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ADVANCE STAGE OF HODGKIN LYMPHOMA SHOWS HIGH DENSITY OF PLASMA CELLS BUT LOW ANGIOGENESIS

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ABSTRACT

Tumor microenvironmental of classical Hodgkin lymphoma (cHL) containing plasma cells and angiogenesis, however their exact roles remained indeterminate. Plasma cells are important prognostic factors of various malignancies. Angiogenesis can be assessed by evaluating of micro vessel density (MVD). Higher MVD is associated with a poor prognosis in various type of malignancies. The objectives of this study were to investigate the density of plasma cells and MVD in the microenvironment of cHL and to determine the association of both components with the stage of cHL. The selected 37 paraffin blocks of cHL cases were sectioned. The clinical staging was performed using modification of Ann Arbor staging system. To assess the density of plasma and endothelial cells, anti-CD138 and anti-CD34 antibodies were employed by immunohistochemistry. The association of plasma cells'densities and MVD with cHL stages was measured by statistical analysis using t-test by STATA version 15 with significant consideration if $p < 0.05$. In our cohort cHL was occurred in slightly older patients (≥40 years; 53.33%), more in male (60%), and majority with nodal location (86.67%). The subtypes was dominated by lymphocyte rich (43.33%); followed by nodular sclerosing (30%) and mixed cellularity (26.67%), while lymphocyte depleted was not found. Statistical analysis revealed that higher density of plasma cells was significantly correlated to the patient higher stages ($p=0.0003$). While density of micro vessels is not significant correlation ($p=0.5564$) to the stages of cHL**.** High densities of plasma cells but not angiogenesis correlate to advance stage of Hodgkin lymphoma.

Keywords**:** Hodgkin lymphoma, angiogenesis, advance stage, CD138, CD34

1. INTRODUCTION

The incidence rate of classical Hodgkin lymphoma (cHL) in underdeveloped countries is lower in comparison to the developed regions, however, the mortality rates is higher in underdeveloped countries.[1] Worldwide incidence and mortality of cHL were 0.4 and 0.2 percent of all sites in 2020.[2] Although majority of cHL patients is curable, almost 20% of patients eventually relapse and succumb to the disease.[3] The tumor microenvironment (TME) has important roles in tumor biology, growth, progression, and resistance to chemotherapy in malignancies including cHL.[4] The diversity of cHL environment is unique

and becoming its characteristics and among this variety angiogenesis and plasma cells are included as important components.[5] The roles of these two components are still unknown. Plasma cells are important prognostic factors of various malignancies including cHL. A recent analysis of the role $CD138^{+}$ plasma cells revealed that greater plasma cell infiltration is correlated with an advanced stage of the disease and poor survival.[6] Angiogenesis is the process of new blood vessel growth, thus this process has important roles in tumor development and tumor progression.[7] It can be measured by evaluating of micro vessel density (MVD) assessment. Higher MVD is associated with a poor prognosis in various type of malignancies.[8-10] However, there is still no clear evidence of the roles of plasma cells and angiogenesis in cHL.[11]

The evaluation of cHL staging has to be accurately performed as an important step for precise treatment selection. The system of staging for cHL patients is determined based on some evidences comprising of the lymph nodes involvement. Firstly, is the location of disease involvement; on the one or both sides of the diaphragm, the second is the number of involved sites, the third is bulky tumor, the fourth is whether there is contiguous extranodal involvement or disseminated extranodal disease, and the last is the present of B symptoms.[12]

The objectives of this study were to investigate the density of plasma cells and MVD in the microenvironment of cHL and to determine the association of these both components with the stage of cHL.

