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Original article

Immunohistochemical expression of VEGF in relation to VEGFR and CD34 in NHL using digital image analysis system

Bassam Mohammad Hameed[1], Hind Shaker Aboob[2], Raad Jaber Musa[3]

1 M.B.Ch.B., Msc. Pathology (haematology), Ph.D. Pathology (haematology) Lecturer at Department of pathology and forensic medicine /collage of medicine /AL-Nahrain university

2 M.B.Ch.B., FIBMS(path/haemaology) Lecturer at Department of pathology and forensic medicine /collage of medicine /AL-Nahrain university

3 M.B.Ch.B., M. Sc. Path. (Haem.); F.I.C.M.S.Path.(Haem.) Professor of Haematology at Department of pathology and forensic medicine /collage of medicine /AL-Nahrain university

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Abstract

Background: Lymphoma growth and progression appear to be promoted by at least two distinct angiogenic mechanisms: autocrine stimulation of tumor cells via expression of Vascular Endothelial Growth Factor(VEGF) and Vascular Endothelial Growth Factor Receptor(VEGFR) by lymphoma cells, and paracrine influences of the proangiogenic tumor microenvironment on local tumor vascularity.

Objectives: To assess autocrine effect of VEGF, by studying the correlation of VEGF expression with its receptor VEGFR expression in NHL. And to assess paracrine effect of VEGF, by studying the correlation of VEGF expression with CD34 expressed on endothelial cells in Non-Hodgkin Lymphoma (NHL).

Materials and Methods: A cross sectional study was designed. A total of 66 bone marrow tissue samples were included in the study, all diagnosed as having NHL according to working formulation. From each block, 3 sections were taken, and were immunohistochemically stained for CD-34, VEGF and VEGFR. Scoring of Immunohistochemical staining was performed using specialized automated cellular image analysis system, Digimizer software, version 3.7.0.

Results: VEGF Immunohistochemical digital parameters named digital labeling index (DLI) was significantly correlated with the followings; VEGFR (DLI) [P =0.042, r =0.324], CD34 stained area (A) [P=0.037, r =0.556]. Also VEGFR (DLI) was significantly correlated with CD34 (A).

Conclusion: Autocrine and paracrine effect of VEGF is evident in NHL, as there is positive correlation between VEGF expression and VEGFR expression, and as tumor vascularity increase with the increase in VEGF expression.

Keywords: NHL, VEGF, VEGFR

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Introduction

Tumor angiogenesis mediated by many mediators in tumor microenviroment, of these VEGF (vascular endothelial growth factor) is the most important factor for angiogenic switch through its interaction with receptors regulating different aspects of tumor angiogenesis. Lymphoma growth and progression as many other tumor promoted by angiogenesis, this occurs by two main mechanisms; autocrine stimulation of tumor cells by expressing both VEGF and its receptor VEGFR, and second by paracrine influences of the pro-angiogenic tumor microenvironment. [1]

Many researchers stated that there is positive correlation between VEGF expression with VEFGR expression in DLBCL lymphoma cells supporting the concept of autocrine-paracrine role for VEGF as growth factor for DLBCL promoting cell survival and proliferation.[2,3]

In this study we tried to study angiogenesis via VEGF, VEGFR and microvessel density in non-Hodgkin lymphoma using digital analysis system, Advantages of using digital image system in that scoring immunohistochemical staining in this method offer objectivity, reproducibility, quantification while overcoming manual scoring method that requires considerable expertise and is susceptible to interobserver variability.[4]

Aims of the study

1. To assess autocrine effect of VEGF, by studying the correlation of VEGF expression with its receptor VEGFR expression, in bone marrow biopsy of non-Hodgkin lymphoma patient using automated image analysis system.
2. To assess paracrine effect of VEGF, by studying the correlation of VEGF expression with CD34 expressed on endothelial cells, in bone marrow biopsy of non-Hodgkin lymphoma patient using automated image analysis system.

Materials and methods

A cross sectional study was designed, a total of 66 tissue samples (paraffin block of bone marrow biopsy) were included in the study. All the samples were taken from the (Medical city/ teaching laboratories), cases presented during the period 2008-2010 as all diagnosed having NHL according to working formulation by histopathological examination for primary lymph node biopsy. From each block, 3 sections of 5µm thickness were taken, each section were immunohistochemically stained for CD-34, VEGF and VEGFR.

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The procedure was carried out according to manufacturer's instructions. Taking sections and mounted on Fisher brand positively charged slides. Than slides deparaffinized, and placed in DAKO antigen retrieval (PH 6 for VEGF, PH 9 for VEGFR, 1700 for CD34) after heating slides with antigen retrieval solution in microwave for 20 min, slides let to cool down. Later on LSAP (DAKO staining kit) used for staining, after blocking endogenous peroxidase, and incubation of primary antibody for 30 min. Scoring of immunohistochemical staining was performed using specialized automated cellular image analysis system, Digimizer software, version 3.7.0.

