Study of prognostic significance of marrow angiogenesis assessment in patients with de novo acute leukemia

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Background: Angiogenesis is the highly ordered formation of new blood vessels from pre-existing vessels. It is seen throughout growth, in wound healing, menses, and is important in cancer, where pro- and antiangiogenic signals can be released by cancer cells, endothelial cells, stromal cells, blood, and the extracellular matrix.

Aim of the study is to use standardized method for counting blood vessels to verify the significance and prognostic value of assessing marrow angiogenesis at diagnosis of de novo acute leukemia.

Subjects and methods: The study included 70 newly diagnosed acute leukemia cases and a control group composed of 35 bone marrow biopsy sections obtained from breast cancer patients. Examination of CD34 immunohistochemically stained sections for the assessment of marrow angiogenesis by quantification of its microvessel density (MVD).

Results: MVD was significantly increased in acute leukemia patients in comparison to control group (*P*-value <0.001). Increased MVD was associated with unfavorable outcome.

Conclusion: The study demonstrated an evidence of increased angiogenesis in acute leukemia detected by high bone marrow MVD which may play a significant role in leukemic process. Understanding its role may help in designing new therapeutic strategies for acute leukemia.

Keywords: Acute leukemia, Angiogenesis, Microvessel density, Prognosis, Clinical outcome

Introduction

Angiogenesis is a critical natural process that occurs in the body both in health and in disease to create new blood vessels from pre-existing vessels. This process occurs physiologically in the uterus during the menstrual cycles and pregnancy. Moreover, angiogenesis is necessary for the repair and regeneration of tissue during wound healing and skeletal growth.[1]

It involves activation of endothelial cells of a mature vessel, localized degradation of the surrounding basement membrane, the movement of adjacent vascular endothelial cells, and proliferation to form tubular structures making a network of new blood vessels.[2]

New blood vessels formation are controlled physiologically through a series of 'on' and 'off' switches[3] in which cytokines, growth factors, and other endogenous modulators act in balance to stimulate or inhibit blood vessel formation. When angiogenic growth factors are created in greater amounts than angiogenesis inhibitors, the balance is tilted in favor of the growth of new blood vessels until the inhibitors exceed the stimulators.[4] Known angiogenic growth factors include, angiogenin, angiopoietin-1, fibroblast growth factors (FGF), granulocyte colony-stimulating factor (G-CSF), and vascular endothelial growth factor (VEGF-A) and its tyrosine kinase receptors that are considered as the best-characterized signaling pathway in developmental angiogenesis.[5] While known angiogenesis inhibitors include, angioarrestin, angiostatin, and endostatin.[6]

Pathological angiogenesis occurs when diseased cells produce abnormal amounts of angiogenic growth factors overwhelming the effects of natural angiogenesis inhibitors. It occurs in diseases such as cancer, diabetic blindness, age-related macular degeneration, rheumatoid arthritis, and psoriasis.[7,8] In these conditions, new blood vessels feed diseased tissues and destroy normal tissues. In cases of cancer, angiogenesis is required for tumor cell proliferation, extracellular matrix invasion, and hematogenous metastasis.[9]

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DOI 10.1179/1607845415Y.0000000012

Insufficient angiogenesis occurs when tissues cannot produce adequate amounts of angiogenic growth factors. It occurs in diseases such as coronary artery disease, stroke, and chronic wounds. In these conditions, blood vessel growth is inadequate, and circulation is not properly restored, leading to the risk of tissue death.[8]

Angiogenesis has been suggested as important factor in the pathogenesis of hematological malignancies. The bone marrow of leukemic patients had shown increased blood vessel content compared to normal counterparts, suggesting that leukemia might be angiogenesis dependent and raising the possibility for a role of antiangiogenic therapy in the treatment of leukemia. Multiple mechanisms of angiogenic pathways regulate acute leukemia (AL) survival and proliferation including direct induction of angiogenesis, expressing receptors for specific angiogenic growth factors (paracrine regulation), and secreting their own angiogenic factors for their own angiogenic growth factor receptors (autocrine stimulation).[10]

To investigate the role of angiogenesis as a prognostic factor and monitoring of disease progression in cancer, an accurate and standardized method should be applied for counting the microvessel density (MVD).