2. METHODS

A cross-sectional method was performed on the paraffin blocks of carefully chosen cases. Initially, we collected 44 cases of Hodgkin lymphoma diagnosed based on the 2016 World Health Organization classification.[13] These patients were registered from 1st January 2018 to 31st December 2021 at Department of Anatomic Pathology, Faculty of Medicine University of Sriwijaya, Dr. Mohammad Hoesin Hospital, Palembang, Indonesia. After watchful selection based on the quality of fixation and laboratory processing which can be assessed by carefully examined the all slides of Hematoxylin-Eosin and Immunohistochemistry, 37 cases were obtained as samples of this research. The remaining 7 samples were excluded because poor quality of tissue fixation which will influence quality of antibody expression of further immunohistochemistry staining. The selected 37 paraffin blocks were sectioned. The clinical staging was performed using Ann arbor staging system.[12]

To assess the density of plasma cells and MVD immunohistochemistry was performed by using anti-CD138 and anti-CD34 antibodies respectively. Plasma cells were assessed using anti-CD138 (EP201, monoclonal, © Medaysis Company, USA). Angiogenesis was assessed using immunohistochemistry using anti-CD34 (Class II, Clone QBEnd 10, DAKO, Denmark) in all the cases. Immunohistochemistry was performedusing standard protocols with streptavidin-biotin complextechnique and microwave heat-induced antigen retrieval incitrate buffer (pH 6.0). The detection system was provided by Lab Vision Corporation (Neo Markers, Fremont, California, USA).

The tissue sections initially were deparaffinized and rehydrated in graded alcohols. The endogenous peroxidase was blocked using 0.3% hydrogen peroxide for 20 min. The antigen retrieval conducted by microwaving the sections in a thermoresistant jar containing citratebuffer pH 6.0 for both antibodies. The primary antibody anti-CD138 and anti-CD34 was then applied to the tissues. The reaction

product was developed using diaminobenzidine tetrahydrochloride for 10 min. The slides were counterstained with hematoxylin, dehydrated, mounted and ready to be measured.

The CD 138 and CD34-stained sections were examinedat 100x magnification to select 5 hot spots of the region with the highest density of CD138 expression and the most intense vascularization of CD34 expression. Furthermore, from five hotspots above, another five hotspots of regions with the densest areas of CD138 and CD34 expression were selected and captured at 400x magnification with each field representing an area of 0.96 mm^2 using light microscope Olympus CX33 with digital camera Indomikro modification. All plasma cells; defined as CD138 positive cells as distinct cell membrane and cytoplasm brown staining (Figure 1A). The expression of CD34 in endothelial cytoplasm and/or cell membrane was carefully evaluated to determine one micro vessel. A micro vessel; defined as distinct CD34 positive cells or cell cluster, irrespective of lumen (Figure 1B).

The expression of CD 138 and CD 34 were calculated as numbers of plasma cells and micro vessels per 0.96 mm2 respectively using Image J software (Java based image processing 1.53c GPLv2, National Institute of Health). The association of densities of plasma cells and MVD with the stages of cHL was measured by statistical analysis using t-test by STATA version 15 (college Station, Texas, 77845 USA) with significant consideration if $p < 0.05$.

3. RESULTS

In this study cHL patients ages were observed with ranges of ages 7 to 77 years with the median age was 40 years.

Table 1. Clinicopathologic characteristics of cHL' patients and staging

Characteristics	n (%)	Stages	
		Advanced (%)	Limited (%)
Ages			
• ≤35 y	18 (48.65)	7 (38.89)	11 (61.110)
• 36-54	15 (40.54)	9 (60.00)	6 (40.00)
• ≥55y	4 (10.81)	1 (25.00)	3 (75.00)
Gender			
• Male	24 (64.87)	12 (50.00)	12 (50.00)
• Female	13 (35.13)	5 (38.46)	8 (61.54)
Locations			
• Nodal	33 (89.19)	16 (48.49)	17 (51.52)
• Extra nodal	4 (11,71)	1 (25)	3 (75)
Variants			
• Lymphocyte rich	14 (37.84)	6 (42.86)	8 (57.14)
• Nodular sclerosing	12 (32.43)	7 (58.33)	5 (41.67)
• Mixed cellularity	11 (29.73)	4 (36.36)	7 (63.64)
Stages			
• Limited disease	20 (54.05)	-	-
• Advanced disease	17 (45.95)	-	-

