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Impact of Cox2, CD163, and Microvessel Density Expression on the Prediction of Relapse and Patients' Outcome in Classical Hodgkin Lymphoma

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Abstract

Background: Tumor microenvironment, specifically tumor-associated macrophages, plays an important role in tumor initiation and progression. CD163 has been recognized as a valuable specific macrophage marker. Cyclooxygenase-2 (Cox2) plays a role in tumor progression. CD31 is reliable for estimation of the density of microvesseles (MVD), which has prognostic importance in several malignant tumors. Thus, the current study was conducted to test the association between CD163, Cox2, and CD31 expression with the prognosis of classical Hodgkin lymphoma (cHL) patients and their potential correlation with clinicopathological variables.

Method: CD163, Cox2, and CD31 expressions were examined in newly diagnosed patients with cHL through immunohistochemistry on tissue biopsy and the results were correlated with the patients' outcome after the median follow-up, which was about 35 months.

Results: 104 patients were included in this study. High CD163 was found in 32.7% of the patients. Cox2 was positive in 42.3% of them. CD 31 with high MVD (≥10%) was found in 51% of the subjects. A significant association was detected between CD163 and Cox2 with tumor stage ($P = 0.001$, and $P = 0.001$) and IPS score. Regarding CD31, we could not find any significant associations with disease parameters, except with histological subtype ($P = 0.001$). A significant relationship was observed between Cox2 and CD163 expression and the relapse rate ($P = 0.001$, $P = 0.01$, respectively). Regarding survival, only Cox2 showed a significant association with disease-free survival ($P = 0.0379$).

Conclusion: These findings suggested that Cox2 and CD163 expression can be used as predicator for early relapse and as new therapeutic targets in cHL.

Keywords: Hodgkin disease, Tumor microenvironment, CD163, Cox2

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Introduction

Tumor microenvironment is an emerging player in several solid tumors, including classical Hodgkin lymphoma (cHL).[1] Tumor-associated macrophages (TAM) are a group of macrophages recruited to the area of the tumor tissue. Macrophages can change their profiles in response to environmental stimuli. Once they are exposed to Th2 cytokines as IL-13 and IL-4, they are polarized to immunosuppressive M2 macrophages.[2, 3] In tumors, TAMs display an M-2-like phenotype which expresses a specific surface marker as CD163. In the microenvironment of tumors, TAMs affect tumors progression in several aspects; they accelerate tumors growth and dissemination, contribute to matrix degradation, and suppress the adaptive immune response against tumor through releasing various factors, including chemokines, inflammatory, and growth factors. Moreover, TAMs are important inducers of the "angiogenic switch", which support tumor growth by expression of hypoxia inducible factor (HIF 1α); this induces the transcription of numerous genes responsible for angiogenesis.[4] Therefore, it is not surprising that increased TAMs infiltration will correlate with reduced patient survival as observed in certain tumors, for instance, follicular lymphoma, and breast and ovarian carcinoma.[5]

On the other hand, detection of CD31 positive vessels through immunohistochemistry (IHC) is reliable for estimation of microvesseles density (MVD) and has a prognostic importance in many malignant tumors, including high grade non-Hodgkin lymphoma, multiple myeloma, and acute and chronic leukemia.[6,7] Several studies have reported a correlation between MVD and biological behavior of cHL.[8,9]

Cyclooxygenase-2 (Cox2) has been confirmed by certain studies as a strong factor in the prognosis of cHL patients. Cox2 has been described as an inflammation-associated enzyme and microenvironment. Certain researchers have described a link between inflammation, angiogenesis, and tumor microenvironment and cancer progression[10,11,12] Nonsteroidal anti-inflammatory drugs that inhibit Cox2 are believed to decrease the number of adenocarcinomas in a mouse model of adenomatous polyposis.[13] Furthermore, many previous investigations have found that overexpression of Cox2 was correlated with progressive disease and a poor prognosis in several tumors, such as colon, cervix, and breast, suggested that Cox2 plays a role in cancer progression.[14,15] However, a few number of studies have investigated the association between Cox2 and Hodgkin lymphoma as its expression by Hodgkin Reed Stemberg (HRS) cells could affect the inflammatory microenvironment of cHL.

We summarized the role and relationship between CD163, CD31, and Cox2 in HL (Figure 1).[10,11]

Therefore, this study aimed to evaluate the expression of CD163, CD31, and Cox2 in cHL with IHC and to determine their correlation with each other and with various pathological and prognostic parameters.