The aim of the present study was to standardize an applicable method for counting MVD and apply it to verify the significance and prognostic value of assessing marrow angiogenesis at diagnosis of AL.

Subjects and methods

Materials

Bone marrow specimens from 70 de novo AL patients were studied. Diagnosis and classification of AL according to their clinical, morphological, cytochemical, and immunophenotypic examinations. Their inclusion criteria were based on the availability of biological material and their Egyptian nationality. All patients were treated with the current chemotherapy protocols. A written consent was obtained from all patients and/or their parents according to institutional guidelines.

A bone marrow core biopsy (iliac crest) was obtained from all patients for examination of hematoxylin and eosin-stained sections, to confirm diagnosis, precise assessment of the marrow cellularity as well as the degree of secondary marrow fibrosis (if present), and examination of immunohistochemical (IHC)-stained sections for the assessment of marrow angiogenesis by the quantification of the MVD. Additional biopsies were obtained to study the response to induction therapy (induction outcome) between days 14 and 28 after induction therapy.

To establish controls, 35 bone marrow biopsy (BMB) sections; previously obtained from breast cancer patients for staging were included in this study. All proved to be morphologically free of any hematological malignancies, having normal reticulin content and distribution, and morphologically negative for any metastatic deposits; confirmed by IHC staining for cytokeratin, mammaglobin, and CA15.3 monoclonal antibodies.

Immunohistochemical staining

Bone marrow specimens were fixed in 10% formal-saline, decalcified, embedded in paraffin, and decalcified with xylene. Serial sections (3–4 μm thick) of each sample were processed for IHC identification using CD34. IHC localization was performed by two-step IHC staining system based on a horse radish peroxidase (HRP)-labeled polymer which is conjugated with secondary antibodies (DAKO Envision™ peroxidase (HRP)/DAB, Mouse, code: K4006). Before staining, tissue sections were de-paraffinized in xylene, rehydrated in descending ethyl alcohol concentrations, pretreated by heat-induced epitope, retrieval for 15–20 minutes in Tris-EDTA buffer solution pH 9.0 in 90–95°C, and blocking endogenous peroxidase was done by applying enough blocking reagent to fully cover the sections for 10 minutes. The diluted primary Ab was applied on the tested sections for 1 hour (simultaneously, for the negative control samples, additional sections from limited number of cases were stained by replacing the primary antibody with phosphate buffer saline (PBS) solution), washed three times in PBS, the HRP-labeled polymer was applied for 30 minutes on tested and control sections, then, the staining was completed by applying freshly prepared (DAB + substrate – chromogen solution) to the sections. The sections were counter stained by hematoxylin stain 5% for 20 minutes.

Evaluation of BM angiogenesis

BM angiogenesis was evaluated by the assessment of marrow MVD that is estimation of the average quantity of microvessels per square millimeter (mm^2) area among the CD34 IHC-stained BM sections.

Modified method of Babarovic *et al.*[11] was used for estimation of MVD through the following steps, using a research binocular light microscope (Leica, DM – 750), the tested immuno-stained slides were initially scanned at ×40 magnification to identify the section area of the slide, check the staining quality, verify the distribution pattern of the highlighted microvessels, and to locate regions of higher vascular concentrations (i.e. hot spots). At higher magnification (×100), several serial images were captured for each BM section and its total area, in square millimeter (mm^2), was estimated by recruiting a full high definition (HD) digital camera (Leica – ICC-50) integrated with the microscope and supplied by a software for digital morphometric image analysis

(Leica Application Suite (LAS) – Image Analysis, version 4.0.0, Leica Microsystems (Switzerland) Limited). Areas of crushed cartilaginous and/or osseous tissues were excluded. Among the estimated total area of the BM section, quantification of microvessels was performed at ×400 magnification according to the following rules:

