Preliminary study of hypoxia markers in diffuse large B-cell lymphoma

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Abstract. While the association of hypoxia has been established in various types of solid cancers, little is known about its presence and existence in diffuse large B-cell lymphoma (DLBCL). The purpose of the present study was to evaluate the expression of hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor A (VEGF-A) in DLBCL and to analyze the association of these factors with several clinical and pathological characteristics. The immunohistochemical protein expression of HIF-1α and VEGF-A was investigated in 34 *de novo* DLBCL tumor samples from January 2017 to December 2017 from the Department of Hematology/Medical Oncology and Anatomical Pathology at Dr Kariadi Hospital (Semarang, Indonesia). The present study revealed by using immunohistochemistry (IHC), that hypoxic markers were overexpressed (88.2% for both HIF-1α and VEGF-A) in the vast majority of patients with DLBCL. Only in 4 tumors, was HIF-1α expression normal, and interestingly VEGF-A was negative as well. There was a significant correlation in the intensity of staining of HIF-1α and VEGF-A using our custom scoring system in surgically resected tissues (r=0.475; P=0.005). Both HIF-1α and VEGF-A were also associated to serum LDH and tumor diameter. Collectively, HIF-1α and VEGF-A were predominantly expressed in the majority of DLBCL tumor cells. The findings of the present study indicate the existence of hypoxia in DLBCL tumors similar to numerous solid cancers, and thus warrants further investigation to clarify its role as a potential pathogenic or prognostic marker in this type of hematological cancer.

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Introduction

Tissue hypoxia commonly occurs in tumors and adaptation to it appears to be one of the important characteristics of malignant cells. Accordingly, hypoxia-inducible factor-1α (HIF-1α) plays a key role in adaptation to hypoxia and regulates the expression of genes responsible for glucose metabolism, cell proliferation, angiogenesis, aggressive behavior and cancer stem cell survival (1). HIF-1α and hypoxia signaling influence a wide variety of pathways including those related to vascular endothelial growth factor A (VEGF-A) (2). VEGF-A is the major angiogenic factor that regulates different aspects of vascular angiogenesis and lymphangiogenesis (3). While hypoxia has been associated with various types of solid cancers (4,5), little is known about its presence and existence in lymphoid cancer, such as malignant lymphoma.

As a consequence of increased cellularity and proliferation, as well as enhanced metabolism within a tumor with relatively abnormal vasculature and blood supply, oxygen concentration within the tumor is generally lower than in adjacent non-neoplastic tissue (6). Cancer, irrespective of its origin, will develop hypoxic regions that express the HIF-1α and VEGF-A protein (2,4). The hypothesis is that diffuse large B-cell lymphoma (DLBCL) tissue would also develop a hypoxic milieu exponentially as the tumor grows, which causes resistance to chemotherapy, angiogenesis and maintenance of cancer stem cells (7), as has been demonstrated in several types of carcinoma, including those of the ovary, breast, prostate, lung, renal, glial cells, as well as melanoma.

There are numerous published studies concerning hypoxia in solid tumors. In various types of cancer, HIF-1α and VEGF-A have prognostic significance. However, there are only a few studies related to DLBCL, a highly proliferating cancer and aggressive lymphoma. A previous study by Evens *et al* (8,9) reported that HIF-1α was highly expressed in ~59-70% of patients with DLBCL. In another study, Pazgal *et al* (10) revealed the expression of VEGF and its receptor in non-Hodgkin's lymphoma. The aim of the present study was to determine the expression of HIF-1α and VEGF-A in DLBCL, as our preliminary data for future research.