Patients and Methods

Study eligibility

This prospective study included 104 de novo patients with cHL, who presented to outpatient clinic of South Egypt Cancer Institute, Assiut University, Egypt, from January 2015 to December 2017. All the stages were included in this study and all the patients received ABVD regimen 4-8 cycles. Radiotherapy with involved field radiation was utilized for the patients with bulky disease following chemotherapy. Pretreatment excisional biopsy was taken from all the patients; demographic data, tumor stage, and IPS (International prognostic score) were also determined. All of the patients signed written consent to participate in this study. This research was approved by the Ethical Committee of South Egypt Cancer Institute under the code of No-SEC-IRBIORG0006563/208.

The follow-up period ranged from 16 to 50 months. During chemotherapy, clinical examination was usually done following every cycle, also computed tomography (CT) was done after four cycles, if radiological CR was confirmed by CT; therefore, we gave the patients another two cycles and stopped chemotherapy (total of

Table 1. Clinicopathologic characteristics of the patients

Variable	Number	Percent %
Age		
median (range)	33(18-63)	
Gender		
Male	55	(52.9)
Female	49	(47.1)
Histological subtype		
Mixed cellularity	49	(47.1)
Nodular sclerosis	44	(42.3)
Lymphocyte rich	11	(10.6)
Ann Arbor stage		
I	7	(6.7)
II	54	(51.9)
III	34	(32.7)
IV	9	(8.7)
Limited	31	(29.8)
Intermediate	30	(28.9)
Advanced	43	(41.3)
B symptoms	39	(37.5)
(IPS)		
IPS <3	60	(57.7)
IPS ≥3	44	(42.3)
ABVD alone	64	(61.5)
ABVD+RTh	40	(38.5)

IPS: International prognostic score

six cycles). Positron emission tomography (PET) scans were mandatory for all the patients after six cycles, if they have confirmed CR by CT to confirm the complete remission state. However, for those patients who have residual disease after 6 cycles we gave them another two cycles to reach the maximum 8 cycles ABVD. However, for the patients with stage I and II and those with favorable prognostic factors and their CR confirmed by CT and PET CT after four cycles, we considered stopping chemotherapy after 4 cycles and starting the follow-up. The follow-up investigation of the patients included; CT every six months and PET every year for the first three years.

On the other hand, if the patient showed progression at any cycles, the ABVD regimen was discontinued.

IHC

IHC staining for CD163, CD31, and Cox2 was performed according to the manufacturer's protocol. Primary antibodies incubation was done using CD163 Ab-1 (Clone 10D6), Cat. #MS-1103-S0, Cox2 cat No. 35-8200 (thermo fisher scientific, Fremont Blvd. Fremont, CA 94538, USA) at a concentration of 1/100 for 16 hours (overnight), and CD31 (PECAM-1) clone GM006 (Genemed Biotechnologies, Inc, South San Francisco, CA 94080, USA) at a concentration of 1/50 for 1 hour.

The slides were incubated for about 10 min at room temperature (25-30 Celsius) with the biotinylated goat antipolyvalent, and then, incubated for about 10 min with steptavidin peroxidase at room temperature. Diaminibenzidine was applied to the slides for about 5 min at room temperature. Finally, the slides counterstained with Mayer's hematoxylin, dehydrated, and mounted.

Negative control slides were prepared by omitting primary antibody. Sections from placenta were utilized as a positive control for CD163. The slides from a case of angiosarcoma were used for CD31 and breast carcinoma for Cox2.

Evaluation of IHC

The stained slides were examined and their

Table 2. Immunohistochemical expression of CD163, CD31, and Cox2 in cHL

Expression of Markers	Number	(%)
CD163		
Low expression (<25%)	70	(67.3)
High expression (≥25%)	34	(32.7)
CD31 (MVD)		
Low MVD (<10%)	53	(51)
High MVD (≥10)	51	(49)
Cox2		
Negative	60	(57.7)
Positive	44	(42.3)

MVD: Microvessels density; cHL: Classical Hodgkin lymphoma; Cox2: Cyclooxygenase-2

positivity were evaluated as follow:

CD163 expression was cytoplasmic. The evaluation was carried out by determining the percentage of positive cells for CD163 to overall cellularity. All the cases were divided into low expression (<25% of positive cells) and high expression (> 25% of positive cells) categories.[16]

For CD31 (microvessels density counting), the evaluation was done in areas with the highest blood vessels density. Counting was done in three hot spots, for each of which 5 high power fields were counted and the average were calculated. Microvessels defined with clear cut lumen and well-defined shapes were considered for counting.[9] For statistical analysis, the MVC were divided to high (≥10) and low counts (<10).