1. Any IHC-stained (CD34 +ve) individually scattered endothelial cell was considered as a single distinct countable microvessel.
2. Any IHC-stained endothelial cells cluster (whether arranged in a complete or incomplete vascular structure, with or without a lumen and clearly separated from adjacent microvessels, blasts, and other marrow elements) was considered also as a single distinct countable microvessel.
3. The presence of a lumen (with or without RBCs) was not necessary for microvessels morphological identification but considered only as a helpful feature.
4. All immuno-stained highlighted microvessels (whether crowded within the 'hot spots' or randomly individually dispersed among the intertrabecular hemopoietic areas) were included into the count. Those encountered within the BM trabeculae were not included into the count.
5. All immuno-stained microvessels (whether located among the malignant hemopoietic infiltrates or among the non-infiltrated normal hemopoietic areas) were included into the count.

For each immuno-stained BM section the total number of microvessels counted per the whole section was divided by the total section area estimated in (mm^2) and therefore the number of microvessels per one square millimeter area (i.e. MVD) was obtained.

Quantification of leukemic blast infiltration and criteria for response to chemotherapy

Quantitative analysis of leukemic blast infiltration was performed in bone marrow aspirates by routine cytological analysis as described by the FAB group.[12] A complete remission was defined as a bone marrow with normal hematopoiesis of all cell lines, less than 5% blast cells, and a peripheral blood count with at least 1500 neutrophils/μL, and 100.000 platelets/μL.[13]

Statistical methods

Data was analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL, USA). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test (Fisher's exact test) was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using Mann–Whitney test (non-parametric *t*-test). Comparison between three groups was done using Kruskal–Wallis test (non-parametric ANOVA) then *post hoc* 'Schefe test' on variable rank was used for pair-wise comparison.

Spearman-rho method was used to test correlation between numerical variables. A *P*-value <0.05 was considered significant.

Results

The present study was conducted on 70 AL cases and 35 normal BMB sections. AML group included eight pediatrics (2–13 years old). ALL group included nine pediatrics (3–11 years old). Demographic, clinical, and laboratory data of the leukemic group are summarized in Table 1.

Results of marrow micro vascular density in then studied groups

Among the studied 22 ALL cases, the marrow MVD range was 10.9–106.8 with a median of 32.5 vessels/mm^2, while among the studied 46 AML cases it was 12.4–221.6 with a median of 30.3 vessels/mm^2 (Fig. 1) and among the control group it was 1.6–16.6 with a median of 5.0 vessels/mm^2 (Fig. 1E). A comparison of marrow MVD was established between each of the studied groups and the control group. Statistically, a highly significant increase was detected in the marrow MVD between each of the studied groups and the control group, *P*-values <0.001 (Table 2).

Patients in the studied groups were further categorized according to their marrow MVD degrees. Four categories were identified and included those with MVD up to high normal, increased up to one fold, increased more than one fold up to two folds and increased MVD more than two folds.

Relationship between marrow MVD and the grades of second marrow fibrosis

In the AL group, among the 10 patients with mild degree of marrow fibrosis (grade I), 5 (50%) showed normal MVD (as controls) and 5 (50%) showed increased MVD (up to one fold). Meanwhile, among the 28 patients with prominent degree of marrow fibrosis (grade III) 17 (60.7%) showed increased MVD (greater than one fold and up to two folds) and 6 (21.4%) showed increased MVD (greater than two folds); *P*-value <0.001(Table 3).

Relationship between marrow MVD and induction outcome

The response to induction therapy (induction outcome) was assessed by BM re-examination between days 14 and 28 after induction therapy, among the 32 patients with unfavorable outcome (partial remission (PR), no response (NR), and induction-related mortality (IRM)), 26 (81.2%) showed increased MVD and only 6 (18.8%) showed normal