Materials and methods

Patients and specimens. Using the database from the Division of Hematology/Medical Oncology and the Department of Anatomical Pathology of Dr Kariadi Hospital (Semarang, Indonesia), 149 patients who were diagnosed with DLBCL from January to December 2017, were identified. After obtaining approval from the Institutional Review Board, the following data were recorded retrospectively from the files: Patient demographic characteristics (notably age), onset of disease, clinical and pathological results, serum lactate dehydrogenase (LDH), hemoglobin (Hb) at presentation, Hb prior to diagnostic procedure, glomerular filtration rate, the National Comprehensive Cancer Network (NCCN) International Prognostic Index (IPI) score (11), nodal/extranodal disease, diameter of the tumor being biopsied, and Ann Arbor staging. No patients had a history of chemotherapy or radiotherapy before surgical sampling and DLBCL diagnosis.

The present study involving human tissue samples was approved by the Medical Ethics Committee of Dr Kariadi Hospital (reference no. 338/EC/KEPK-RSDK/2019). Written informed consent from patients was also obtained for the study of the resection specimens and for the use of their clinical data. However, informed consent was obtained only from patients with eligible samples (n=34) for data collection and publication purposes.

Clinical characteristics. Pre-treated DLBCL samples were collected from 34 patients with archived data, from the Division of Hematology/Medical Oncology and the Department of Anatomical Pathology patient databases of Dr Kariadi Hospital. The histological sections were reviewed by two pathologists to verify the histologic diagnosis. Diagnosis was based on the World Health Organization Classification of Tumours of Hematopoietic and Lymphoid Tissue (12). Characteristics of all patients are shown in Table I. Clinical staging was determined according to the Ann Arbor system (stage I, involvement limited to a single lymph node area; stage II, involvement of more than one lymph node in the regional area; stage III, multiple involvement of lymph node areas on both sides of the diaphragm; stage IV, generalized involvement) as well as the presence of 'B' symptoms such as unintentional weight loss, low grade fever and drenching night sweats (13).

Cases preoperatively treated with radiation or chemotherapy and those with incomplete clinical data were excluded. A total of 34 samples were eventually evaluated in the clinical and histological study. There was a similar proportion of male and female patients, and all patients had not undergone any treatment at the time of biopsy. Only samples measuring >2 cm from the tumor excision were considered. Immunohistochemical staining for CD10, MUM-1, BCL-6 to germinal center B-cell-like (GCB) and non-GCB subtyping and Ki-67 were available for all specimens as per respective regular practice for DLBCL diagnosis and assessment of proliferation index of Dr Kariadi Hospital.

Histological assessment. All archival specimens were fixed in 10% neutral-buffered formalin for 24 h at room temperature and embedded in paraffin using the routine method. For this study, the specimens were cut into 4-μm-thick sections and subjected to immunohistochemical analysis conducted via the avidin-biotin-peroxidase complex method.

Both the H&E slides and the immunohistochemical stains were evaluated in a blinded fashion separately by two pathologists (HI and DP). In the case where there was a discrepancy, the slides were reviewed together on a double-headed scope. For all immunohistochemical markers, the percentage of cell staining was recorded. Tumors were considered positive if >10% of cells evaluated expressed the antibody. Overexpression was further categorized into groups by the percentage and intensity of cells stained.

Immunohistochemistry (IHC). Immunohistochemical techniques were used to evaluate the expression of HIF-1α and VEGF-A using specific antibodies: Human anti-HIF-1α (cat. no. MAB1536-SP; R&D Systems, Inc.) and human anti-VEGF (cat. no. 298-VS; R&D Systems, Inc.), both at a 1:100 dilution and their respective IgG isotype control (cat. no. BZ-0840590F-AP; Bioenzy, Inc.). After recovering the antigen from the slides, they were briefly placed in a 0.01 M sodium citrate solution and incubated in a 40-50˚C water bath for 20 min. The sections were then blocked with 5% pig serum (Biocare Medical) for 2 h at room temperature and the antibodies of interest were added. The preparation was incubated with the primary antibody overnight at 4˚C in a humid chamber. The universal biotinylated link (JAN code 4987582002362; Dako; Agilent Technologies, Inc.) and streptavidin-conjugated with horseradish peroxidase (HRP) (cat. no. HP604; Biocare Medical) were used as secondary antibodies, incubated also at room temperature for at least 30 min. Color was generated by adding the substrate diaminobenzidine (DAB) for 1 to 2 min and counterstaining was performed with hematoxylin for 5 min. Finally, the cells were dehydrated and covered with resins.

