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Expression of Silent Information Regulator-1 (Sirt1), CD163 and Tryptase; Implications for Immune Dys-regulation, Prognosis and Therapeutic Targeting in Classical Hodgkin Lymphoma

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Abstract

Background: Many studies have assessed the relation between cHL histology and its clinical outcome but the results are still controversial. The most recent issue was the discovery of the methods of interaction between the elements of benign reactive inflammatory cells and their effect on cHL metabolism. Silent information regulator-1 (Sirt1) is a class III HDACs family member, it was expressed in T-cell lymphoma and was considered a recently discovered therapeutic target, we tried to detect that the significance of Sirt1 expression in malignant Reed-Sternberg cells and surrounding inflammatory infiltrate in cHL. Cluster of Differentiation 163 (CD163) is a scavenger receptor cystein-rich (SRCR) family member which identifies monocytes and macrophages, Tryptase is the secretory granules that were serine proteinase derived and it has been used as a mast cell activation marker. The tumor-infiltrating macrophages and mast cells have been found to have different roles in malignancies of many organs but their role in cHL prognosis had not been sufficiently clarified.

Aim of the work: To assess the influence of Sirt1 expression in the malignant cells and the surrounding tumor microenvironment CD163 positive macrophages and tryptase positive mast cells on the pathological parameters and clinical outcome in cHL patients.

Methods: We evaluated clinicopathological and prognostic significance of Sirt1, CD163 and tryptase expressions in sections from fifty paraffin blocks of cHL using immunehistochemistry.

Results: The expression of Sirt1 in cHL was associated with advanced stage of the disease, presence of B symptoms, splenic affection, (p<0.001), bone marrow infiltration (p=0.002) and the presence of bulky mediastinal lymph node (p=0.004). The expression of CD163 and tryptase in cHL was associated with advanced stage of the disease and the presence of bulky mediastinal lymph node (p<0.001). High Sirt1, tryptase and CD163 expression was associated with poor response to therapy, high incidence of relapse after successful therapy and poor three-year overall survival rate (p<0.001).

Conclusion: High levels of expression of Sirt1, CD163 and tryptase were found to be markers of poor prognosis in cHL.

Introduction

Classical Hodgkin lymphoma (cHL) forms ninety percent of all Hodgkin lymphomas and it is categorized into 4 subtypes; lymphocyte-rich (LR), nodular sclerosis (NS), mixed cellularity (MC) and lymphocyte-depletion (LD) subtype [1]. CHL is a treatable cancer [2], but still few patients are resistant to therapy or they may have lymphoma recurrence after successful therapy [3]. Novel researches had focused on recent therapeutic targets detection to improve the response to therapy and to decrease the incidence of lymphoma recurrence. CHL is composed of malignant giant cells i.e. Reed-Stenberg (RS) cells that are surrounded by reactive inflammatory infiltrate [4], e.g. lymphocytes, eosinophils, mast cells, macrophages which interact with RS cells via many cellular pathways and by production of cytokine by RS cells that affect the benign reactive inflammatory cells via autocrine and paracrine pathways [5,6]. Previous studies have focused on the cellular composition of cHL microenvironment to detect their clinicopathological and prognostic role, but conflicting results were detected. Silent information regulator-1 (Sirt1) is a class III HDACs family member that has a role in the gene silencing of yeast [7]. It was expressed in T-cell lymphoma and was considered a recent therapeutic target [8]. Our study attempted to acquire more

understanding of the pathogenesis of cHL. As previously found that the RS cells and the surrounding reactive lymphocytes could express Sirt1 [9] we tried to detect the clinicopathological role of such expression in our patients. A more understanding of cHL microenvironment, may allow better management via restoration of the immune balance that could allow its better remission. Cluster of Differentiation 163 (CD163) is a protein that is encoded by the *CD163* gene [10], it is a scavenger receptor cystein-rich (SRCR) family member [11,12], which has been found to mark macrophages [13]. Tryptase enzyme is detected in the secretory granules that were serine proteinase derived, were

contained in mast cells and it has been used as a mast cell activation marker [14,15]. Increased numbers of tryptase positive mast cells had found to accompany lympho-plasmacytic lymphoma (LPL) [16]. Researchers had described the different roles of mast cells infiltration in cHL and the relations between increased mast cell infiltration and disease progression [17], but still conflicting results were found. Little information was proved regarding Sirt1 expression in cHL [18]. The tumor-infiltrating macrophages have been found to have different roles in malignancies of many organs, but their role in cHL prognosis had not been sufficiently clarified [19], and the mast cells infiltration in cHL has been found to have different conflicting roles in the prognosis in adults' patients [20,21].