Table 1 Characteristics for the studied groups

Parameter	AML ($n = 46$)	ALL ($n = 22$)	MPAL ($n = 2$)
Age (years)			
Median (range)	39 (2–73)	22.5 (3–65)	*
Sex			
Frequency (%)			
Male	18 (39.1%)	16 (72.7%)	*
Female	28 (60.9%)	6 (27.3%)	
M:F ratio	1:1.6	2.7:1	
Organomegaly			
Frequency (%)			
Present	17 (37%)	14 (63.6%)	*
Absent	29 (63%)	8 (36.4%)	
Lymphadenopathy			
Frequency (%)			
Present	6 (13%)	12 (54.5%)	*
Absent	40 (87%)	10 (45.5%)	
TLC ($\times 10^3$/cmm)			
Median (range)	27.6 (0.8–245)	18.3 (1.4–488)	*
Hb (g/dL)			
Median (range)	7.5 (3.3–10.3)	7.9 (5.2–12)	*
Platelets ($\times 10^3$/cmm)			
Median (range)	44.5 (14–146)	52 (15–275)	*
PB blasts (%)			
Median (range)	39 (0–98)	45 (0–95)	*
BMA blasts (%)			
Median (range)	56.5 (2–94)	68.5 (22–97)	*
Overall marrow cellularity			
(%) Median (range)	95 (48–100)	95 (40–100)	*
Marrow infiltration pattern			
Frequency (%)			
D	18 (39.1%)	8 (36.4%)	*
DP	7 (15.2%)	8 (36.4%)	
I	4 (8.7%)	1 (4.5%)	
N/I	17 (37%)	5 (22.7%)	
Second marrow fibrosis:			
Frequency (%)			
(+)	7 (15.2%)	3 (13.6%)	*
(++)	21 (45.7%)	10 (45.5%)	
(+++)	18 (39.1%)	9 (40.9%)	
FAB classification:			
Frequency (%)			
	M0: 1 (2.2%) M1 + M2: 32 (39.6%) M3: 4 (8.7%) M4 + M5: 9 (19.6%) M6: 0 M7: 0	L1: 0 L2: 20 (90.9%) L3: 2 (9.1%)	*
Immunophenotyping:			
Frequency (%)			
	Myeloid: 37 (80.4%) Myeloid with monocytic: 9 (19.6%)	Pre B: 14 (63.6%) B-ALL: 4 (18.2%) C-ALL: 1 (4.5%) T-ALL: 3 (13.6%)	*

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; MPAL, mixed phenotype acute leukemia; MPAL, mixed phenotype acute leukemia; D, diffuse; DP, diffuse packed; I, interstitial; N/I, nodular/interstitial.
*The statistical analysis could not be performed for this group because it is composed of a very small number of cases.

MVD (as controls), however, among the 38 patients with favorable induction outcome (CR) still 34 patients (89.5%) showed increased MVD and only 4 (10.5%) showed normal MVD (as controls); P-value = 0.05, which is nearly significant (Table 4).

Discussion

For evaluation of angiogenesis, Babarovic *et al.*[11] noticed that the best IHC results were obtained with anti-CD34 monoclonal antibody compared to CD31 and factor VIII which are expressed in a large population of bone marrow cells, including megakaryocytes and myeloid cells.

Functional pluripotent CD34+ hematopoietic cells express VEGFR-2 antigen and these cells are known as precursors of both human hematopoietic and endothelial lineage.[14] The methodology applied for assessment of MVD in the previous studies of Mesa *et al.*,[15] Padro *et al.*,[16] Noren-Nystrom *et al.*,[17] and Todorovic *et al.*[18] was depending on the calculation