Image capture

Using a light microscope, each immunohistochemically stained slide was scanned with 10 × objectives for the positive brown immunostaining, and with 40× objective three fields that reflect the best of the overall immunostaining of the entire slide were chosen and captured using a Sony digital camera (cyber-shot DSC-W510). Captured images of 4000×3000 pixels were saved on PC in an uncompressed JPG format.

Image analysis

Each image was analyzed by Digimizer software (Version 3.7.0). Determination of immunostaining intensity was done by using the Magic Wand tool in the toolbar menu in digimizer program (see figure 1). The tolerance level of the Magic Wand tools was adjusted so that the entire positive cells were selected. The measurements comprised:

1. Color Intensity (I): which measures the average intensity of the brown color for the selected objects depending on the expression of antigens in the cells.
2. Fractional area stained (A) = [(mean area× Number of objects) / area of a single image field] × 100%
3. Digital Labeling Index (DLI): first used by Al-Sinjery, G. M. [5], this tool is calculated according to the following formula:

DLI= [Fractional area(A) × reverse Intensity(I)].

This digital parameter, is the best representative for the expression because it combines both the Fractional area and the Intensity of immunohistochemical staining.

Statistical analysis:

Statistical analysis was performed with **SPSS** (statistical package for social sciences) version 16 and **Excel 2007** programs.

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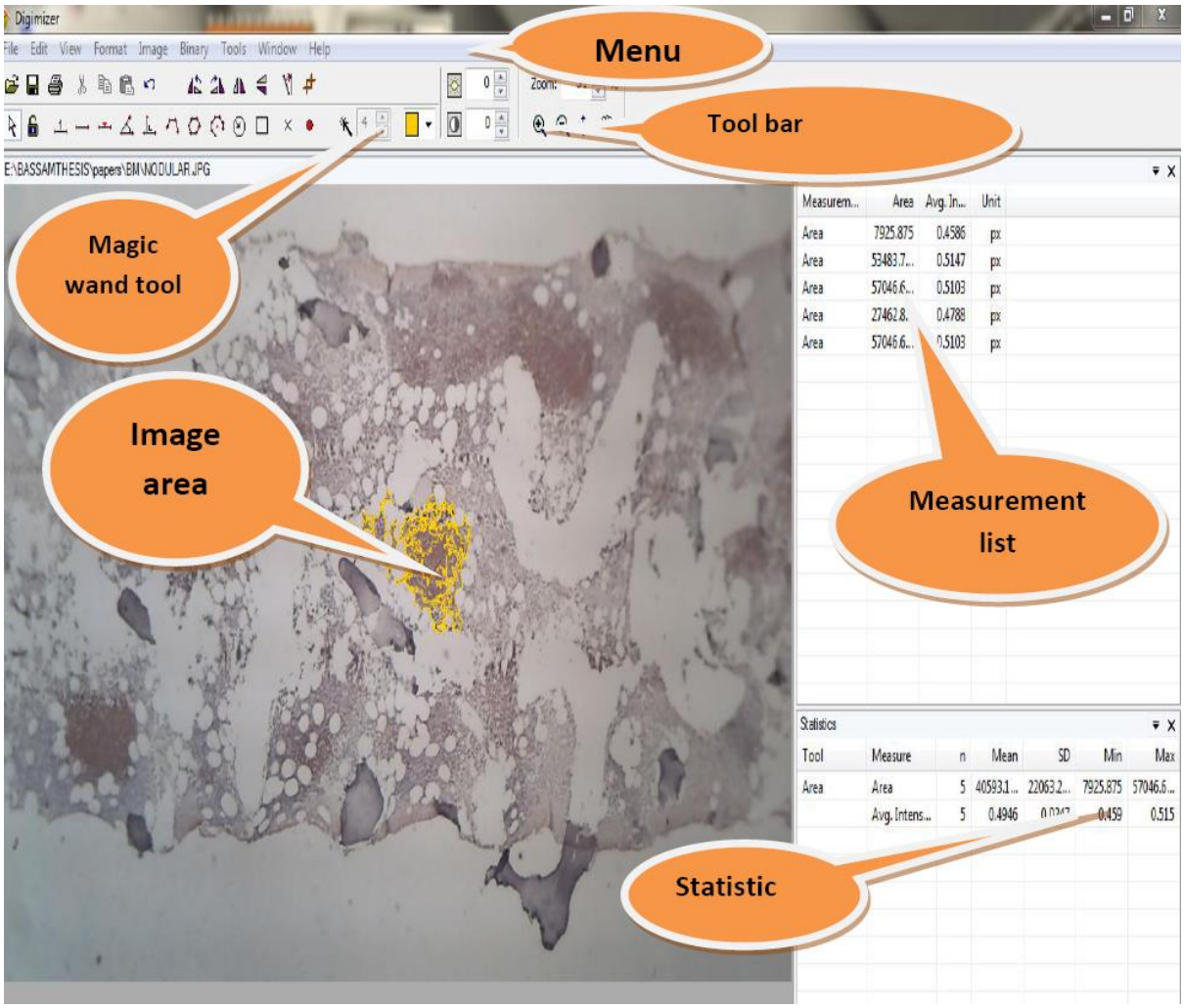