Cox2 expression was evaluated in HRS cells. Cytoplasmic and/or membranous staining was considered as positive expression.[17]

Statistical analysis

Statistical analysis was done employing SPSS version 16. Chi square test was applied to explore the associations. Spearman correlation coefficient was used to determine correlations. Survival analysis was done utilizing Kaplan-Meier method and Log-Rank testing.[18] Overall survival (OS) was calculated from the date of diagnosis to the date of death due to any causes. Disease-free survival (DFS) was calculated as the interval from the date of diagnosis to the date of disease progression, relapse or death due to any causes. The results were evaluated and $P < 0.05$ was considered to be significant.

Results

Patients' characteristics

Between January 2015 and December 2017, 104 newly diagnosed patients were included in this study. Table 1 represents the clinical and pathological characteristics at diagnosis. The mean age at diagnosis was 33 years (18 to 63 years). With slightly male predominance, 44 patients (42.3%) had an IPS score of over 3, and 39 (37.5%) had B symptoms (Table 1). All the patients were treated with ABVD regimen, followed by radiotherapy in 38.5% of the cases. After the first-line chemotherapy ± RT, 93 subjects achieved complete remissions (89%) and four (4%) achieved partial remission, seven (7.6%) were considered to be non-responders, three had stationary disease, four had progression, 26 (25%) relapsed after achieving complete remission, eight (7.5%) had early relapse, and 18 (17.5%) had late relapse. All the 34 patients with primary failure or relapse received a second-line chemotherapy regimen, among whom only six were treated with high-dose chemotherapy with autologous stem-cell support.

Histological features

The most frequent histological subtype was

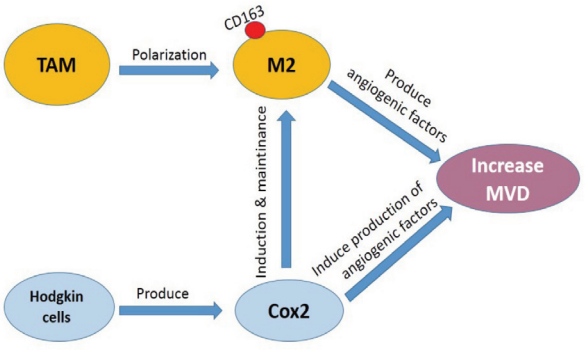

Figure 1. This figure shows the diagrammatic representation of the relation between CD163, Cox2, and MVD.
TAM: Tumor associated macrophage, MVD: Microvessels density

via mixed cellularity (49/104; 47.1%), then nodular sclerosis (44/104; 42.3%), followed by lymphocyte rich variant (11/104; 10.6%) (Table 1).

Correlation of expression of CD163, CD31, and Cox2 with clinicopathological variables

The high expression of CD163 was found in 34 patients (32.7%) (Table 2, Figure 2A). Cox2 expression was positive in 44 patients (42.3 %). The mean MVD of all the cases was 10. 51 samples had high MVD (Figure 2E), while the remaining 53 had low MVD (Figure 2F). A significant correlation was detected between CD163 and Cox2 ($P = 0.001$ r = 0.357); however, we did not find any correlations between CD31 and either CD163 or Cox2 ($P = 0.892$) ($P = 0.572$) (Table 4). Immunohistochemical expression of CD163, Cox2, and CD31 and their correlation with each other are depicted in tables 3 and 4 and figure 2.

CD31 showed a significant correlation with histological subtype ($P = 0.001$) as mixed cellularity subtype showed higher MVD count