Aim of the work

To assess the influence of Sirt1 expression in the malignant cells and the surrounding tumor microenvironment CD163 positive macrophages and tryptase positive mast cells on the pathological parameters and clinical outcome in cHL patients.

Patients and Methods

We evaluated expressions of Sirt1, CD163 and tryptase in sections from fifty paraffin blocks retrieved from fifty cases of cHL using immunohistochemistry. We have collected the paraffin blocks from Pathology Department, Faculty of Medicine, Zagazig University in the period from December 2010 to December 2015. We used REAL/WHO and Cotswolds-modified Ann Arbor staging system for classification and staging of cHL [22,23]. We detected the data of patient as sex, age, lymphoma size, stage and the presence of B symptoms by retrospective examination of the patient's and the slide files in pathology department.

Most patients had follow-up records for 5 years in both Medical Oncology and Clinical oncology & nuclear medicine departments, Faculty of Medicine, Zagazig University. We followed all the fifty patients till death or till the most recent follow-up contact with them and we have finished data collection in December 2016.

Immunohistochemical staining

We performed the immunohistochemical staining by using streptavidin-peroxidase method [24], where we incubated our slides with primary rabbit monoclonal anti- Sirt1 antibody (ab32441) (Abcam, Cambridge, MA, USA) at 1:150 dilution, anti-CD163 antibody (ab87099) (Abcam, Cambridge, MA, USA) at 1/500 dilution and primary mouse monoclonal anti-mast cell tryptase antibody (ab81703) at 1:2000 dilution. We used colon carcinoma, spleen and tonsil sections as positive controls for Sirt1, CD163 and tryptase respectively and we have replaced the primary antibodies by the non-immune serum for the negative controls.

Evaluation of immunohistochemical expression of Sirt1

We evaluated the staining extension & intensity independently in 5 random fields in our entire examined slides [25]. We graded the stain intensity as follows: negative, 1+, 2+, and 3+. Then we evaluated the extent of positively stained cells as follows: zero (0-1%); one (1–25%); two (25–50%); three (50–75%); and four (>75%). To reach the final immunopositivety score we multiplied both the intensity & extent of the stain that has given values from zero to twelve. We used a cutoff of 4 above which was considered as high Sirt1 expression and below which was low expression. Sirt1 expression either as high or low, was it based on the expression by both the malignant tumor cells (RS) and the small reactive lymphocytes.

Evaluation of immunohistochemical expression of CD163 and Tryptase

For the CD163 staining, we counted 3 variable areas of the tumor in high power field x 400 magnifications and we scored the percentage of CD163 positivity in the cell membrane and cytoplasm of macrophages [26]. We have counted mast cells positive for tryptase in ten randomly selected high power fields x 200 magnifications [6]. We used a cutoff of twenty five percent for CD163 positive macrophage and tryptase positive mast cells above which was considered high expression and below which was low expression of both markers and that was applied for all patients.

Statistical Analysis

The statistics were evaluated by using SPSS 22.0 for windows (SPSS Inc., Chicago, IL, USA) and MedCalc windows (MedCalc Software bvba 13, Ostend, Belgium). Continuous variables that were expressed as the mean ± SD & median (range), were checked for normality by using Shapiro-Wilk test. Mann Whitney U test was used to compare between two groups of non-normally distributed variables. Kruskal Wallis H test was used to compare between more than two groups of normally distributed variables. Stratification of OS and RFS was done according to all clinicopathological features and immunohistochemical markers. These time-to-death distributions were estimated using the method of Kaplan-Meier plot, and compared using two-sided exact log-rank test. All tests were two sided. A p-value <0.05 was considered significant.

Results

Patient data

The clinical data of our patients with cHL that were included in the current study were summarized (Table 1). We included 28 (56%) males and 22 (44%) females with age ranged from (20-80) years (Mean: 47.5 ± 20.7 years), 31 (62%) cases were having mixed cellularity subtype, 14 (28%) cases were of nodular sclerosis subtype and 5 (10%) cases were of lymphocyte rich subtype.