The cases were observedslightly more in older patients (in the age group of 40 years and over; 53.33%), higher in male (60%), and majority with nodal location (86.67%). The cHL subtypes was dominated by lymphocyte rich (43.33%); followed by nodular sclerosing (30%) and mixed cellularity (26.67%), while lymphocyte depleted cHL was not found among our patients (Table 1). In addition, the total number of patients with advanced and limited stages among the age groups of ≤35 years and 36-54 years were not much different. While in group of ≥55 years although the limited stage was 3 times than the advanced one, however, the number of patients in this group was too small. Similar trend can be seen in the group of locations, nodal and extranodal. Likewise, the total number of patiens in gender group, showed not much different between advanced and limited among male-female groups. Similar trend can also be seen among variant of cHL (Table 1).

According to the Ann Arbor staging system and its Cotswolds' modification, 14 cases (37.84%) were included of stage IA, 12 cases (32.43%) were of stage II, 8 cases (21.62%) were of stage III, and 3 cases (8.11%) were of stage IV. For statistical purposes, stage I and II cases were considered as a limited disease, whereas stage III and IV cases were considered as an advanced disease. Our cohort showed slightly more of limited disease (54.05%) in comparison to the advanced stage of disease (Table 1). Statistical analysis using t-test (STATA version 15) revealed that higher density of plasma cells was significantly correlated to the patient higher stages (p=0.0003). While density of micro vessels showed no significant correlation (p=0.5564) to the stages of cHL. In addition, density of plasma cells has no correlation with angiogenesis (p=0.396).

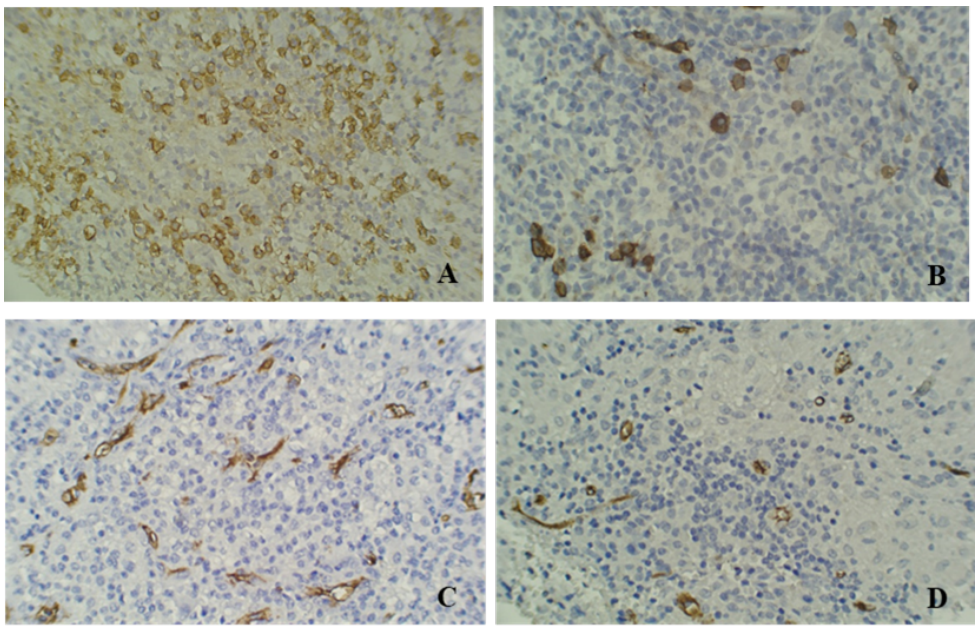

Figure 1. The expression of CD138 and CD34 on classical Hodgkin lymphoma. The expression of CD138 is found membranous localization in plasma cells of cHL microenvironment which showed high density (A) or low density (B). Immunostaining of endothelial cells by anti-CD34 antibody showed membranous staining, on high density (C) and low density (D).

