Immunohistochemical study of bone marrow angiogenesis in multiple myeloma and its correlation with clinicopathological factors

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Summary:
Background: In recent years, bone marrow angiogenesis was reported to be involved in the pathogenesis and progression of certain hematological malignancies like multiple myeloma, leukemias, and lymphomas. Recent studies have suggested that bone marrow angiogenesis plays an important role in the pathogenesis and prognosis of multiple myeloma.
Objectives: At the present study, bone marrow angiogenesis in multiple myeloma was examined using immunohistochemical staining for CD34, and correlated with various pathological and clinical parameters.
Patients and methods: This is a retrospective study; where by archival paraffin-embedded tissue blocks along with the clinical and hematological records of fifty-two patients with multiple myeloma and twenty controls were obtained from the Department of Hematology of the Medical City Teaching Laboratories in the period from January 2010 to January 2013. Bone marrow angiogenesis was studied by immunohistochemical staining for CD34 to identify microvessels.
Results: There was a significant association between higher angiogenesis grades and advanced clinical stage of the disease (p = 0.002), and higher plasma cell percent in the bone marrow (p = 0.03), and increasing immaturity of the plasma cell (p = 0.001), and diffuse pattern of bone marrow infiltration by plasma cells (p = 0.007).
Conclusions: Patients with increased plasma cell burden, immature plasma cell morphology, and diffuse pattern of infiltration had a higher microvessel density.
Key words: Multiple myeloma; angiogenesis; CD34.

Introduction:
Angiogenesis is the formation of new vessels from an existing network of vasculature, and it plays a significant role in a variety of physiologic and pathophysiologic processes like tumor growth and spread.1 Angiogenesis is recognized as a crucial component in the growth and metastatic spread of solid tumors, and it is a complicated development in the disease process that involves the degradation of extracellular matrix proteins and the activation, proliferation, and migration of endothelial cells and pericytes in a multistep manner.2 It has been recognized that dysregulation of angiogenesis constitutes an important step in the development and progression of hematologic malignancies and that these malignancies may invade and proliferate in the bone marrow (BM) and other organs by mechanisms similar to those reported in solid tumors. Measurement of microvessel density by immunohistochemistry like CD34 has been widely used for assessing the extent of angiogenic activity.3

Patients and methods:
This is a retrospective cross-sectional study; whereby archival paraffin-embedded bone marrow (BM) blocks along with the clinical and hematological records of fifty-two (52) patients with multiple myeloma (MM) (34 males and 18 females) were obtained from the Department of Hematology of the Medical City Teaching Laboratories in the period from January 2010 to January 2013. The patients were newly diagnosed and did not receive prior treatment. The BM biopsies were performed at diagnosis. All relevant clinical and laboratory data available for all patients were reviewed, and the peripheral blood and marrow aspirate smears (stained with Leishman stain) were re-examined carefully. BM biopsy sections stained with H&E were also re-examined. All patients were selected according to the WHO diagnostic criteria, 4 which include the followings: Symptomatic myeloma: M-protein in serum or urine, Bone marrow clonal plasma cells increased, related organ or tissue impairment (e.g. hypercalcemia, renal insufficiency, anemia, bone lesions, hyperviscosity and recurrent infection). The Salmon-Durie staging system 5 was used in this study for clinical staging of the patients. Paraffin-embedded BM blocks of twenty control individuals (14 males and 6 females (age

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and sex matched) along with their hematological reports were also collected. All the control BMs were negative for infiltrative lesions and were obtained from patients with anemia and/or thrombocytopenia. BM angiogenesis was studied by immunohistochemical staining for CD34, in the Central Public Health Lab/Baghdad, to identify microvessels. Microvessel Staining: bone marrow biopsy specimens used in this study were prepared from paraffin-embedded blocks. Immunohistochemical staining for CD34 was performed by labeled streptavidin-biotin peroxidase method on the automated immunohistochemistry stainer (Danemark) using buffers and detection reagents supplied by the manufacturer. The primary antibody CD34 class 2 (DAKO Denmark) monoclonal mouse (QBEnd 10) IgG1 Kappa; diluted against 0.05 mol/L Tris/Hcl was incubated with tissue sections for 20 min. The LSAB + detection kit (DAKO) was used for antigen visualization; sections were counter stained with a light hematoxylin and then coverslipped. Paraffin sections of Hemangioma were run with each batch to serve as a positive control, and a section stained with nonimmune rabbit immunoglobulin was used as a negative control for each sample tested. Estimation of microvessel density and its grading: slides were first scanned at 100× magnification, and five areas of maximum microvessels density (MVD) called hot spots were identified at 200× magnification on each slide. In each of these hot spots, microvessels (capillaries and small venules) were counted at 400×. In each case, means of the hot spots were counted. Microvessels were identified as endothelial cells either singly or clustered in nests or tubules, clearly separated from one another, with or without lumen not exceeding 10 mm (i.e., not more than 1.5 times the endothelial cell nucleus) in transverse diameter. Areas occupied by larger vessels and vessels in the periosteum were excluded. Each slide (cases as well as controls) were assigned an angiogenesis grade. Grading of test cases was performed taking into account the MVD of controls, such that the MVD of all the controls included was Grade 1 MVD of the test cases. MVD was graded as follows 6:
1. Grade I: ≤ 25 microvessels. 2. Grade II: > 25 microvessels but ≤ 50 microvessels. 3. Grade III: > 50 microvessels/high power field (>40).