All immunohistochemical reactions were conducted using formalin-fixed and paraffin-embedded samples. Specific immunoreactivity was observed in the cytoplasm and the nuclei of the tumor cells. Expression of both hypoxia markers was assessed by analyzing at least 1,000 tumor cells from representative tumor fields using a microscope at a magnification of x250, and the labeling index was calculated as the percentage of labeled nuclei of the total number of tumor cells counted.

Scoring criteria. The extent of staining was categorized into six semiquantitative scales based on the percentage of positive tumor cells: 0 (<1% positive cells), 1 (1-10% positive cells), 2 (11-24% positive cells), 3 (25-49% positive cells), 4 (50-74% positive cells), and 5 (≥75% positive cells). The cut-off for HIF-1α percentage staining with IHC was based on a previous study by Evens *et al* (8,9), where a 10% value was defined as overexpression of a DLBCL tumor. The intensity of staining was also determined semi-quantitatively on a scale 0 to 3 as follows: 0 (negative), 1 (weakly positive), 2 (moderately positive) and 3 (strongly positive). Multiplication of the percentage score and intensity gave rise to the final score of a maximum of 8 points (Table I). For statistical analysis, tumors were categorized according to their final staining score as negative or normal or low expression (score, +2), mild overexpression (score, 3-4), moderate overexpression (score, 5-6), and high overexpression (score, 7-8).

Table I. Customized scoring system used in the present study.

	Positive distribution score		Intensity score	
Score 0	No cells stained	Negative	Score 0	Negative
Score 1	>0 to 10% of cells stained	Normal	Score 1	Weak intensity
Score 2	>10 to 30% of cells stained	Overexpression	Score 2	Intermediate intensity
Score 3	>30 to 50% of cells stained	Overexpression	Score 3	Strong intensity
Score 4	>50 to 75% of cells stained	Overexpression		
Score 5	>75% of cells stained	Overexpression		

The total score is the sum of the positive distribution and intensity score, with a maximal score of 8.

Statistical analysis. Differences were evaluated using SPSS v.21 (IBM Corp.). The association between staining intensity and clinicopathological patterns was assessed. Spearman correlation test was used to examine the correlation between clinical characteristics, tumor size, and IHC. Spearman rank test was used to investigate whether the scores of HIF-1α and VEGF-A immunohistochemical labelling were correlated with age, tumor diameter, and serum LDH. Statistical tests were two-sided and correlation was considered significant for a P-value of <0.05.

Results

Patient and tumor sample characteristics. A total of 34 samples were included in the current analyses. The clinical and pathological characteristics of patients with DLBCL are shown in Table II. There were 17 men and women with a mean age of 51.2 years (ranging from 24 to 77 years old). The histological diagnosis in all patients was DLBCL, based on the WHO classification (12). The mean time from onset of disease to first diagnostic revelation was 5.1 months. There was more limited-stage DLBCL compared to advanced-stage disease. However, the majority of samples had high-intermediate and high prognostic risk according to the National Comprehensive Cancer Network (NCCN)-IPI score (11). The majority of tumors were sampled based on excisional biopsy from nodal disease (n=25) while the rest were examined from extranodal tumors from the gastrointestinal tract (n=5), nasopharyngeal (n=3), and central nervous system (n=1). With regard to cell-of-origin subtype, this study involved both GCB and non-GCB in a relatively comparable proportion, that is 47.1 and 52.9%, respectively.

HIF-1α protein expression. Within positive tumors, the extent, intensity, intracellular location, and distribution of staining observed with the antibodies were heterogenous. The number of positive tumors did not correlate with the intensity of staining and ranged from <1% to 90% of tumor cells. In the cases examined, HIF-1α nuclear staining was found in <10% of tumor nuclei in 11.8%, between 10-25% in 11.8%, between 26-50% in 35.3%, between 51-75% in 44.1% and in >75% in 8.8% of tumors. The intensity of nuclear immunoreactivity for each antigen in different tumor cells varied. To illustrate these points, examples of immunostaining are shown in Fig. 1A.