4. DISCUSSION

The microenvironment of cHL consists of many components; plasma cells, macrophages, eosinophils, T regulatory cells, and blood vessels including endothelial cells, stromal cells and others. Reed-Sternberg and Hodgkin cells produce cytokines which induce plasma cells in microenvironment of cHL.[14-15] Plasma cells roles in cHL microenvironment is still unclear but may be correlated to B-symptoms and have an inferior effect on prognosis.[16]

The limitation of our study that we have no information of B symptoms and history of patients' therapy as well as follow up post therapy. Our finding showed that higher density of plasma cells was related to more advanced stages of cHL. We did not identify isotypes of immunoglobulins of plasma; therefore, it was not sure whether $CD138^+$ plasma cells among TME of cHL in our study have their terminal differentiation since certain immunoglobulin isotype such plenty IgG1 but little amount of IgA among TME showed positive correlation with overall survival.[17] Advanced-stage disease of cHL is associated with short overall survival, hence, it could be possible that plasma cells among TME of cHL might be prevented from terminal differentiation which may facilitated the tumor cells to escape from the immune attack, thus allowing the tumor microenvironment most likely becoming favorable for disease progression.[17] Or may be certain isotypes other IgG1 and IgA could relate to worse survival or higher grade of cHL, however, since we did not identify the isotype, we were unsure this possibility.

Angiogenesis, is the formation of new blood vessels or neovascularization or neoangiogenesis from pre-existing vascular network, commonly occurs in two ways. Firstly, is known as the sprouting angiogenesis, which is induced by budding endothelial sprouts from host vasculature in response to the angiogenic stimulus. The next one is called as intussusceptive angiogenesis or splitting angiogenesis, originates by splitting a single vessel into two. While vasculogenesis, is the de novo process of neovascularization by endothelial cells or circulating bone marrow derived endothelial progenitor cells.[7,18] In cHL the formation of microvessels reaches the highest density at the early stages.[19] The progress the stages of cHL microvascular network reduces as found in our cohort, where the higher cHL stage, the lower density of MVD. This was most likely correspond to dominant roles of stimulating factors of blood vessels differentiation such as cytokines namely endothelial progenitor cells (EPCs), vascular endothelial growth factor (VEGF-A) in comparison to the such factors promoting the formation of new blood vessels for instance CD34.[18,19] Endothelial cells proliferation, sprouting and tube formation was induced by VEGF-A.[20]

Neovascularization or angiogenesis is a complex and important process in cHL growth which is involved angiogenic cytokines, matrix proteins, growth factors, and other important mediators for instance VEGF family which be induced by many characteristics of tumors, most importantly hypoxia.[20] A study showed that concentration of EPCs and VEGF-A were significantly higher in cHL patients' blood before the initial treatment, suggest stimulation of new blood vessels formation, which may in turn contribute to tumor growth and metastasis in these patients in comparison to healthy patient.[21] Another study revealed VEGF was higher in newly diagnosed cHL patients, in compared to the patients with a complete remission and healthy controls.[22]

Since several limitations of this study including unknown patient therapy and their follow up, no data of patients' B symptoms in our study, also isotypes of immunoglobulin, further work, could include more information of patients' outcome and all clinical data of patients as well as the isotypes of immunoglobulin. Future research should be performed to explore various isotypes of immunoglobulin in the microenvironment of cHL. In addition, several factors of neovascularization such as endothelial progenitor cells (EPCs), vascular endothelial growth factor (VEGF-A) could be included.

In conclusion, we determined that advance stage of classical Hodgkin lymphoma correlate with high densities of plasma cells and lower angiogenesis. We are certain that our findings could provide an improvement of knowledge of tumor microenvironment particularly in cHL Thus, enable to recommend improved therapeutic strategies and prognosis for cHL patients.

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