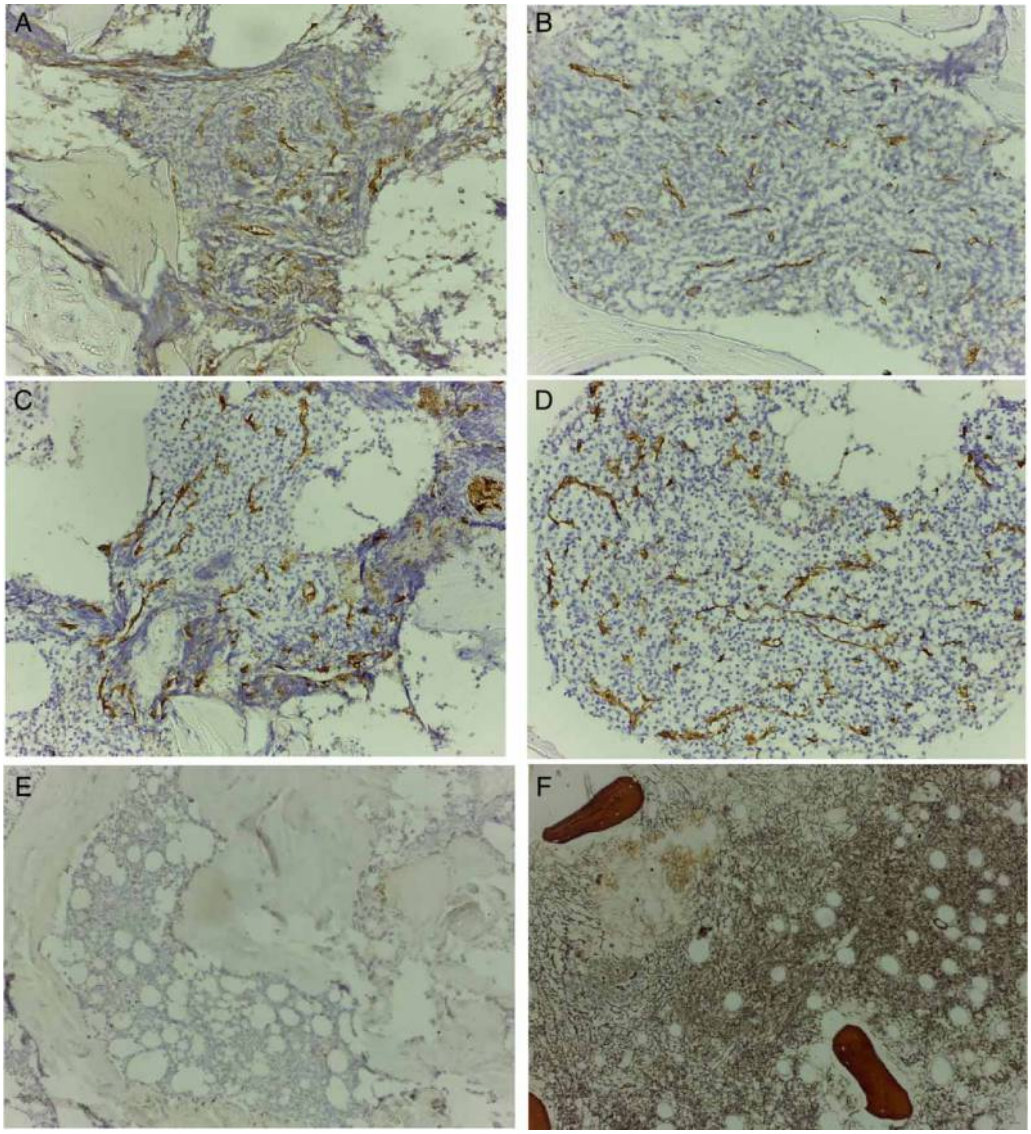

Figure 1 CD34–IHC-stained BMB sections showing: (A and B) ALL (L2) showing increased marrow MVD (57.8 vessels/mm²) × 200. (C and D) Stained BMB from a case of AML (M3) showing increased marrow MVD (221.6 vessels/mm²) × 200. (E) A case from the control group marrow MVD (2.7 vessels/mm²) × 100. (F) Reticulin stain showing marked BM fibrosis ×200.

of mean of microvessel numbers counted in at least three independent most vascularized hemopoietic areas 'hot spots' per section; using a fixed frame of known area for counting per each hot spot.

Table 2 Comparison of marrow MVD between each of the studied groups and the control group

	MVD (vessels\mm²): Median (range)	Control group (*n* = 35) MVD (vessels\mm²): Median (range)	*P*-value
AL (*n* = 70)	31.1 (10.9–221.6)	5.0 (1.6–16.6)	<0.001
AML (*n* = 46)	30.3 (12.4–221.6)		
ALL (*n* = 22)	32.5 (10.9–106.8)		

AL, acute leukemia; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; MVD, microvascular density.
P-value <0.001 is statistically significant.