VEGF-A protein expression. Hypoxia upregulates the expression of a variety of genes important in cancer biology, including VEGF (3,10). Immunohistochemical staining was also performed to determine whether the pattern of HIF-1α protein expression observed was correlated with the distribution of VEGF-A protein. Signals were predominantly observed at non-necrotic and viable tumor margins (Fig. 1B), as has been previously reported in solid tumors (14).

Approximately 88.2% of DLBCL samples had overexpression of VEGF-A with 32.4% of the neoplastic cells exhibiting high immunoreactivity (score of 7 or 8). The pattern of labelling was diffuse and cytoplasmic. Similar proportions of the tumor cells also showed HIF-1α expression; a mild overexpression (score, 3-4) was recorded in 35.3%, a moderate overexpression (score, 5-6) in 44.1%, and high overexpression in 8.8% of the samples. The pattern of labelling was primarily diffuse and cytoplasmic, and, in one case, perinuclear. The proportion of HIF-1α and VEGF-A immunohistochemical scores in DLBCL are presented in Fig. 2.

HIF-1α and VEGF-A correlation with clinical characteristics. In univariate analysis, the patterns of HIF-1α and VEGF-A expression were analyzed with the following clinical parameters: Age, tumor diameter, Hb values, serum LDH, and tumor Ki-67 (Table III). The following results were obtained: i) HIF-1α and VEGF-A: Based on the IHC total score (the sum of the percentage of stained cells and intensity), HIF-1α and VEGF-A were revealed to reflect the degree of overexpression and a moderate positive correlation was observed between them; ii) age of the patients: Both HIF-1α and VEGF-A expression did not appear to be affected by age; tumor diameter: A significant positive correlation between HIF-1α and VEGF-A with tumor size was noted (the larger the DLBCL tumor was, the higher both HIF-1α and VEGF-A expression levels were); Hb level: HIF-1α was negatively correlated with the Hb value (P=0.165) while VEGF-A appeared to less likely be associated with the Hb value; serum LDH: LDH was significantly correlated with HIF-1α and VEGF-A expression as well as the tumor diameter; and Ki-67 tumor: This proliferation index was not correlated with HIF-1α VEGF-A, age, tumor diameter, Hb values, and serum LDH.

Discussion

Since DLBCL is the most common subtype of aggressive lymphomas in humans, previous research has attempted to identify

Table II. Characteristics of patients with DLBCL.

Variables	No. of patients (%)
Age (years)	51.2±14.2
Age, min-max (years)	24-77
Sex (male/female)	17/17
From onset to hospital admission, months	5.1±2.2
Presence of B symptoms	21 (61.8%)
Laboratory values	
Hb value at presentation (g/dl)	12.1±2.2
Pre-operative Hb (g/dl)	12.6±1.4
eGFR (ml/min)	83.1±26.7
Pre-operative LDH (IU/l)	829.5±551.5
LDH range, min-max (IU/l)	277-2,322
Disease information	
Ann Arbor staging	
Limited stage (Ann Arbor I + II)	22 (64.7%)
Advanced stage (Ann Arbor III + IV)	12 (35,3%)
NCCN-IPI Score	
Low and low-intermediate risk (0-2)	10 (29.4%)
High-intermediate and high risk (3-5)	24 (70.6%)
Tumor characteristics	
Tumor diameter (min-max), sampled (in cm)	2.9±0.8 (2.0-5.4)
DLBCL subtype	
GCB	16 (47.1%)
Non-GCB	18 (52.9%)
Ki-67 expression	49.1±13.2
Ki-67, min-max	30-77.5
HIF-1α expression	
Normal	4 (11.8%)
Overexpression	30 (88.2%)
VEGF-A expression	
Normal	4 (11.8%)
Overexpression	30 (88.2%)