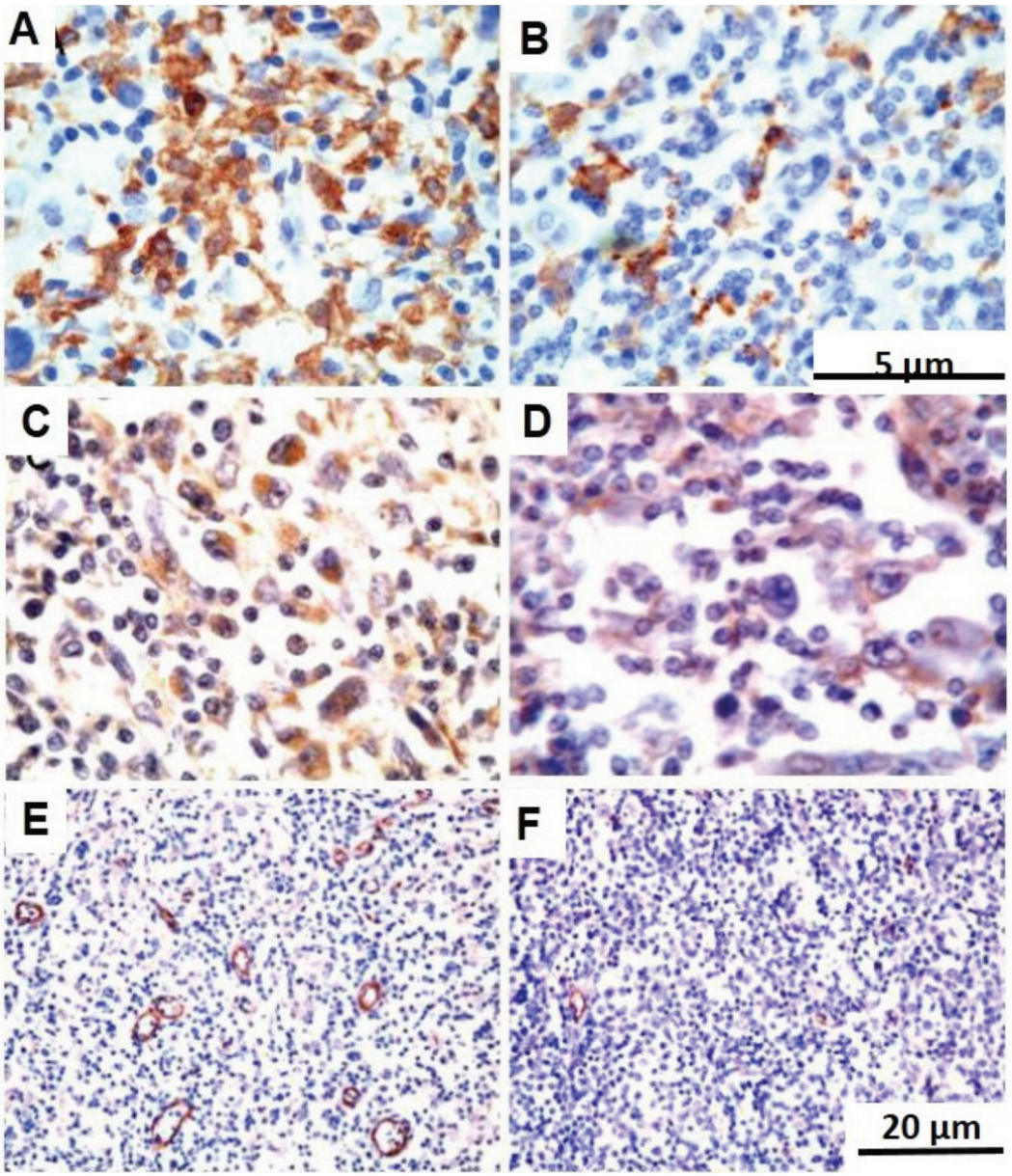

Figure 2. This figure shows the immunohistochemical expression of CD163, CD31, and Cox2 in classical Hodgkin lymphoma. A: High CD163 expression. B: Low CD163 expression. C: Positive Cox2 in HRS cells. D: Negative Cox2 in HRS cells. E: High MVC by CD31. F: Low MVC by CD31. (A, B, C, D ×1000, E, and F, ×200).
Cox2: Cyclooxygenase-2; HRS: Hodgkin Reed Sternberg cells, MVC: Microvessels density