Figure 1. showing snap shot for digimizer softwear window

Results

Correlation between digital parameters of digimizer softwear of VEGF and other angiogenic markers in NHL: Digital parameter for VEGF digital labeling index (DLI) which combines staining intensity and area staining was significantly correlated with the followings; VEGFR digital labelling index (DLI), CD34 staining area (A), as well as VEGFR (DLI) was significantly correlated with the CD34 staining area (A). figure (II)

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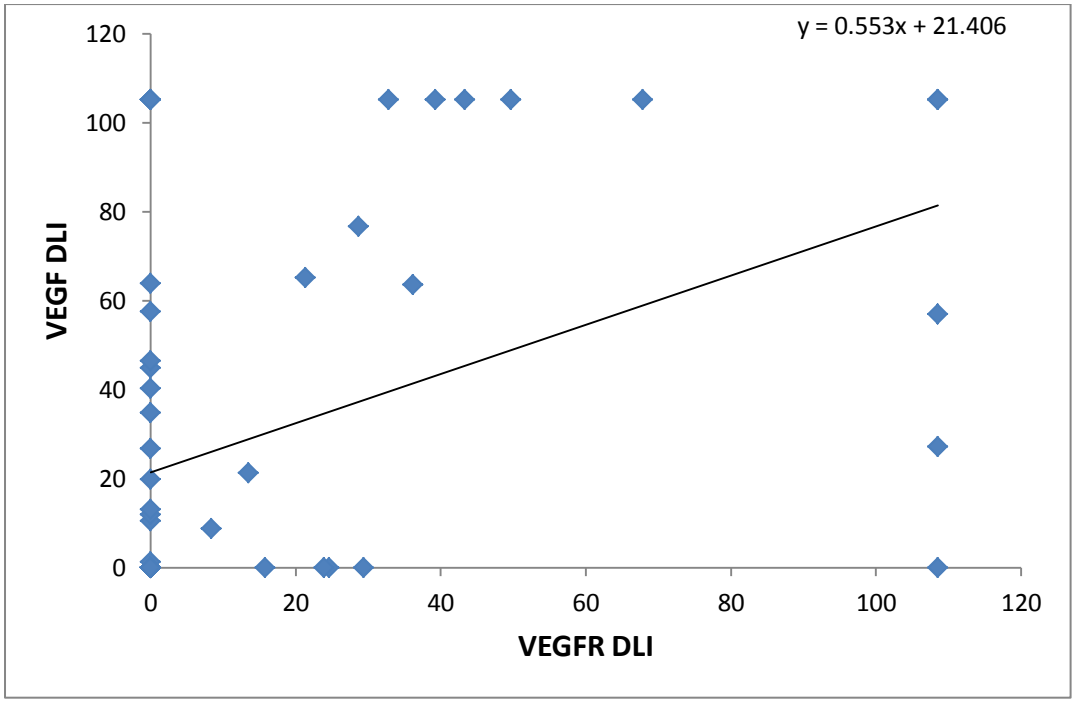

Figure II. Correlation chart shows positive correlation between VEGF (DLI) and VEGFR-1(DLI),(P =0.042, r =0.324).