DLBCL, diffuse large B-cell lymphoma; Hb, hemoglobin; eGFR, estimated glomerular filtration rate; LDH, lactate dehydrogenase; NCCN-IPI, National Comprehensive Cancer Network-International Prognostic Index; GCB, germinal center B-cell-like; non-GCB, non-germinal center B-cell-like; HIF-1α, hypoxia-inducible factor-1α; VEGF-A, vascular endothelial growth factor A.

early markers of this condition (12), which would be helpful to detect the aggressiveness of the cancer. The presence of hypoxic regions within tumors as the result of tumor growth and imbalance of vasculature has long been reported to be associated with a poor survival (14). Because hypoxia stabilizes HIF-1α, which then triggers the expression of target genes (1,4,7), the present study focused on HIF-1α and VEGF-A (one of its downstream products). Previous studies by Evens *et al* (8,9) and Pazgal *et al* (10) have already addressed this topic, but results have been largely controversial. This is the first time, to the best of our knowledge, that the impact of both markers were evaluated in DLBCL.

The hypothesis is that DLBCL tissue develop a hypoxic milieu exponentially as the tumor grows, which causes resistance to chemotherapy, angiogenesis and maintenance of cancer stem cells, as has been shown in several types of carcinomas including those of the ovary, breast, prostate, lung, renal, glial, as well as melanomas (7). The association between protein expression levels in this 'solid-like tumor' hematological malignancy and its various clinicopathological features were therefore examined.

A major finding in the present study of high-grade malignant lymphoma was the up-regulation of HIF-1α and VEGF-A protein levels in tumors. Since the HIF-1α subunit is unstable in oxygenated tissue, it should be kept in mind that these findings in fixed tissue will represent the *in vivo* situation for every detail. The present study extends these findings in demonstrated upregulation of HIF-1α as well as VEGF-A, which raises an important question about both the mechanisms of protein upregulation and its consequences for the tumor, and ultimately the impact to overall medical management of DLBCL.

The present study revealed that 88.2% of DLBCL tumor samples exhibited VEGF-A overexpression. With regard to VEGF-A expression, Shahini *et al* (15) demonstrated a high proportion of VEGF-A in DLBCL from low positivity to high positivity with only 3% staining negative (n=30). Notably, the level of VEGF-A expression was correlated with IPI prognostic score and microvascular density. In another study, overexpression of VEGF, its VEGF-receptor, and microvessel density were reported to be correlated with a poorer response related to systemic chemotherapy (16). A systematic meta-analysis also concluded that tumor VEGF expression was associated with worse survival in non-Hodgkin's lymphoma (17).

The main stimulus for VEGF expression is hypoxia through the HIF-1α pathway (1,3). The results of the present study were similar to those previously described for DLBCL, with moderate to strong cytoplasmic and nucleoplasmic staining of HIF-1α antibody observed in DLBCL cells (10). In previous studies, the HIF-1α positivity rate ranged from 56.0 to 67.3% (8,9). The present study revealed a higher amount (>80%) of HIF-1α overexpression in DLBCL. The present study also revealed that the expression of HIF-1α may differ according to age and Hb value ($P>0.05$). The association between HIF-1α and VEGF was examined in the present study and it was determined that both transcriptional factors were significantly correlated with a higher tumor diameter and more advanced stage.

The HIF-1α transcriptional factor plays an essential role in oxygen homeostasis and high expression of HIF-1α protein has been found to be associated with both tumor aggressiveness and unfavorable prognosis of various types of cancers. In contrast to VEGF-A data, HIF-1α overexpression was found to be an important independent favorable prognostic factor for survival in patients with DLBCL treated with standard chemotherapy R-CHOP (9,10).

