Original Article

Immunohistochemical analysis of CD34 to evaluate angiogenesis in chronic lymphocytic leukemia

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Summary:

Background: Chronic lymphocytic leukemia (CLL) results from a progressive accumulation of long-lived, functionally incompetent, nonproliferating lymphocytes.

Angiogenesis is defined as the formation of new capillaries from pre-existing blood vessels and plays an important role in the progression of solid tumors as well as several hematologic malignancies like CLL.

Patients and methods: A retrospective cross-sectional study done on 68 patients with CLL compared with 15 control individuals (anemic patients), all recruited at the Medical City Teaching Laboratories from January 2005 to December 2008. The bone marrow biopsy (BMB) of each was re-examined histologically. Immunohistochemical (IHC) technique was performed on BMB sections utilizing monoclonal mouse CD34 class II antibody.

Results: This study revealed higher microvessel density (MVD) in BM of CLL patients than normal control marrows. CLL patients had a mean MVD of 10.78 VS 4.26 miorovessel count/high power field (MVC/HPF) in control subjects. Significant inverse correlation was found between MVD and PCV levels and platelet count. A Significant positive correlation was found between MVD and the percentage of lymphocytes in the bone marrow, BM infiltration pattern and the modified Rai stage of CLL patients

Conclusions: Angiogenesis in CLL BM was significantly higher than control marrows and was more accentuated with advanced clinical stage of the disease.

Key words: Chronic lymphocytic leukemia; CD34; IHC

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Introduction:

CLL is the most common leukemia in adults. It is representing about 25-30% of all leukemia in the Western countries.1 CLL is characterized by the accumulation of mature long lived B-cells which are blocked in early G1 of the cell cycle and are unable to undergo programmed cell death (apoptosis).2

Angiogenesis, the branching of new microvessels from pre-existent larger blood vessels, is of major importance in normal embryogenesis and in physiologic processes such as ovulation and the menstrual cycle.3 Abnormal angiogenesis has been identified in a number of hematologic malignancies. Although studies are limited, an increasing body of evidence supports the existence of increased tissue site angiogenesis in CLL.4 An increase in the MVD, which is considered as an index of angiogenic activity and defined by the number of microvessels per microscopic HPF, was noted in CLL BMs.5 The degree of angiogenesis is correlated with disease stage and progression free survival in CLL.5.

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Patients, Materials and Methods

Selection of the patients: This is a retrospective cross-sectional study; included the collection of archival clinical and hematological records along with paraffin-embedded BM tissue blocks of seventy five (75) patients who were diagnosed as CLL at the Department of Hematology of the Medical City Teaching Laboratories in the period from January 2005 to December 2008. The cases were selected on the basis of the availability of BMB performed at the time of diagnosis. Out of these cases only (68) had adequate sections on paraffin blocks, and were thus included in this study. The pattern of BM infiltration (non -diffuse or diffuse) was evaluated according to Rozman et al.6 Furthermore, clinical staging was performed using modified Rai clinical staging system of CLL.7

Paraffin-embedded tissue blocks of fifteen control individuals (age and sex matched) along with their hematological reports were also collected. All the control bone marrows were negative for infiltrative lesions and were obtained from patients with anemia due to iron or vitamin B12 deficiencies.

CLL patients were diagnosed according to the criteria (1

and 2) of the International Workshop on CLL (IWCLL): 8

1- Peripheral Blood: Persistent absolute lymphocytosis more than 10,000/µL mature-appearing lymphocytes in the peripheral blood.

2- BM examination: The aspirate smear must show more than or equal to 30% of all nucleated cells to be lymphoid.

3- Peripheral blood lymphocytosis that have B-cell phenotype of CLL.

All patients had peripheral blood prolymphocytes of less than 10%.

IHC: New 5 µm thick sections from each were made on positively charged slides (Thermo) and subjected for IHC procedure to detect CD34 marker. All CLL and control group cases were included in the IHC study. Primary antibody CD34 class II monoclonal antibody together with IHC detection kit: The EnVision+ Dual Link system-HRP were purchased from DAKO (Denmark). Preparation of BMB sections and reagents were conducted according to manufacturing company leaflet. The positive tissue slide was also used which included sections from sclerosing hemangioma of skin already positive for CD34. The EnVision+ Dual Link system-HRP is a two-step IHC staining technique. This technique basically uses a labelled primary antibody, which binds to its corresponding antigen, followed by a polymeric conjugate in sequential steps. This procedure results in an antibody-antigen interaction and an enzymatic reaction that can be detected by DAB+ substrate-chromogen which results in a brown-colored precipitate at the antigen site.