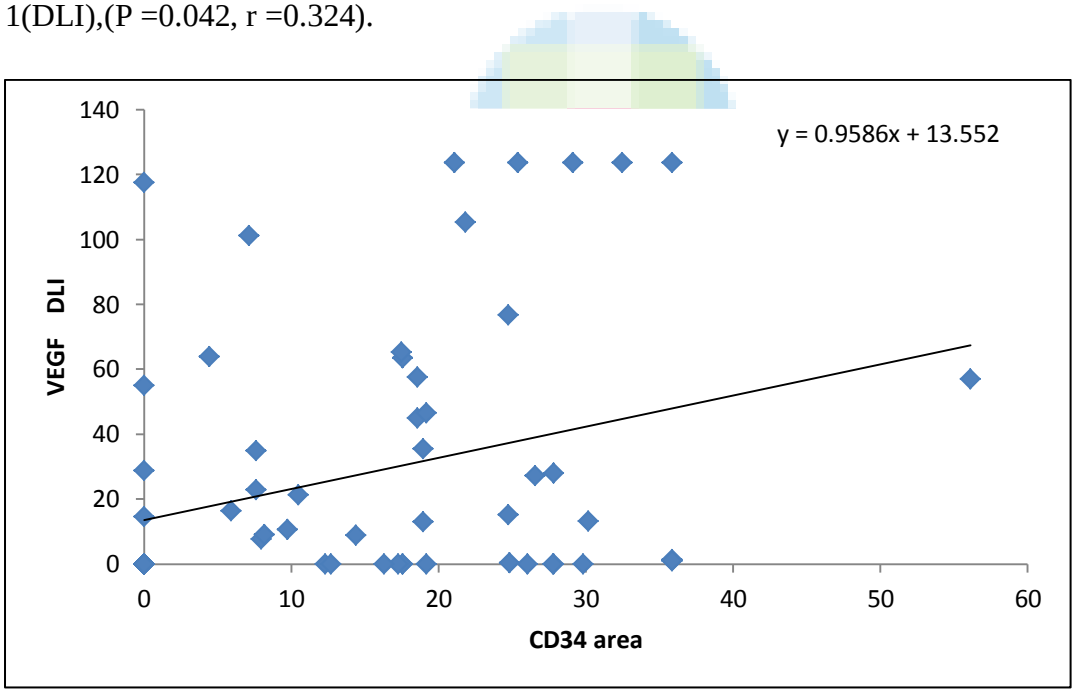

Figure III. Correlation chart shows positive correlation between VEGF (DLI) and CD34 (A),(P=0.037, r =0.556).

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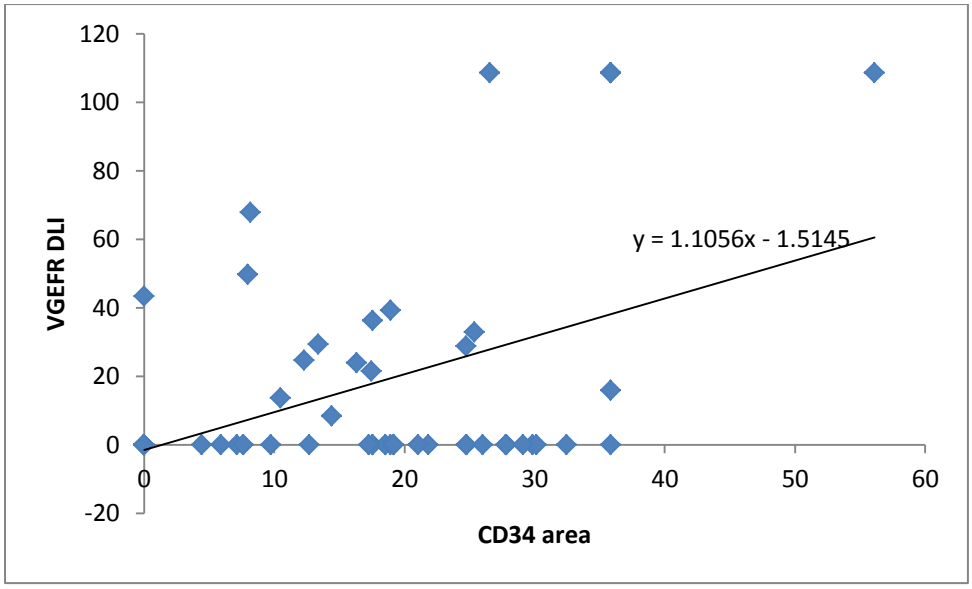

Figure IV. Correlation chart shows positive correlation between VEGFR-1 (DLI) and CD34(A),(P=0.041, r = 0.303).