It is now understood that the metabolic reprogramming in cancer is driven by several oncogenes and tumor suppressors (18). Some of the identified oncogenes, namely protein kinase B (PKB/Akt), Ras, and von Hippel-Lindau (VHL), act via the HIF-1α protein, resulting in non-hypoxic expression of HIF-1α. In normal cells, HIF-1α becomes stabilized in a hypoxic environment (1,4). Hypoxia is also a condition that

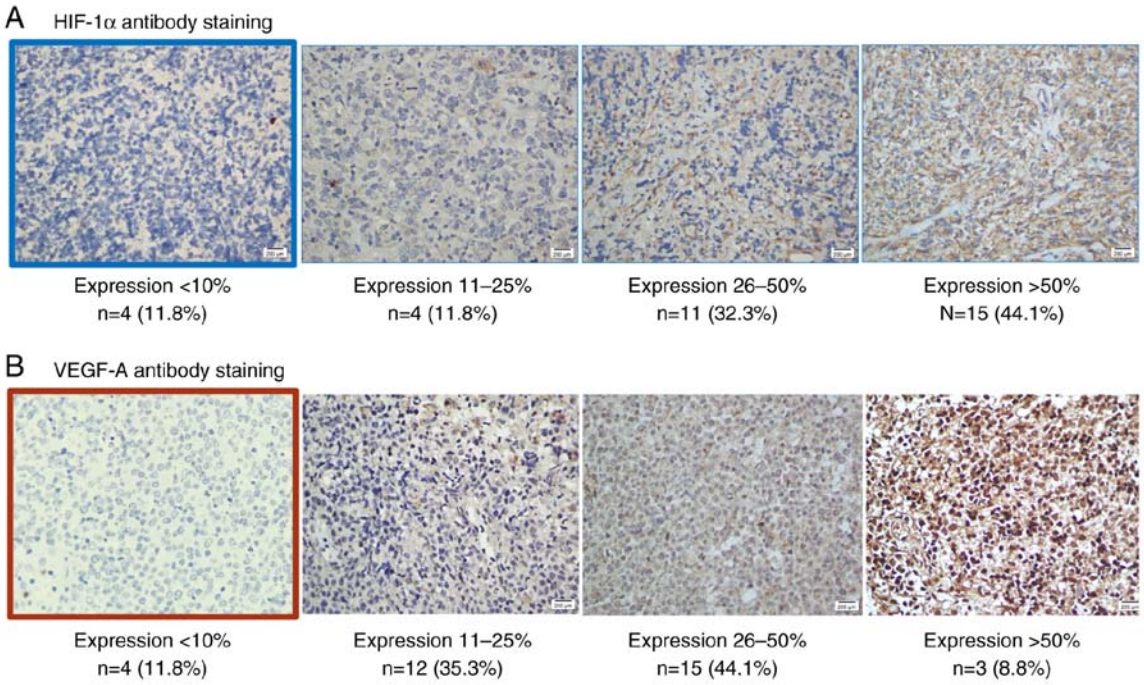

Figure 1. Representative images of immunohistochemical expression of HIF-1α and VEGF-A. (A) A section of DLCBL tissue samples showing expression of HIF-1α, negative staining is on the very left panel. (B) A section of DLBCL tissue samples showing expression of VEGF-A. Magnification, x400. HIF-1α, hypoxia-inducible factor-1α; VEGF-A, vascular endothelial growth factor A; DLCBL, diffuse large B-cell lymphoma.