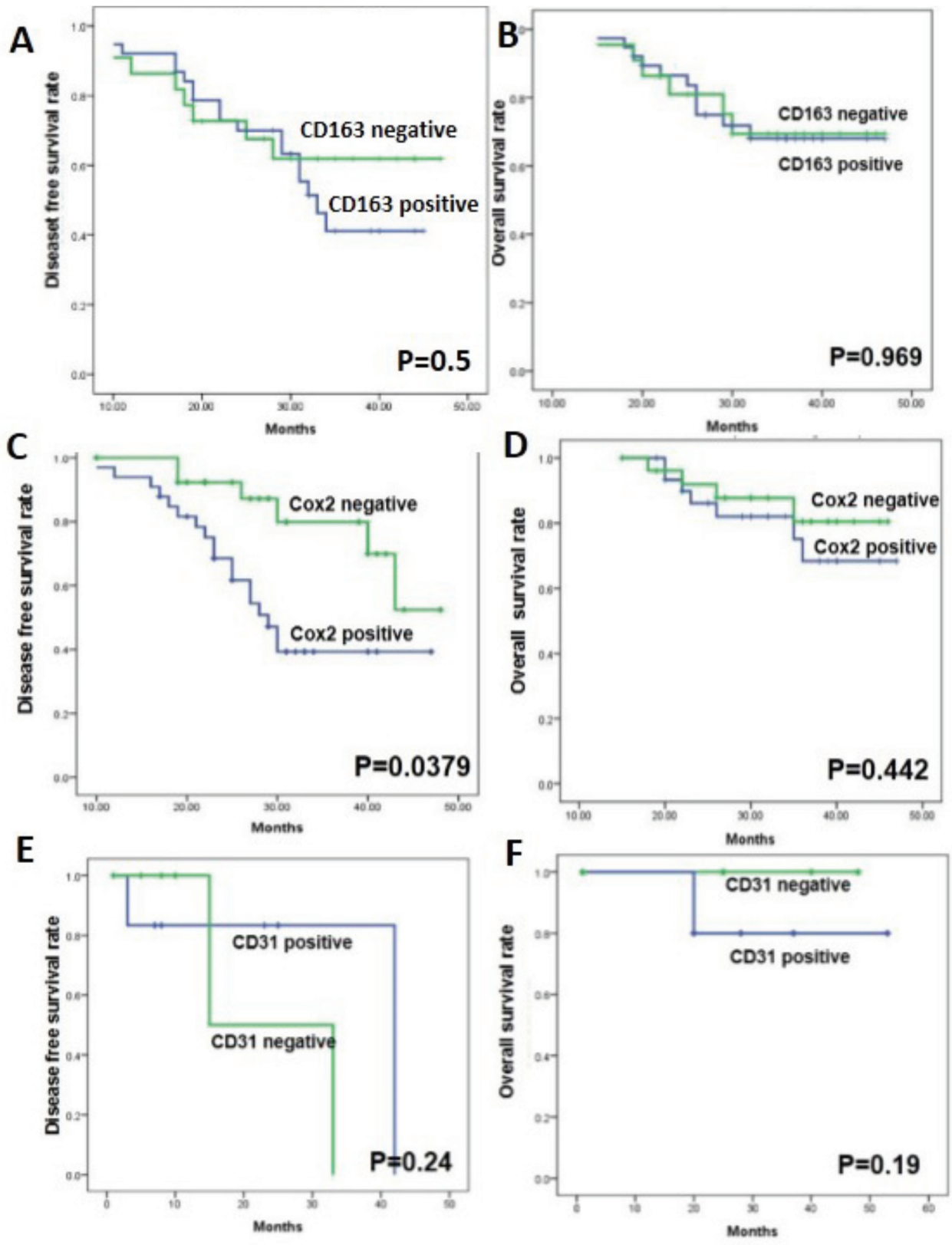

Figure 3. This figure shows disease-free survival and overall survival with the expression of CD168, MVD, and Cox2.
MVD: Microvessels density; Cox2: Cyclooxygenase-2

Table 3. CD163, CD31 (MVD), and Cox2 association with clinical and pathological variables