However, the counting methodology in these previous studies was not standardized for all the cases as the number of examined hot spots per section was not fixed for all cases because of the variation in lengths found among their obtained BM sections. Moreover, in the present study, many AL cases showed that the marrow microvessels distribution pattern mostly occurred simultaneously in crowded hot spots as well as in random diffuse dispersion pattern among the whole section. Both patterns occurred more frequently than the former one alone.

Therefore, the methodology of the present study was applied according to the method of Babarovic *et al.*[11] in which several serial images were captured for each BM section and its total area was estimated by digital morphometric image analysis and among the estimated total area of the BM section, quantification of microvessels was performed, then the total number

Table 3 Grades of second marrow fibrosis among MVD-related categories of patients with AL

Total number = 70	Grades of second marrow fibrosis			
	I ($n = 10$)	II ($n = 32$)	III ($n = 28$)	*P*-value
MVD-related categories of AL patients				
Up to high normal (≤16.6) ($n = 10$)	5 (50%)	5 (15.6%)	0	<0.001
Increased up to one fold (16.7–34) ($n = 29$)	5 (50%)	19 (59.4%)	5 (17.9%)	
Increased >one fold and up to two folds (35–70) ($n = 24$)	0	7 (21.9%)	17 (60.7%)	
Increased >two folds (>70) ($n = 7$)	0	1 (3.1%)	6 (21.4%)	

AL, acute leukemia; MVD, microvascular density; %, percent within second marrow fibrosis grades.
P-value <0.001 is statistically significant.

of microvessels counted per the whole section was divided by the total section area estimated in mm²; therefore the number of microvessels per one square millimeter area (i.e. MVD) was obtained.

In the present study, we found a highly significant increase in the marrow MVD between each of the studied groups and the control group, *P*-values <0.001.

As regards AML, our results are in agreement with Kuzu *et al.*[19] who measured MVD in BMB of 36 AML patients based on CD31 and CD34 expressing vessels (CD31: $P = 0.004$, CD34: $P < 0.001$).

Similar results were detected by Padro *et al.*[16] and Hussong *et al.*[20] who estimated MVD using endothelial cell markers (thrombomodulin, ULEX-E, and vonWillebrand factor (vWF)) respectively.

As regards ALL, all previous studies detected highly significant increase in the marrow MVD.[17,18,21,22]

Table 4 Relationship between marrow MVD and induction outcome in studied groups

	Induction outcome	MVD (vessels/mm²) median	*n*	Range	*P*-value
ALL	CR:	38	30.3	10.9–221.6	0.953
$n = 70$	PR/NR/IRM:	32	34.9	11.8–156.1	
AML	CR:	24	26.5	14.5–221.6	0.231
$n = 46$	PR/NR/IRM:	22	38.8	12.4–156.1	
ALL	CR:	12	48.5	10.9–74.5	0.254
$n = 22$	PR/NR/IRM:	10	21.4	11.8–106.8	

AL, acute leukemia; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; MVD, microvascular density; CR, complete remission; PR, partial remission; NR, no response; IRM, induction-related mortality.
P-value <0.001 is statistically significant.

A borderline association was detected in the present study between MVD and sex in the ALL group (*P*-value = 0.08). However, statistical analysis did not reveal any significant association between MVD and sex as reported by Padro *et al.*[16] and Noren-Nystrom *et al.*[17] in AML and ALL, respectively.

The present study did not reveal any significant difference in MVD in the studied groups related to the presence of organomegaly or lymphadenopathy (*P*-values >0.05). The importance of this relation was to detect if high MVD was associated with bad prognostic sign like extramedullary infiltrations. To the best of our knowledge, no previous studies addressed the relation of the previous parameters with MVD in AL.

Statistical analysis did not reveal any significant differences in MVD between the AML FAB-subtypes as reported by Padro *et al.*[16] and Hussong *et al.*[20]

In the present study, high microvessel counts were not correlated with percentage of BM blast cells in AML and ALL.

These results are in accordance with Padro *et al.*[16] and Kuzu *et al.*[19]

This observation is in line with the findings in multiple myeloma in which the degree of bone marrow angiogenesis did not correlate with the percentage of plasma cell infiltration.[23] However, this correlation was documented by Hussong *et al.*,[20] De Raeve *et al.*,[24] and Zhang *et al.*[25] They gave an explanation for the positive synergistic relationship between leukemic cells and endothelial cells and provided evidence that MVD is directly related to the malignant cell burden.

In the present study, there was no correlation between MVD and peripheral WBCs, platelet count, or MB cellularity. That was in consistent with previous researches.[17,19]

Because marrow angiogenesis and fibrosis could be augmented by the same stimulators like FGF, platelet-derived growth factor, and transforming growth factor (TGF-beta) which found to be secreted in higher levels by marrow stromal cells in hematological malignancies,[26] we hypothesized that both processes are positively related. Consistently with this hypothesis, a highly significant association was detected between the degree of marrow MVD and the grade of second marrow fibrosis in AML and ALL (*P*-values <0.001). This relation was only investigated and proved before in multiple myeloma.[11] Subsequently, use of antiangiogenic drugs like anti-FGF in leukemia could suppress both marrow angiogenesis and fibrosis. The effect of this double action on disease outcome and rate of relapse should be widely investigated.

As regards response to treatment in the studied AL, AML, and ALL groups, no significant associations were detected between the degree of marrow MVD and post induction therapy outcome. However,

because of the short follow-up period due to loss of many participants, we could not analyze the prognostic value of MVD at presentation (day 0) for event-free and overall survival.

Padro *et al.*[16] suggested that BM MVD at diagnosis cannot be used to predict the clinical outcome in terms of achieving a complete remission after induction chemotherapy. In contrast, Kuzu *et al.*[19] and Noren-Nystrom *et al.*[22] found that patients with high MVD had an unfavorable outcome and shorter overall survival.

Todorovic *et al.*[18] found that the initial values of MVD had a positive correlation with overall survival and leukemia-free survival. We can conclude that even if high MVD does not affect results of induction therapy, it could increase rate of relapse. These results are encouraging for inclusion of MVD enumeration in bone marrow examinations of AML and ALL patients at diagnosis as an additional prognostic factor.

Understanding its role may help in designing new therapeutic strategies for ALs.

Detection of the therapeutic effect of different anti-angiogenic drugs in AL patients and studies that combine antiangiogenic factors with conventional treatment of AL to detect if this combination has additive therapeutic effect over conventional therapy alone are highly recommended to stratify patients into responders and non-responders.

Disclaimer statements

Contributors No other contributors.

Funding None.

Conflicts of interest None.

Ethics approval Ethics approval was received.

References

1 Folkman J. Clinical applications of research on angiogenesis. N Engl J Med. 1995;333:1757.
2 Dedhar S, Hannigan GE, Rak J, Kerbel RS. The basic science of oncology. Tannock IF, Hill RPIII, (eds.) Vol. 9. London: McGraw-Hill NY, St Louis; 1998. pp. 197–205.
3 Folkman J. Role of angiogenesis in tumor growth and metastases. Semin Oncol. 2002;29:15–8.
4 Koster A, Raemaekers JM. Angiogenesis in malignant lymphoma. Curr Opin Oncol. 2005;17:611–6.
5 Schliemann C, Bieker R, Padro T. Expression of angiopoietins and their receptor Tie2 in the bone marrow of patients with acute myeloid leukaemia. Haematologica 2006;91:1203–11.
6 Dvorak HF. Vascular permeability factor\vascular endothelial growth: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. J Clin Oncol. 2002; 20:4368–80.
7 Carmeliet P. Angiogenesis in life, disease and medicine. Nature 2005;438:932–6.
8 Konopleva M, Tabe Y, Zeng Z, Andreeff M. Therapeutic targeting of microenvironmental interactions in leukaemia: mechanisms and approaches. Drug Resist Update 2009;12(4): 103–13.
9 Vacca A, Ribatti D, Presta M, Minischetti M, Iurlaro M, Ria R, *et al.* Bone marrow neovascularization, plasma cell angiogenic potential and matrix metaloproteinase-2 secretion parallel progression of human multiple myeloma. Blood 1999;93:3064.
10 Trujillo A, McGee C, Cogle C. Angiogenesis in acute myeloid leukaemia and opportunities for novel therapies. J Oncol. 2012;ID: 128608, 9 pages.
11 Babarovic E, Valkovic T, Stifter S, Budisavlijevic I, Seili-Bekafigo I, Duletic-Nacinovic A, *et al.* Assessment of bone marrow fibrosis and angiogenesis in monitoring patients with multiple myeloma. Am J Clin Pathol. 2012;137:870–8.
12 Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, *et al.* Proposals for the classification of the acute leukaemias (FAB cooperative group). Br J Haematol. 1976;33: 451–8.
13 Cheson BC, Cassileth PA, Head DR, Schiffer CA, Bennet JM, Bloomfield CD, *et al.* Report of the National Cancer Institute-sponsored workshop on definitions of diagnosis and response in acute myeloid leukemia. J Clin Oncol. 1990;8:813.
14 Ziegler BL, Valtieri M, Porada GA. KDR receptor: a key marker defining hematopoietic stem cells. Science 1999;285: 1553–8.
15 Mesa RA, Hanson CA, Rajkumar SV. Evaluation and clinical correlations of bone marrow angiogenesis in myelofibrosis with myeloid metaplasia. Blood 2000;96:3374–80.
16 Padro T, Ruiz S, Bieker R, Burger H, Steins M, Kienast J. Increased angiogenesis in the bone marrow of patients with acute myeloid leukemia. Blood 2000;95(8):2637–44.
17 Noren-Nystrom U, Heyman M, Frisk P, Golovleva I, Sundstrom C, Porwit A. Vascular density in childhood acutelymphoblastic leukemia correlates to biological factors and outcome. Br J Haematol. 2009;146:521–30.
18 Todorovic M, Balint B, Radisavljevic Z, Andjelic B, Todorovic V, Perunicic-Jovanovic M, *et al.* Microvessel density evaluation in acute lymphoblastic leukemia – a study of angiogenesis. Bilt Transfuziol. 2011;57(1–2):49–51.
19 Kuzu I, Beksac M, Arat M. Bone marrow microvessel density (MVD) in adult acute myeloid leukaemia (AML): therapy induced changes and effects on survival. Leuk Lymphoma 2004;45:1185–90.
20 Hussong JW, Rodgers GM, Shami PJ. Evidence of increased angiogenesis in patients with acute myeloid leukemia. Blood 2000;95(1):309–13.
21 Perez AR, Sallan SE, Tedrow U, Connors S, Allred E, Folkman J. Spectrum of tumor angiogenesis in the bone marrow of children with acute lymphoblastic leukaemia. Am J Pathol. 1997; 150(3):815–21.
22 Noren-Nystrom U, Roos G, Bergh A, Botling J, Lönnerholm G, Porwit A, *et al.* Bone marrow fibrosis in childhood acute lymphoblastic leukemia correlates to biological factors, treatment response and outcome. Leukemia 2008;22:504–10.
23 Vacca A, Ribatti D, Roncali L. Bone marrow angiogenesis and progression in multiple myeloma. Br J Haematol. 1994;87:503.
24 De Raeve HR, Vermeulen PB, Vanderkerken K, Van Marck E. Microvessel density, endothelial cell proliferatin and carbonic anhydrase IX expression in hematologicalmalignancies, bone marrow metastases and monoclonal gammopathy of undetermined significance. Virchows Arch. 2004;445(1):27–35.
25 Zhang Y, Pillai G, Gatter K. Expression and cellular localization of vascular endothelial growth factor A and its receptors in acute and chronic leukemias: an immunohistochemical study. Human Pathol. 2005;36:797–805.
26 Dong M, Blobe GC. Role of transforming growth factor-β in hematologic malignancies. Blood 2006;107:4589–96.