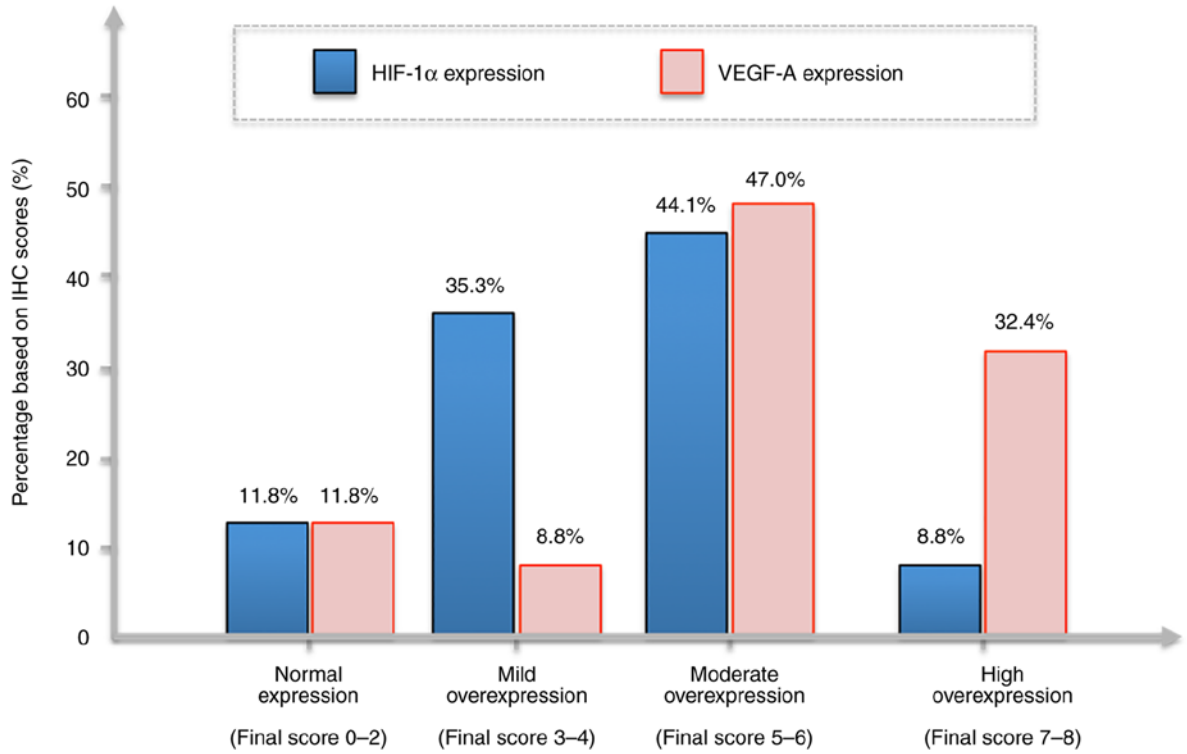

Figure 2. Percentages of HIF-1α and VEGF-A according to various degrees of expression using total immunohistochemical scores (the sum of the staining percentage and staining intensity as described in the *Scoring criteria* section of the Materials and methods). HIF-1α, hypoxia-inducible factor-1α; VEGF-A, vascular endothelial growth factor A.

almost certainly occurs in the tumor microenvironment when there is tumor expansion leading to an imbalance between vascular growth and increased oxygen demands. This finding is now accepted as a universal finding in numerous types of solid cancers (2,4,5,19), and is also characteristic of highly proliferative cancers such as DLCBL (20).

Further research incorporating HIF-1a and/or VEGF-A or some other factor in the hypoxia pathway will be undertaken

Table III. Cross correlation between certain clinical variables, laboratory value and hypoxia markers.

	HIF-1α	VEGF-A	Age	Tumor diameter	Hb value	Serum LDH	Ki-67
HIF-1α	N/A	r=0.475; P=0.005[a]	r=0.085; P=0.631	r=0.388; P=0.023[a]	r=-0.244; P=0.165	r=0.662; P<0.001[a]	r=0.220; P=0.211
VEGF-A	r=0.475; P=0.005[a]	N/A	r=0.131; P=0.461	r=0.341; P=0.049[a]	r=0.020; P=0.910	r=0.498; P=0.003[a]	r=0.182; P=0.304
Age	r=0.085; P=0.631	r=0.131; P=0.461	N/A	r=0.256; P=0.145	r=0.212; P=0.229	r=0.089; P=0.617	r=0.172; P=0.332
Tumor diameter	r=0.388; P=0.023[a]	r=0.341; P=0.049[a]	r=0.256; P=0.145	N/A	r=-0.127; P= 0.475	r=0.364; P=0.034[a]	r=0.301; P= 0.085
Hb value	r=-0.244; P=0.165	r=0.020; P=0.910	r=0.212; P=0.229	r=-0.127; P=0.475	N/A	r=-0.266; P=0.128	r=-0.138; P=0.436
Serum LDH	r=0.662; P<0.001[a]	r=0.498; P=0.003[a]	r=0.089; P=0.617	r=0.364; P=0.034[a]	r=-0.266; P= 0.128	N/A	r=0.055; P=0.756
Ki-67	r=0.220; P=0.211	r=0.182; P=0.304	r=0.172; P=0.332	r=0.301; P=0.085	r=-0.138; P=0.436	r=0.055; P=0.756	N/A