Statistical analysis: statistical analysis was performed with the SPSS 20 statistical software program. Univariate data were summarized using standard descriptive statistics, tabulation of categorical variables and histograms of numerical variables. Associations between categorical variables were assessed via cross-tabulation and chi-square. T-test and ANOVA were used to compare means of continuous variables. Spearman correlation was used to correlate variables when at least one variable was ordered. Pearson Correlation was used to measure the association between two continuous variables. Exact tests were used to calculate the p value. In all statistical analyses, a p value < 0.05 was considered to indicate statistically significant difference.

Results:
Fifty-two patients with multiple myeloma, 34 males and 18 females were included in this study. Ages ranged between 37 and 90 years with mean age ± SD (60.54 ± 11) years with an M:F ratio of 1.9:1. The BM microvessel density was markedly and significantly increased with multiple myeloma patients (Mean = 36.21 ± 14.9) compared with BM of controls (Mean = 7.5 ± 2.4) P =0.0001, figure 1 A & B.

Figure-1: Bone marrow section (×400) showed few microvessels in control group (A) and numerous microvessels in MM patients (B) (×400) [HIC, CD 34].

A significantly larger number of patients, with higher angiogenesis were associated with advanced clinical stage (stage II and III) of the disease than patients with low angiogenesis grade (p = 0.002, table-1) and the mean MVD was significantly higher in advanced stages of the disease than early stage (p = 0.0001, figure-2). At the same time there was a significant positive linear correlation between MVD and advancing clinical stage of the disease (p < 0.0001) (Figure 3).
Table 1: Association between the angiogenesis grades and stage of the disease

<table>
<thead>
<tr>
<th>Angiogenesis grade</th>
<th>Stage-I</th>
<th>Stage-II</th>
<th>Stage-III</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade-I</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Grade-II</td>
<td>3</td>
<td>21</td>
<td>9</td>
<td>34</td>
</tr>
<tr>
<td>Grade-III</td>
<td>0.0%</td>
<td>36.4%</td>
<td>63.6%</td>
<td>11</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>28</td>
<td>16</td>
<td>52</td>
</tr>
</tbody>
</table>

P = 0.002

Figure 2: Comparison between MVD in different stages of MM.

Figure 3: Correlation between MVD and Stages of the Disease in MM.

There was a significant association between higher angiogenesis grades and higher plasma cell percent in BM (p = 0.03, table 2), and the mean MVD was significantly higher in patients with BM plasma cell more than 50% (Mean = 40.31 ± 13.54) than patients with BM plasma cell less than 50% (Mean = 31.04 ± 15.16), p = 0.027. Also there was a significant positive linear correlation between plasma cell percentages in the bone marrow and MVD (p = 0.004).

Table 2: Association between angiogenesis grades and plasma cell percent in BM

<table>
<thead>
<tr>
<th>Plasma cell % (BM)</th>
<th>Angiogenesis grade</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less than 50%</td>
<td>Grade-I</td>
<td>Grade-II</td>
</tr>
<tr>
<td>7</td>
<td>30.4%</td>
<td>47.8%</td>
</tr>
<tr>
<td>More than 50%</td>
<td>17</td>
<td>11</td>
</tr>
<tr>
<td>3.4%</td>
<td>58.6%</td>
<td>37.9%</td>
</tr>
<tr>
<td>Total</td>
<td>15.4%</td>
<td>53.8%</td>
</tr>
</tbody>
</table>

P = 0.03

There was a significant association between high grades of angiogenesis and decreasing maturity of the plasma cell (p = 0.001, table 3).