Variable	CD163			MVD			Cox2		
	Low	High	*P*	Low	High	*P*	Negative	Positive	*P*
Histological Type									
MC (N=49)	30 (61.3%)	19 (38.7%)	NS	15 (30.6%)	34 (69.4%)	≤0.001	26 (53%)	23 (46.9%)	NS
NS (N=44)	33 (75%)	11 (25%)		28 (63.6%)	16 (36.3%)		30 (68.1%)	14 (31.8%)	
LR (N=11)	7 (62.7%)	4 (36.3%)		10 (90.9%)	1 (9%)		4 (36.3%)	7 (62.7%)	
Ann Arbor Stage									
I (N=7)	6 (85.7%)	1 (14.2%)	≤0.01	3 (43.%)	4 (57%)	NS	7 (100%)	0 (0%)	≤0.001
II (N=54)	43 (79.6%)	11 (20.3%)		29 (53.7%)	25 (46.2%)		39 (72%)	15 (28%)	
III (N=34)	19 (55.8%)	15 (44.1%)		16 (47%)	18 (52.9%)		12 (35.2%)	22 (64%)	
IV (N=9)	2 (22.2%)	7 (77.7%)		5 (55.5%)	4 (44.5%)		2 (22.2%)	7 (77.7%)	
IPS									
IPS<3 (N=60)	50 (83.3%)	10 (16.6%)	≤0.001	26 (43.3%)	34 (56.6%)	NS	42 (70 %)	18 (30%)	≤0.01
IPS≥3 (N=44)	20 (45.4%)	24 (54.5%)		17 (38.7%)	27 (61.3%)		18 (40.%)	26 (60.%)	
Response									
ST,Pro N=7	4 (57.1)	3 (42.8)	0.021	3 (42.6)	4 (57.1)	NS	3 (42.6)	4 (57.1)	0.043
CR (N=93)	64 (68.9%)	29 (31.1%)		48 (51.6%)	45 (48.3%)		56 (60.2%)	37 (39.7%)	
Relapse	9	17	≤0.01	14	12	NS	5	21	≤0.001

IPS: International prognostic score; MC: Mixed celurirty; LR: Lymphocytic rich; MVD: Microvessels density; NS: Nodular sclerosis; NS: Non-significant; ST: Stable disease; Pro: Progression of the disease

than other types. However, no significant differences regarding CD163 and Cox2 expression were observed with histological subtype (P = 0.412 and P = 0.112, respectively) (Table 3).

Additionally, there were no statistically significant differences between the expression of the three markers with age and gender.

Prognostic significance of CD163, CD31, and Cox2

High expression of CD163 and Cox2 was correlated with both advanced stage and high IPS score; however, we did not observe any significant correlations between CD31 with either of the stages and IPS score (Table 3).

All the patients received ABVD regimen; three received four cycles, 71 received six cycles, 30 received eight cycles, and 40 subjects with bulky disease were consolidated with involved filed radiotherapy.

Seven participants had refractory disease, while 26 patients out of 104 relapsed over the period of follow-up ranging from 12 to 60 months. A statistically significant association was found between higher CD163 expression and response rate (P = 0.021) and relapse rate (P = 0.01) (Table 2). Nevertheless, we could not find any significant differences between the level of CD 163 and DFS (P = 0.512). Moreover, we did not find any significant effects on OS (P = 0.969) (Figure 3).

On the other hand, on subanalysis based on the stages, there was a significant association between higher expression and poor DSF in patients with stage III and IV only, yet no effects on OS survival.

Furthermore, no significant associations were found between CD31 expression with response rate (P = 0.913), relapse rate (P = 0.522), DFS (P = 0.241), and OS (P = 0.190) (Figure 3). Moreover, while performing the subanalysis by stage, we did not find any effects on survival in the patients with higher stages.

On the other hand, the only significant

Table 4. Correlation between expression of CD163, CD31, and Cox2 with each other

Correlations	*P* value	r value
CD163 and Cox2	0.001	0.357
CD163 and CD31	0.892	0.131
Cox2 and CD31	0.574	0.572

Cox2: Cyclooxygenase-2

relationship between expression of a marker and the patients' survival outcome was observed between Cox2 expression and response rate ($P = 0.043$), relapse rate ($P = 0.001$), DFS ($P = 0.0379$), that was also clearly evident in advanced stages of the disease.

However, Cox2 expression did not show significant association with OS ($P = 0.442$) (Table 3 and Figure 3).

Discussion

Despite the fact that classical Hodgkin disease is assumed to be curable, still a lot of patients show resistance against the first-line therapy or relapse after a period of complete remission.[19] The are three different prognostic scores, developed by different working groups like (esEORTC),[20] including German Hodgkin Study Group[21] and National Cancer Institute,[22] all of them based on clinical and laboratory data alone. The absence of an agreement on the stratification of early-stage HL is itself indicative of the limitation of these scores.