HIF-1α, hypoxia-inducible factor-1α; VEGF-A, vascular endothelial growth factor A; Hb, hemoglobin; LDH, lactate dehydrogenase; N/A, not applicable.

in a future study. The primary hypothesis is nonetheless that an additional therapeutic target in combating tumor hypoxia, angiogenesis and progression will ultimately lead to improvement of the outcome of DLBCL.

The VEGF gene and several other genes involved in the homeostatic response to oxygen levels are under the control of HIF-1α, a transcriptional activator mediating changes in gene expression in response to changes in cellular oxygen concentrations (3,6). HIF-1α expression in numerous human cancers has been demonstrated to be correlated with tumorigenicity and angiogenesis (5,21). With all samples taken into account, Table III revealed that the IHC HIF-1α score was correlated with VEGF-A. Notably, the degree of overexpression as depicted in Fig. 2 indicated that perhaps at some point, the regulation of both markers was different and independent of each other. An explanation for this finding cannot be provided; however, the degree of tumor diameter, the proportion of nodal vs. extranodal involvement, disease stage, and patient performance status may be considered to modify each marker expression and the relationship between them.

Immunohistochemistry is inherently a subjective assessment method to quantify tumor proteins or markers. The scoring system used in the present study delineates the expression and intensity against both HIF-1α and VEGF-A antibodies in tumors, aiming to enhance its objectivity. Another possible objective tool used for assessment is RT-qPCR. However, such technology was unavailable, and along with our small sample size, were limitations of the present study. No attempt was made to correlate HIF-1α with VEGF-A due to the nature of this semiquantitative data. The number of samples in the present study was also relatively small and this was a single center study, thus it may not sufficiently be representative of DLBCL tumors, especially since tumor size was not taken into account in HIF-1α and VEGF-A quantification due to technical issues related to diagnostic sample availability.

In conclusion, HIF-1α and VEGF-A expression levels were elevated in patients with DLBCL in a significant proportion of the samples obtained in January 2017 to December 2017 at Dr Kariadi Hospital. These findings may have implications for the understanding of DLBCL biology and potential treatment strategies with regard to the hypoxic milieu and either HIF-1α and/or VEGF-A expression. The present study ultimately provides preliminary data to confirm the clinical significance of HIF-1α and VEGF-A expression for routine application. Further investigations of this pathway should be performed both *in vivo* and *in vitro* to determine whether the HIF-1α/VEGF-A pathway is clinically useful for either prognosis or as a therapeutic target for an improved approach in patients with DLBCL.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

EAP and PW designed of the study, and performed patient inclusion and follow-up. RMN helped write the first draft of the manuscript, and organized and searched for the relevant literature. HI and DP participated in the immunohistochemical staining, interpreting and analyzing the results, and then providing their expertise in pathological evaluation. BS and DS were involved in reviewing and critically revising the article for important intellectual content. CS also designed the study, analyzed and interpreted the results and supervised the course of the study. EAP and PW confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Human samples used in the present study were obtained from patients who had provided written informed consent. The study was approved by the Ethics Committee of Dr Kariadi Hospital (Semarang, Indonesia), and was conducted according to The Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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