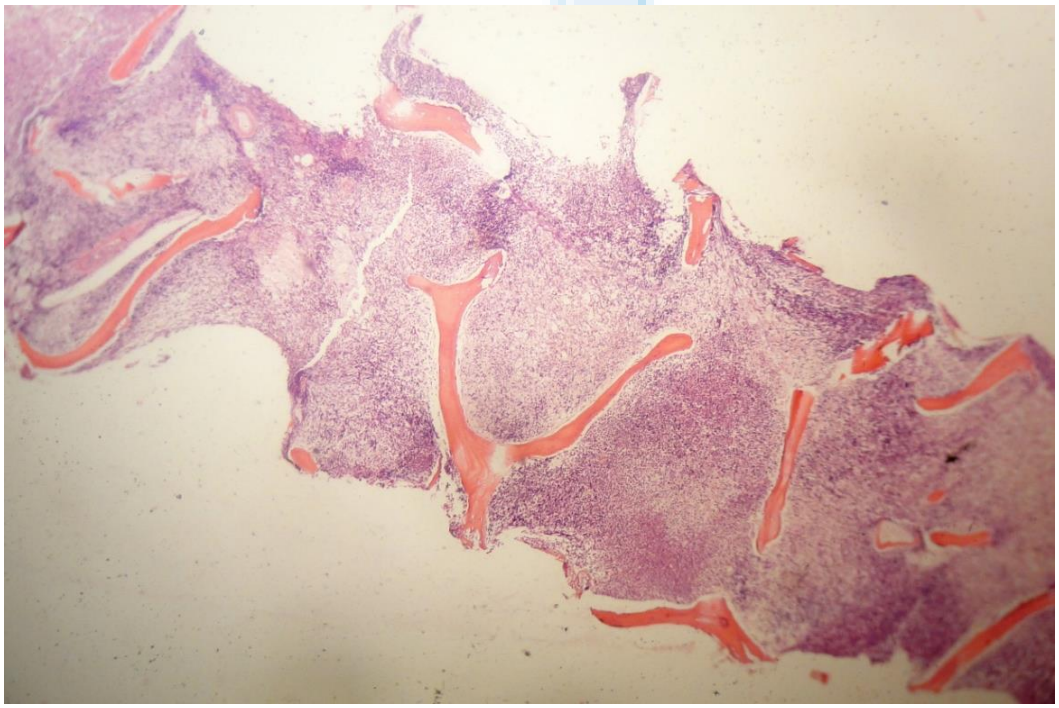

Figure V. Trephine biopsy section from a patient with Folliculer lymphoma showing paratrabecular infiltration& random focal . H&E, x 4 objective.

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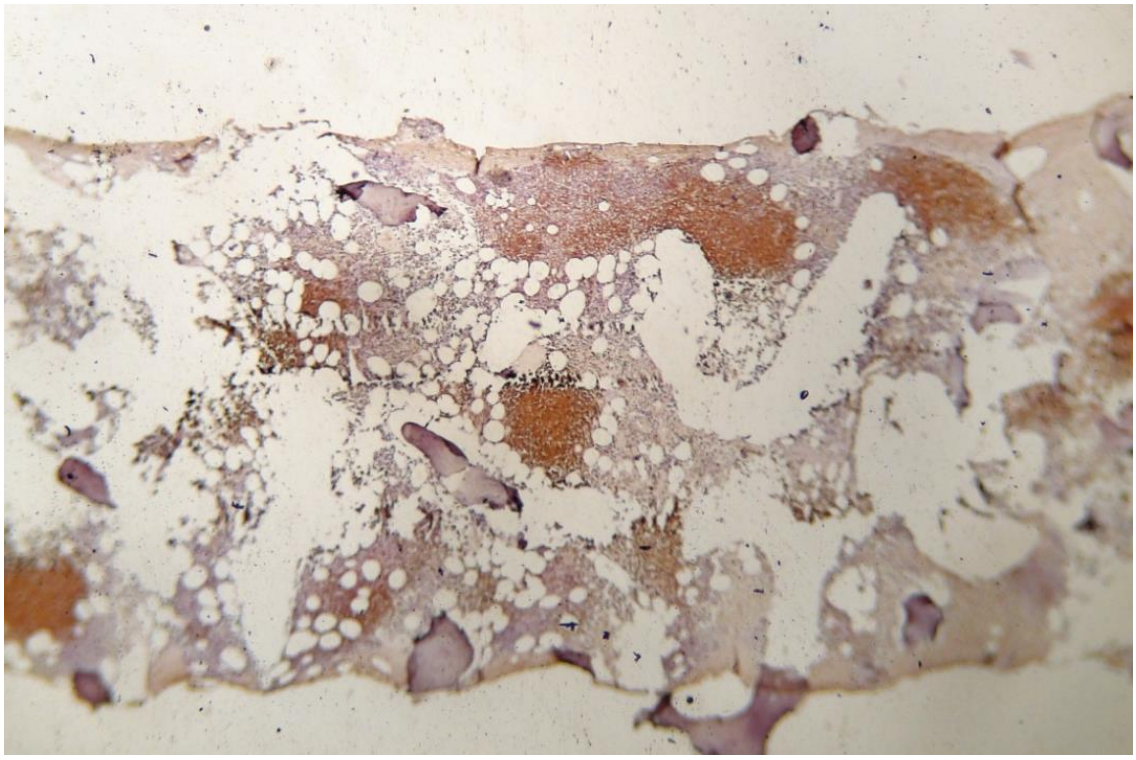

Figure VI. Trephine biopsy section from a patient with B-SLL showing nodular infiltration . with D20 + immunohistochemistry (X 4 objective.).