Table 3: Association between Angiogenesis grade and plasma cell morphology

<table>
<thead>
<tr>
<th>Plasma cell morphology</th>
<th>Angiogenesis grade</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature (Plasmacytic)</td>
<td>Grade-I</td>
<td>Grade-II</td>
</tr>
<tr>
<td>7</td>
<td>41.2%</td>
<td>52.9%</td>
</tr>
<tr>
<td>Mixed (Plasmacytic and Plasmablastic)</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>0.0%</td>
<td>55.2%</td>
<td>44.8%</td>
</tr>
<tr>
<td>Immature (Plasmablastic)</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>16.7%</td>
<td>50.0%</td>
<td>33.3%</td>
</tr>
<tr>
<td>Total</td>
<td>15.4%</td>
<td>53.8%</td>
</tr>
</tbody>
</table>

P = 0.001

There was a significant association between high angiogenesis grades and diffuse pattern of bone marrow infiltration by plasma cells (p = 0.007, table 4).
Table-4: Association between the angiogenesis grade and BM infiltration pattern.

<table>
<thead>
<tr>
<th>BM infiltration pattern</th>
<th>Angiogenesis grade</th>
<th></th>
<th></th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Grade-I</td>
<td>Grade-II</td>
<td>Grade-III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-diffuse</td>
<td>7</td>
<td>8</td>
<td>4</td>
<td>21.1%</td>
<td>19</td>
</tr>
<tr>
<td>Diffuse</td>
<td>1</td>
<td>20</td>
<td>12</td>
<td>36.4%</td>
<td>33</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>28</td>
<td>16</td>
<td>30.8%</td>
<td>52</td>
</tr>
</tbody>
</table>

P = 0.007

Discussions:
Multiple Myeloma (MM) occurs with variable frequencies throughout the world, with the highest rates in the USA black populations and least in Japan and some Eastern European countries.1,2,7 Its prevalence rate in Iraq constitutes about 0.75% of all cancers registered,8 a figure which is comparable to figures reported from Western countries.1,2,9 Immunohistochemistry has provided an objective method for assessing degree of neovascularization. It has been shown to be a significant and independent prognostic indicator of various malignancies including myeloma.3,10 MM was the first hematological malignancy in which a significant correlation of angiogenesis with prognosis and survival could be identified.11In this study the mean age of 60.54 years is similar to that reported previously in Iraq which was 60.08 years,12,13 and also similar to other non-Iraqi studies, in which the mean age was about 60 years.9,14 The male/female (M:F) ratio of 1.9:1 in this study is similar to other previous Iraqi studies,12,13 and also similar to other non-Iraqi studies.14In this study, the BM-MVD was markedly increased in MM patients compared with BM-MVD of controls. This result is in accordance with the results of previous studies.6,15,17There was a significant association between higher angiogenesis grade and advanced stage of the disease. These findings suggest that dysregulation of angiogenesis is common in MM, and as such may represent an early event in pathogenesis of the disease. Since the clinical course of MM may last many years, the acquisition of other genetic mutations may augment the earlier dysregulation of angiogenesis, accounting for the increased CD34 positivity seen in higher stage individuals. It also indicates that MM demonstrates upregulated angiogenesis and thus may contribute to disease progression and a poor prognosis.6,15,16 Our findings are similar to the results reported by previous studies.6,15,16This study revealed a significant association between higher angiogenesis grades and higher plasma cell percent in the BM. It was postulated that higher plasma cell percentage is associated with active myeloma and these patients tend to have higher intramural vascularity, and increased vascular endothelial growth factor in the neoplastic cells.6,17 This result is in accordance with the results of previous studies.6,15-17 However; other workers found no correlation between plasma cell burden and MVD.18,19There was a significant association between high grades of angiogenesis and decreasing maturity of plasma cells in BM. The increase in MVD in MM of immature cytologic type may also be related to the release of additional angiogenic cytokines, such as IL-8 and granulocyte-macrophage colony stimulating factor, by the host BM cells. In addition, increased production of vascular endothelial growth factor (VEGF) by MM cells may contribute to the increased intratumoral vascularity seen in these patients.6,20 Our results are similar to previously reported studies.6,15-17,20In this study, there was a significant association between high angiogenesis grades and diffuse pattern of bone marrow infiltration by plasma cells. It was postulated that diffuse pattern of infiltration was associated with active myeloma and these patients tend to have higher intramural vascularity, and increased VEGF in the neoplastic cells.6,15-17,20 Our results are similar to previously reported studies.6,15-17,20

Conclusions:
Angiogenesis was increased in patients with MM as compared to controls. Patients with increased plasma cell burden, immature plasma cell morphology, and diffuse pattern of infiltration had a higher microvesSEL density.Since these factors are known independent prognostic factors and angiogenesis correlate with them, therefore, angiogenesis may be included as one of the prognostic factors for multiple myeloma; however more extensive follow up studies are needed to confirm this.

Author contribution:
Hasan Abdulrazaq Buraid: Postgraduate (M.Sc.) Student: Study conception, acquisition of data, interpretation of data, Abdulkareem Mohammad Jaafar: Supervisor: Study design, data analysis, drafting of manuscript, critical revision. Rana Zuhair Naji: Consultant Supervisor: Drafting of manuscript.
References: