].

During the counting process, large vessels, tortuous vessels, and vessels in the periosteum or bone and open

sinusoids were excluded. Areas of staining with no discrete breaks were counted as single vessels, and the presence of a Lumen was not required.

The Computerized image analysis (CIA) system used in this study is an automatic morphometry machine that analyse semi-automatic features using computerized pixel counting. Microvessel surface area was determined and expressed as the percent of the area showing the stained blood vessel in relation to the total tissue area which is the Microvessel Density (MVD) [11].

Lille Scoring System (Dupriez Score) [12] was done for all the patients; accordingly, three distinct prognostic groups were identified depending on three adverse prognostic factors as shown in the following table:

Table 1: The Lille Scoring System (Dupriez score) for Predicting Survival in MMM [12].

Number of adverse prognostic factors*	Risk Group	Median Survival(months)
0	Low	93
1	Intermediate	26
2	High	13

*Adverse prognostic factors: Hb less than 10 g/dL and WBC count less than 4 or greater than 30 x 10^9/L.

Statistical analysis was done using descriptive statistics, the Student's unpaired t-test, and analysis of variance (ANOVA). In addition to applying the rules of correlation and regression (r). P value of < 0.05 was considered statistically significant.

Results

This study was conducted retrospectively on 31 cases of MMM and 10 aged matched control cases Table 2. The [Hb], WBCs count, and Platelets count were obtained from the patients' data sheets and are shown in table 3.

Lille Scoring System (Dupriez Score) was able to identify three distinct prognostic groups according to three adverse prognostic factors as shown in table 3.

The results of the staining procedure with CD31 and CD34 were compared to that of the appropriate control sections & a cytoplasmic reddish - brown coloration of endothelial lined vessels was considered as a positive reaction (Figures 1 and 2).

In this study the mean of the MVC/Area, using light microscope, was significantly higher using both CD31 and CD34 in patients with MMM (394.42±155.74 no./mm^2, 455.1474±168.95 no./mm^2 M±SD respectively) than in the control cases (16.2±18.73 no./mm^2, 18.55±17.88 no./mm^2 M±SD respectively) ($p < 0.05$), using t-test (Table 4).

By using the Computerized image analysis (CIA) the microvessel density (MVD) was significantly higher with both CD31 and CD34 in patients with MMM (7.93% ± 4.38%, 9.64% ± 4.47% M± SD respectively) than in the control cases (1.50%±0.91%, 1.57%±0.74% M±SD respectively) ($P < 0.05$), using t-test (Table 4).

In this study there was a significant positive relation between age of the patient and MVD using both CD31($r=0.67$, $P<0.05$) and CD34($r=0.62$, $P<0.05$).

By using Analysis of variance (ANOVA) there was a significant

statistical association between MVD and MVC/Area and the three grades of Dupriez Score using both markers, as shown in tables 5(A&B) & 6(A&B).

This study revealed that there was no relation between angiogenesis, by using both markers and the following parameters in MMM patients ($p>0.05$): Sex of the patient, Hb concentration, peripheral blood platelets, and peripheral WBCs count.

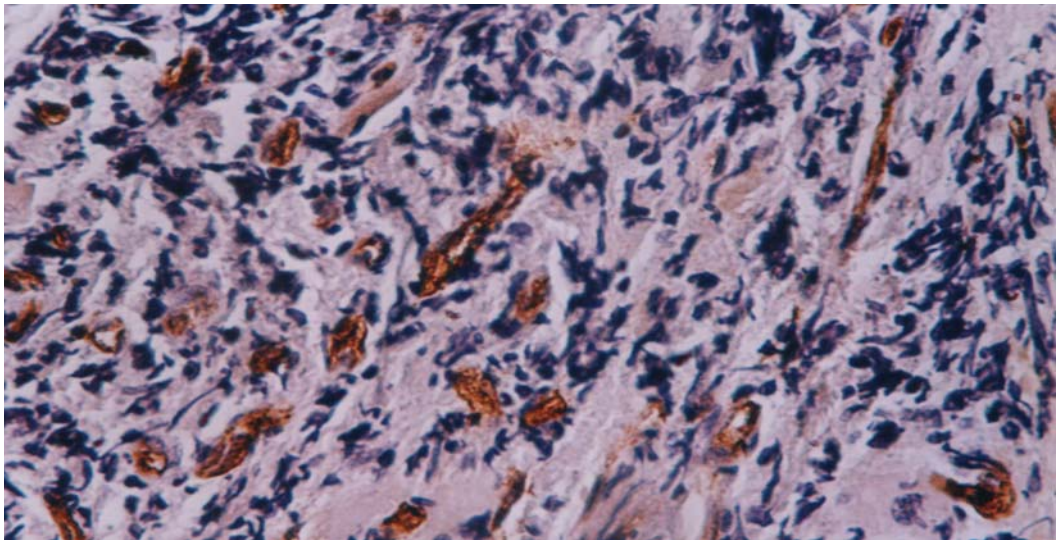

Figure 1: Positive CD31 stained blood vessels (400X).