Evaluation of IHC results: The degree of angiogenesis in CLL was quantified by measuring the microvessel numbers in BM trephine biopsy sections and comparing it to normal control BM sections. The blood vessels in BM trephine sections were highlighted by IHC using antibodies to CD34. Positive staining of CD34 showed brown particles located in endothelial cell cytoplasm and membrane. Microvessels were defined as any endothelial cell or group of endothelial cells, distinct from other endothelial cells, non-endothelial cells, and connective tissue. The presence of a lumen was not considered necessary for defining a microvessel, and the length of the microvessel was not a factor in this definition. The microvessel counts were performed at high power magnification X400 for the entire BM trephine biopsy section. MVD was evaluated according to Kinihod5 with slight modification by the number of X400 high power field instead of X600 in kinihod study and was expressed as MVC/HPF.

Results:

This study revealed more microvessels in BMB sections of CLL patients compared to normal control marrows (Figure 1). The mean MVD in CLL bone marrows was 10.78 (±7.48) MVC/HPF, which was significantly higher than the MVD in control bone marrows 4.26 (±3.33) MVC/HPF, (P< 0.001) (Table 1).

The mean MVD for males was 10.99 (±7.83) MVC/HPF while that for females was 10.27 (±6.72) MVC/HPF. There was no significant difference between males and females (Table 2).

Significant inverse correlations were found between MVD and each of PCV levels (P = 0.023) and platelet count (P = 0.006). A significant positive correlation was found between MVD and lymphocyte percentage in the BM (P = 0.009) (Table 3). A significant positive correlation was found between MVD and the BM infiltration pattern of CLL patients (P = 0.008) (Table 4). There was also a significant positive correlation between MVD and the modified Rai stage (P = 0.005) (Figure 2).

Table 1 MVD in BM sections of control group and CLL patients

Subject	No.	Range of MVD (MVC/HPF)	Mean (±SD) of MVD (MVC/HPF)
Patients	68	2.50-34.00	10.78±7.48
Controls	15	1.20-9.60	4.26±3.33

P < 0.001

Table 2 MVD in CLL patients according to sex

Sex	No.	Range of MVD (MVC/HPF)	Mean (±SD) of MVD (MVC/HPF)
Male	48	2.50-34.00	10.99±7.83
Female	20	4.00-29.33	10.27±6.72

P= N.S

Table 3 Correlation between MVD and certain hematological parameters in CLL patients

MVD		
PCV	r	-0.276
	P	0.023
Platelet count	r	-0.333
	P	0.006
BM % of lymphocytes	r	0.315
	P	0.009