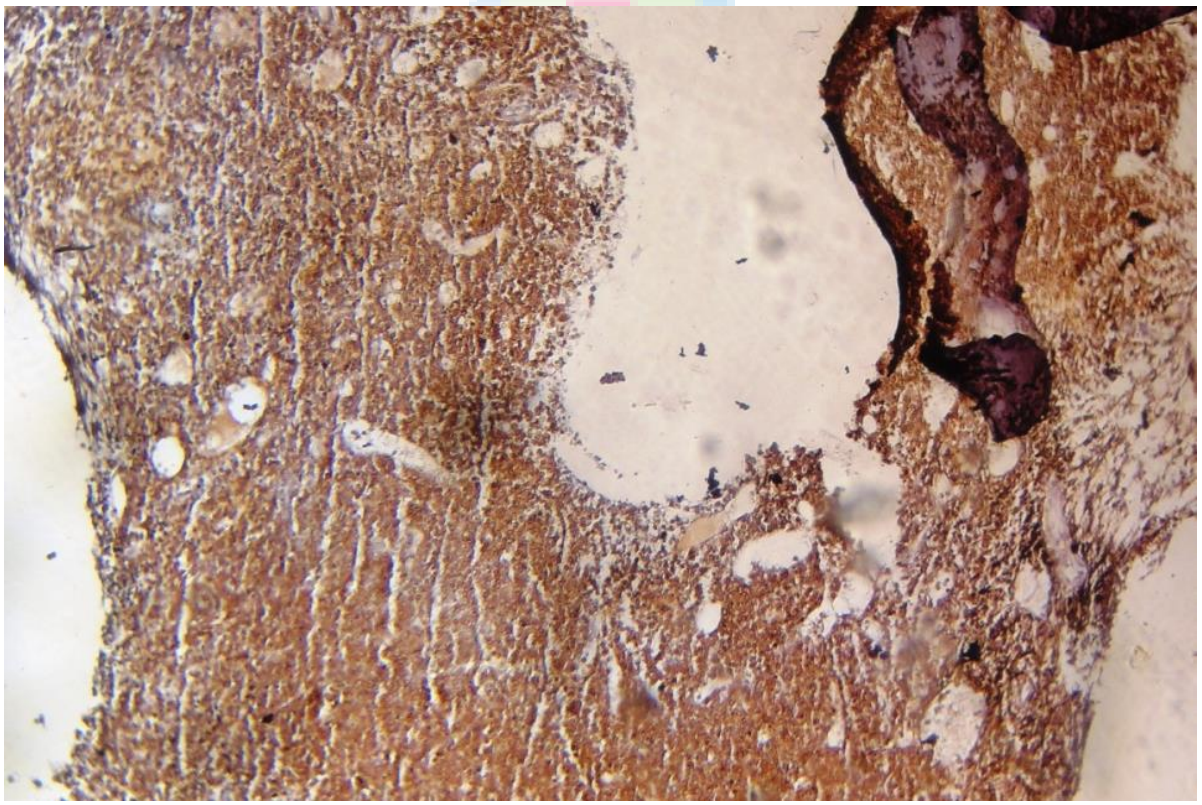

Figure VII. Trephine biopsy section from a patient with B-DLL showing diffuse infiltration. With CD20 + immunohistochemistry (X 10 objective.)

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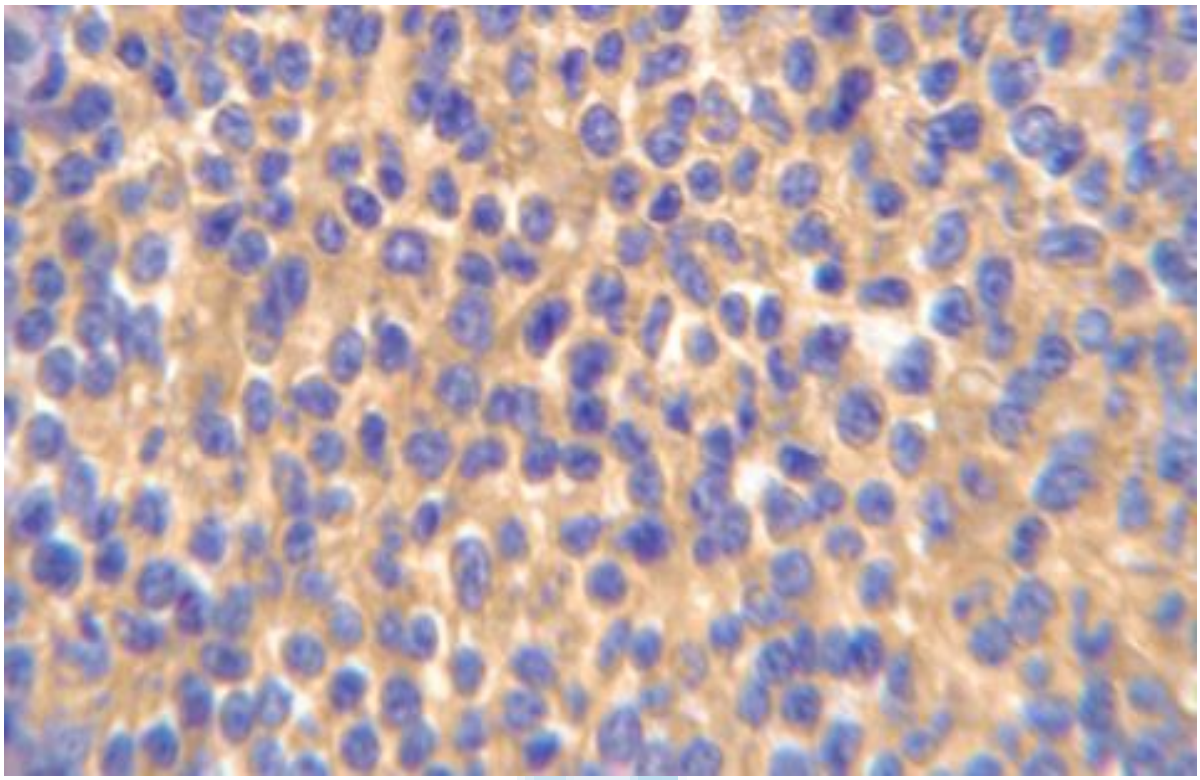