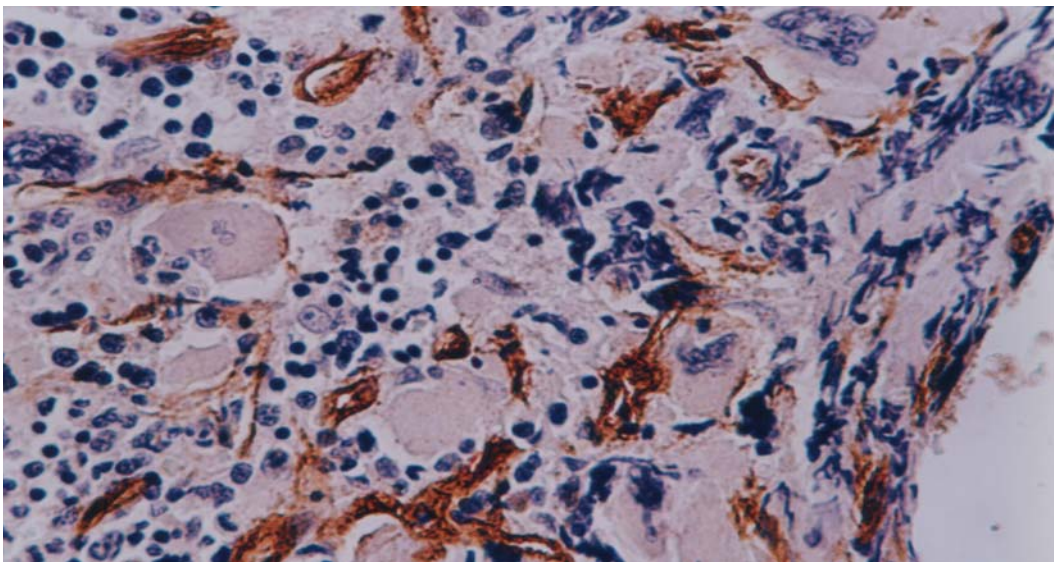

Figure 2: Positive CD34 stained blood vessels (400X).

Table 2: Sex and Age distribution of MMM and Control cases

	MMM patients		Controls	
	No.	%	No.	%
Males	22	71	6	60
Females	9	29	4	40
♂:♀	2.4 : 1		1.5 : 1	
Age range (Years)	38 - 65		38 - 53	
Age Median (Years)	57		44.5	
Age mean ± SD (Years)	55.48 ± 7.66		45.3 ± 5.1	

Table 3: Haematological descriptive profiles of the 31 cases with MMM.

Clinicopathological parameters		Number	Mean	Median	Range	%
Spleenomegaly		31	-	-	-	100%
Haemoglobin (Hb) g/dl		-	9.82 ± 1.59	9.5	6 - 13	-
WBC x10^9/l	**Low (<4 x10^9/l)**	3	12.2 ± 9.16	8.8	3.2 - 34	-
	Normal (4-11 x10^9/l)	19				
	High (>11x10^9/l)	9				
Platelets x 10^9/l	**Low (<150 x10^9/l)**	15	157.5 ± 96.2	150	25 - 450	-
	Normal (150-400 x10^9/l)	15				
	High (>400x10^9/l)	1				
Dupriez Score	**0**	10	-	-	-	32.25%
	1	19	-	-	-	61.29%
	2	2	-	-	-	6.45%

Table 4: Descriptive statistics of MVC/Area and MVD with both CD31 and CD34 in MMM and Control cases

parameters	CD31				CD34			
	MVC/Area (no./mm^2)		MVD (%)		MVC/Area (no./mm^2)		MVD (%)	
	MMM	*Control*	*MMM*	*Control*	*MMM*	*Control*	*MMM*	*Control*
Mean	394.42	16.2	7.93	1.50	455.1474	18.55	9.64	1.57
Standard Deviation	155.74	18.73	4.38	0.91	168.95	17.81	4.47	0.74
Median	389.44	13.39	6.59	1.41	445.75	26.32	8.82	1.16
Range	133.1-636.2	0 - 53	2.5 -19.2	0.44 -2.95	133.8 -730.9	0 – 52.5	3.8 -20.2	0.8 - 2.6

P< 0.05 comparing MVD and MVC with control cases

Table 5 –A-: MVD in the three grades of Dupreiz score using CD31 stained vessels

Groups	Number of Cases	MVD (%) Average
Score 0	10	5.61
Score 1	19	8.18
Score 2	2	17.20

Table 5 –B-: MVD in the three grades of Dupreiz score using CD34 stained vessels

Groups	Number of Cases	MVD (%) Average
Score 0	10	6.44
Score 1	19	10.45
Score 2	2	17.91

Table 6 –A-: MVC/Area in the three grades of Dupreiz score using CD31 stained vessels

Groups	Number of Cases	MVC/Area(no./mm$^{2)}$ Average
Score 0	10	275.7168
Score 1	19	437.1617
Score 2	2	581.8891

Table 6 –B-: MVC/Area in the three grades of The Dupreiz score using CD34 stained vessels

Groups	Number of Cases	MVC/Area(no./mm$^{2)}$ Average
Score 0	10	345.0611
Score 1	19	488.4451
Score 2	2	689.2499

Discussion

In this study the use of two immunohistochemical staining markers had increase the sensitivity of the staining technique and both of them found to be sensitive , reproducible and reliable for assessing neovasculization and their results were similar and comparable to other techniques as anti Factor VIII related antigen immunostaining[13]. In spite CD34 marker can be detected on myeloid progenitors as well, but the number of cells stained was sufficiently small as not to interfere with our analysis.

This study has revealed that MVD using both markers was significantly higher in MMM compared to control cases (Table 4),

This increase in neo-vascularization was in agreement with Lundberg LG.*et al* [(14)] and Panzoni M. *et al* [(8)] study, which had revealed that there was an increase in vascular density in the bone marrow of MMM compared to the bone marrow of healthy subjects.

Moreover Mesa RA. *et al* [(15)] and Pruneri G *et al* [(16)] had found a substantial increase of marrow vascularity in most patients with CMD compared with normal control and with patients with other myeloid disorders such as MDS and AML, and the extent of the abnormality was more pronounced in patients with MMM than in those with either PV or ET.

Moreover this study have found that the architecture of the vasculature clearly differs from the normal architecture in that the vessels in MMM are more tortuous and branched (Figures 1, 2), which was noticed by Lundberg LG. *et al* as well[14]who had concluded that these vessels changes were closely related to the disease activity .

In this study there was a significant positive correlation between MVD and age but not with sex and this was in agreement with Al-Sayegh Z. [(17)] and Hammoudi AT [10] studies.

Many authors had stated that bone marrow angiogenesis might represent the earliest histo-morphological event in the temporal progression towards myelofibrosis and osteosclerosis, and the neo angiogenesis as expressed by increased MVD and elevated in vascular endothelial growth fator (VEGF) level , was detected in early (prefibrotic) stage of MMM therefore they had consider that neoangiogenesis can reflect disease activity[(18-20)].

This study revealed that there was a significant positive correlation between angiogenesis and Dupriez score which is closely related to prognosis in MMM as shown in table 5(A&B) and table 6 (A&B), therefore we may propose that neoangiogenesis in MMM is closely related to these prognostic parameters thus it maybe used as a valuable tool to assess the disease severity as well as its activity[(20,21)].

In this study all patients had splenomegally, which is the result of extramedullary hematopoiesis and since antiangiogenic agent reduce the size of the spleen and liver therefore we may propose that the extramedullary hematopoiesis in the spleen is closely related to the increase in marrow neo-vascularization [(14)].

The increased marrow vascularity in MMM is consistent with the current understanding of the pathogenesis of this disease. The proliferation of an aberrant clone, most likely of megakaryocytic or of monocytic origin (or of both), is believed to be the underlying cause for the induction of an abnormal cytokines

release , mainly transforming growth factor –B_1,b-fibroblast growth factor , platelet derived growth factor and vascular endothelial growth factor , that have fibrogenic ,angiogenic and osteogenic potential [21-23].

In conclusion this study had revealed that microvessel proliferation or neoangiogenesis is a major component of the mixed stromal reaction in MMM which may reflect the disease activity and severity.Also we may predict that the degree of increased angiogenesis may have an independent prognostic value which could serve as an additional variable in clinical prognostic parameters.

Moreover there was no significant statistical difference in the staining sensitivity between CD31 and CD34. And whether angiogenesis was estimated by visual count using light microscope or by computerized imaging system, they gave reliable and comparable results.

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