When inflammatory cells, like macrophages, neutrophils, and lymphocytes, interact with cancer cells, the angiogenic factors started to release.[23,24] Specifically TAMs release most of proteolytic enzymes, cytokines, inflammatory mediators, and growth factors.[25] Among these, the members of the VEGF family and angiogenic peptides induce direct angiogenic effects on target endothelial cells or their bone marrow-derived precursors TAMs are closely associated with VEGF expression and MVD in solid tumors.[26] Cox2 regulates cell proliferation, cell adhesion, inhibition of apoptosis, immune surveillance, and angiogenesis through synthesis of prostaglandin E2.[27]

Our results revealed the importance of expression of a high number of CD163+ since it is correlated with high IPS >3. This could also be useful in predicting relapse in patients as we observed a significant association between response rate and relapse rate. Even though, our study failed to show any significant correlations between DFS and OS, this is probably not in accordance with previous studies which proved the significant association of the high expression of CD163 with patient survival outcome.[27,28,29] However, the results are in line with other studies that also reported a lack of correlation between CD163 and prognosis in cHL patients.[16,30] In particular, a study by Azambuja et al., who failed to confirm a relationship between high expression of CD68+ and CD163+ macrophages with clinical outcomes in terms of DFS in a study, included 265 patients.[31] Moreover, Kayal et al. confirmed the lack of a significant association between levels of other macrophage markers, like CD68 expression, and PFS or OS.[30] The possible explanation for these conflicting data is different clones used for IHC and/or the construction of tissue microarrays instead of whole-section evaluation. Another explanation published by Kamper et al. reported an association between higher levels of CD68 and CD163 expression with the presence of EBV in patients with HL; the EBV positivity by itself also showed a correlation with bad outcome.[16]

As we did not test the EBV positivity at diagnosis; therefore, we could not make sure if there is a correlation between macrophage markers expression level and EBV.

Furthermore, a recent study by Ahmed H et al. tested CD 163 with both IHC and genetic testing on seven SNPs, six SNPs did not show significant associations with relapse rate and survival, but only one SNP rs75608120 was significantly associated with relapse rate. Hence, it is possible that our patients had further

expression of that SNP.[27]

Our results also implied a significant association between CD163 and MVD, which was not surprising since inflammatory cells as TAM release several growth factors, inflammatory mediators, and cytokines including VEGF family and other angiogenic peptides that act on endothelial cells. Moreover, TAMs serve as bridge cells or cellular chaperones that guide the fusion of endothelial cells tips to perform anastomosis and facilitate vascular sprouting.[32-34] In addition, macrophages interact with cancer cells and secrete angiogenic factors, which affect other surrounding cells and blood vessels.[27]

Nonetheless, several studies have emphasized the role of angiogenesis in malignancies since it would determine the risk of progression of tumors.[35] We did not find any significant associations between CD31 expression and disease stages or between IPS and patient survival or diseases stage. Our results were in agreement with those reported by Schmid T et al.,[24] who reported a lack of association between CD31 and patient outcome. Moreover, Panico et al. stated a lack of correlation between MVD and clinical outcomes of classical Hodgkin lymphoma.[34] They explained this lack of association by describing that TAMs may contribute to disease progression through mechanisms other than VEGF release or angiogenesis, which may overshadow the effects of angiogenesis. Since TAMs contribute to extracellular matrix remodeling, promotion of cancer cell proliferation, invasion, and metastasis, they suppress the adaptive immune response.[36, 37] Moreover, VEGF-positive patients are likely to have the nodular sclerosis or mixed cellularity disease subtypes, which are associated with a better overall prognosis than other cHL subtypes;[38] whereas, CD163 expression did not show any such predilection.

Ultimately, regarding COX-2 expression, we found that it was the only marker in our study associated with poor prognosis. This relationship between COX-2 expression and poor prognosis has been confirmed in solid tumors since Cox2 induces angiogenesis, chemoresistance by promoting antiapoptotic mechanisms, such as upregulation of BCL-2, resistance to Fas, and stimulation of invasion through induction of some metalloproteinases.[35]

Cox2 expression in our study indicated a significant correlation with MVD similar to previous works.[35] This was expected because Cox2 affects angiogenesis through production of many angiogenic factors, such as VEGF, basic fibroblastic growth factor, and platelet derived growth factors, which stimulate microvesels production.[35, 39]

Conclusion

In conclusion, tumor microenvironment was found to have an impact on tumor progression in Hodgkin lymphoma as expression of CD163 and Cox2 might be helpful to predict relapse in patients with Hodgkin lymphoma. Cox2 expression could be used to identify a subgroup of cHL patients at high risk of recurrence, which could be prevented with aggressive treatments to this subgroup from the beginning.

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Conflict of Interest

None declared.

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