Figure VIII. Trephine biopsy section from a patient with B-DLL stained with immunohstochemistry showing positive VEGF-A lymphoma cells (arrows). With cytoplasmic brown staining (40X).

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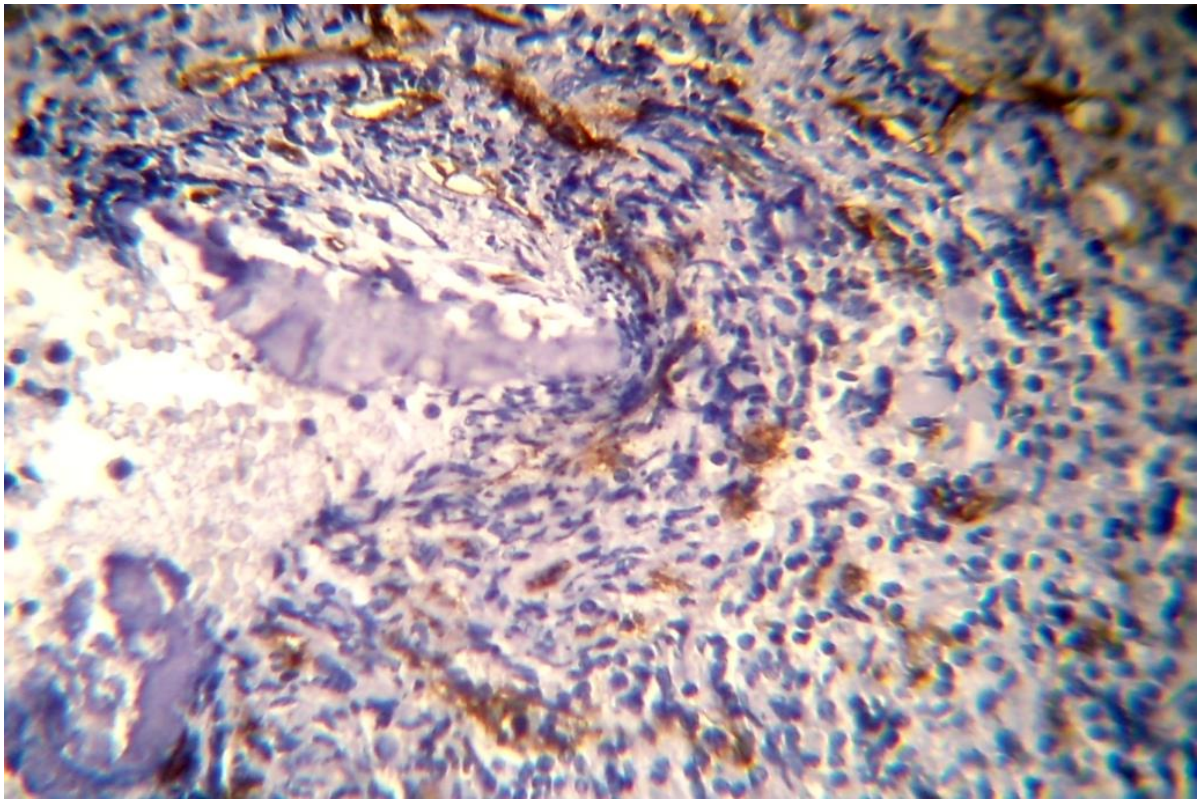

Figure IX. Trephine biopsy section from a patient with B-DLL showing endothelial cells positive for CD34 stained with immunohstochemistry (40X).

Discussion

In Iraq many researchers studied angiogenesis in different malignancies like prostate adenocarcinoma, multiple myeloma , renal cell carcinoma, gastric adenocarcinoma.[6-9] Kareem and Jaafer studied angiogenesis in haematolymphoid tumors, Kareem studied MVD in NHL, while Jaafer studied VEGF in CLL.[10,11]

But most of these studies used microvessel density as a marker of angiogenesis, with manual count, restricting their result to blood vessel count. Another study done by Qasim B. et al pushed the work step forward by using digimizer software for CD34+ blood vessels , this make assessment more subjective and computer based.[12]

This work tried to assess angiogenic markers in NHL, by studying VEGF expression and its autocrine effect via its receptor VEGFR expression and the paracrine effect via CD34+ vessel expression, using Digimizer software to analyze their immunohistochemical expression, instead of manual count for blood vessels and manual score for VEGF/VEGFR expression.

Many researcher suggest that VEGF may play dual roles in tumor angiogenesis, first through signaling to endothelial cells

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promoting them to form new blood vessels resulting in angiogenesis and second via signaling to tumor cells as an autocrine/paracrine growth factor.[2] This research assessed the interaction among vascularity (CD34+ area) and local expression of VEGF and VEGFR in NHL cases.

VEGF expression had positive correlation with VEGFR expression in different digimizer softwear parameters (intensity "I", area "A", digital labeling index "DLI"). This may be due to autocrine effect of VEGF. Both VEGF and VEGFR had positive correlation with lymphoma vascularity expressed by CD34 area "A", also reinforcing the concept of paracrine effect of VEGF. These findings are similar to findings of previous work.[10,13-18]

The VEGF immunohistochemistry is reflective of effective local VEGF signaling. It is expected that MVD increases with VEGF expression. Average lymphoma vascularity labeled by CD34+ endothelial cells did increase with strength of VEGF staining with statistical significant in the present work, and this was also demonstrated in the study by Berthold et al . [19] Dita et al have shown that higher MVD is present in DLCL specimens expressing higher levels of VEGF.[16] This finding is consistent with a paracrine role of VEGF elaborated by lymphoma cells in tumor angiogenesis.[3]

Conclusion

Autocrine and paracrine effect of VEGF is evident in NHL, as there is positive correlation between VEGF expression and VEGFR expression, furthermore as tumor vascularity increases expressed by CD34 stained area with the increase in VEGF expression.

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Correspondence to

Dr. Bassam Mohammad Hameed

Department of pathology and forensic medicine /collage of medicine /AL-Nahrain university

Email: bassammhammad@colmedalnahrain.edu.iq

Mobile: 07700664914

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التقيم المناعي النسيجي للمعامل (VEGF) وارتباطه ب (VEGFRوCD34) في الاورام اللمفاوية عدا هوجكن باستخدام نظام رقمي لتحليل الصور

م.د.بسام محمد حميد 1, م.د.هند شاكر عبعوب 2 , ا.د.رعد جابر موسى 3

1 مدرس في فرع علم الامراض والطب العدلي\كلية الطب\جامعة النهرين

2 مدرس في فرع علم الامراض والطب العدلي\كلية الطب\جامعة النهرين

3 استاذ في فرع علم الامراض والطب العدلي\كلية الطب\جامعة النهرين

الملخص

الخلفية: ان نمو الأورام اللمفاوية والتقدم بتكوينها يمكن ان يتم من قبل اثنين على الأقل من الآليات المرضية: التحفيز autocrine الخلايا السرطانية عن طريق التعبير الأوعية الدموية غشائي عامل النمو (VEGF) والأوعية الدموية غشائي عامل نمو مستقبلات (VEGFR) من قبل خلايا سرطان الغدد الليمفاوية، والتأثيرات نظير الصماوي من المكروية الورم proangiogenic على الأوعية الدموية السرطانية المحلي.

الأهداف: تقييم تأثير autocrine من VEGF، من خلال دراسة علاقة التعبير VEGF مع مستقبلات التعبير VEGFR في NHL. ولتقييم تأثير نظير الصماوي من عامل نمو بطانة الاوعية، من خلال دراسة علاقة التعبير VEGF مع CD34 أعرب عن الخلايا البطانية في سرطان الغدد الليمفاوية غير هودجكين (NHL).

المواد والطرق: تم تصميم دراسة مقطعية. أدرجت ما مجموعه 66 من عينات الأنسجة لنخاع العظم في الدراسة، نم ال تشخيص ل NHL وفقا working formulation. من كل عينة أخذت 3 أقسام، وصبغت immunohistochemically لCD-34، عامل نمو بطانة الاوعية وVEGFR. تم تنفيذ الترقيم باستخدام نظام متخصص الي وتحليل صورة الخلوية ببرنامج Digimizer، الإصدار 3.7.0.

النتائج :ارتبط VEGF المناعى المعلمات الرقمية اسمه مؤشر العلامات الرقمية (DLI) إلى حد كبير مع ما يلي،: [P = 0.042 VEGFR (DLI)، ص = 0.324]، CD34 منطقة الملون (A) [P = 0.037، ص = 0.556]. أيضا VEGFR (DLI) ارتبط بشكل ملحوظ مع A) CD34).

الخلاصة: تأثير Autocrine ونظير الصماوي من عامل نمو بطانة الاوعية هو واضح في NHL، كما أن هناك علاقة إيجابية بين التعبير VEGF والتعبير VEGFR، وكما زيادة الأوعية الدموية للورم يتناسب مع زيادة في التعبير VEGF.

ألكلمات المفتاحية: NHL، VEGF، VEGFR