Table 4 correlation of BM infiltration pattern with MVD

MVD		
BM infiltration pattern	R	0.317
	p	0.008

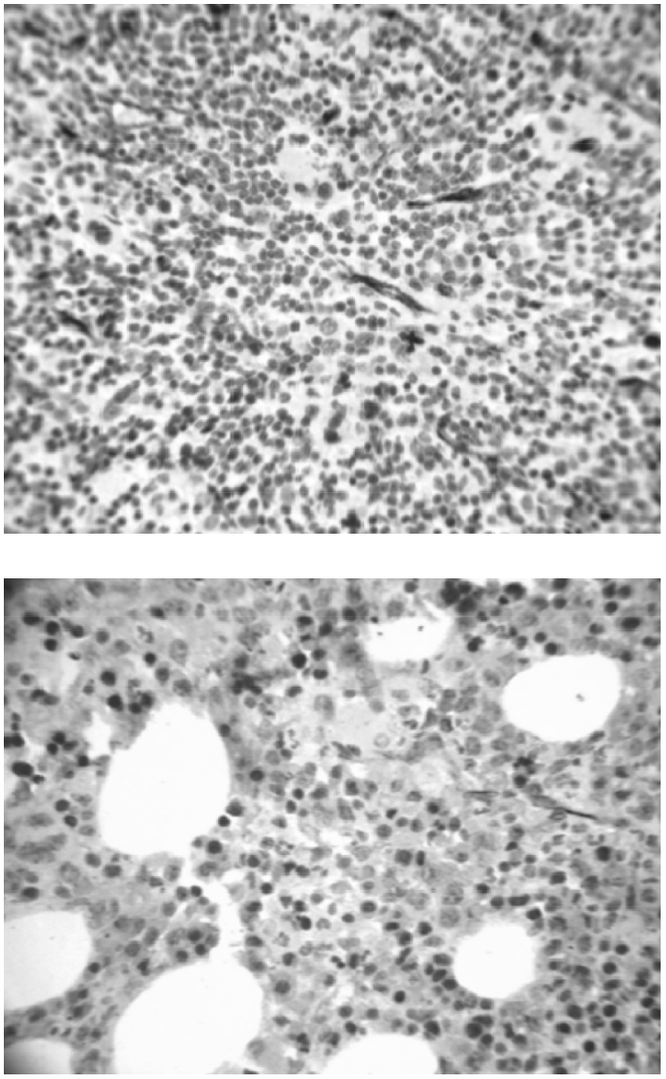

Figure 1 Increased MVD in CLL as compared to normal marrows. There are numerous CD34+ microvessels in a CLL section (Up) (X400). In comparison, control bone marrow section had fewer microvessels (down) (X400)

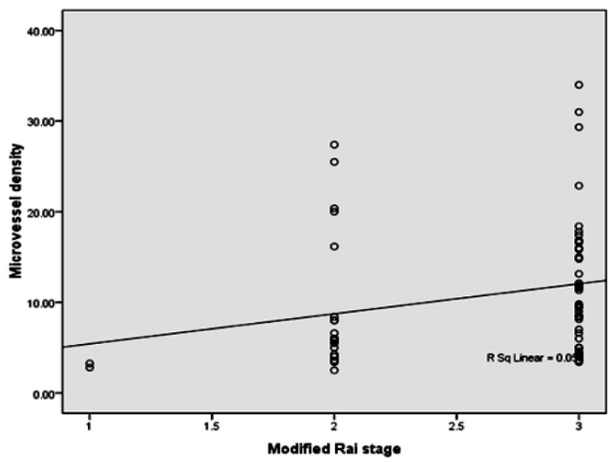

Figure 2 Scatter/dot shows significant positive correlation between MVD and clinical stage (P = 0.008)

Discussion:

This study confirmed a significant increase of BM angiogenesis in CLL compared with control subjects (Figure 1). This was similar to that reported by other workers who found high microvessels by IHC; using modified Weidner method where the results expressed as MVD,4,5 or by using computerized techniques where the results are expressed as microvessel surface area.9,10 These observations indicated that dysregulated angiogenesis is a common phenomenon in CLL. An inverse significant relationship was found between MVD and both PCV level and platelet count of CLL patients. Frater et al referred to the role of hypoxia induced by low PCV level in the upregulation of VEGF and increased microvessel production in bone marrow.4 Also low PCV level and low platelet count are indicators of high-risk clinical stage of the disease.11, 12 This study indicated there was a significant positive correlation between the microvessel counts and extent of BM involvement by CLL lymphocytes (Table 3). This means that CLL cells may be angiogenic in BM.5 Also we found that MVD is positively correlated with the clinical stage of the disease. This indicates that patients with higher MVD were more likely to have advanced disease.14 Similar results were reported by kini et al who pointed to a positive correlation between the microvessel count and each of the clinical stage (according to the Rai system) and the percentage of BM lymphocytes.5 Also we found that there was a positive correlation between the microvessel counts and BM infiltration pattern. Many researchers were unable to correlate BM infiltration pattern with MVD as virtually all studied patients had a non-diffuse BM histology.13 This result was in contrast to the study of Aguayo et al., 14 which did not show a statistically significant difference in microvascular densities between CLL patients and control subjects.

Conclusion:

Angiogenesis in CLL BM was significantly higher than control marrows and was more accentuated with advanced clinical stage of the disease.

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