Sirt1 expression and its correlation with clinicopathological criteria of our patients

Sirt1 was overexpressed in the nuclei of both malignant cells and the background of tumor infiltrating lymphocytes in 29 cases out of 50 (58%) cases of cHL, and in the remaining 42% of cases there were no lymphocytes expressing Sirt1 at all. The expression of Sirt1 in was significantly positively correlated with advanced stage, presence of B symptoms, splenic affection, presence of extranodal spread, low serum albumin, low hemoglobin (p<0.001), presence of bone marrow infiltration (p=0.002) and the presence of bulky mediastinal lymph node involvement (p=0.004) (Table 1 and Figure 1A-D).

correlation clinicopathological features of our patients

High expression of CD163 in tumor infiltrating macrophages was detected in in 23 out of 50 (46%) cases of cHL.

High expression of tryptase in tumor infiltrating mast cells was detected in 22 out of 50 (44%) cases of cHL. The expression of CD163 and tryptase in cHL was significantly positively correlated with advanced stage, presence of B symptoms, splenic involvement, presence of extra-nodal spread, low albumin, low hemoglobin, bone marrow infiltration and the presence of bulky mediastinal lymph node (p<0.001) (Table 1 and Figures 2A-C, 3A-C).

Characteristics	All (N=50)	Sirt1		p-value	CD163		p-value	Tryptase		p-value
		Low (N=21)	High (N=29)		Low (N=27)	High (N=23)		Low (N=28)	High (N=22)	
Age (years)										
Mean ± SD	47.5 ± 20.7	35.9 ± 17.6	55.8 ± 18.8	0.001	37.7 ± 17.8	59 ± 18	<0.001	38.6 ± 18.2	58.7 ± 18.4	0.001
Median (Range)	50 (20-80)	29 (20-72)	60 (20-80)		30 (20-70)	64 (20-80)		31 (20-70)	64 (20-80)	
≤ 45 years	24 (48%)	16 (66.7%)	8 (33.3%)	0.001§	19 (79.2%)	5 (20.8%)	0.001§	19 (79.2%)	5 (20%)	0.002§
>45 years	26 (52%)	5 (19.2%)	21 (80.8%)		8 (30.8%)	18 (69.2%)		9 (34.6%)	17 (65.4%)	
Sex										
Male	28 (56%)	9 (32.1%)	19 (67.9%)	0.111§	13 (46.4%)	15 (53.6%)	0.226§	14 (50%)	14 (50%)	0.335§
Female	22 (44%)	12 (54.5%)	10 (45.5%)		14 (63.6%)	8 (36.4%)		14 (63.6%)	8 (36.4%)	
B symptoms										
Absent	35 (70%)	21 (60%)	14 (40%)	<0.001§	27 (77.1%)	8 (22.9%)	<0.001§	28 (80%)	7 (20%)	<0.001§
Present	15 (30%)	0 (0%)	15 (100%)		0 (0%)	15 (100%)		0 (0%)	15 (100%)	
Bulky Med. LN										
Absent	37 (74%)	20 (58.8%)	17 (45.9%)	0.004§	26 (70.3%)	11 (29.7%)	<0.001§	27 (73%)	10 (27%)	<0.001§
Present	13 (26%)	1 (6.3%)	12 (92.3%)		1 (7.7%)	12 (92.3%)		1 (7.7%)	12 (92.3%)	
Number of nodal sites										
One site	7 (14%)	7 (100%)	0 (0%)	<0.001‡	7 (100%)	0 (0%)	<0.001‡	7 (100%)	0 (0%)	<0.001‡
Two sites	10 (20%)	10 (100%)	0 (0%)		10 (100%)	0 (0%)		10 (100%)	0 (0%)	
Three sites	3 (6%)	0 (0%)	3 (100%)		3 (100%)	0 (0%)		3 (100%)	0 (0%)	
Four sites	6 (12%)	1 (16.7%)	5 (83.3%)		3 (50%)	3 (50%)		3 (50%)	3 (50%)	
Five sites	6 (12%)	1 (16.7%)	5 (83.3%)		3 (50%)	3 (50%)		3 (50%)	3 (50%)	
>Five sites	18 (36%)	2 (11.1%)	16 (88.9%)		1 (5.6%)	17 (94.4%)		2 (11.1%)	16 (88.9%)	
Spleen involvement										
Absent	28 (56%)	21 (75%)	7 (25%)	<0.001§	23 (82.1%)	5 (17.9%)	<0.001§	23 (82.1%)	5 (17.9%)	<0.001§
Present	22 (44%)	0 (0%)	22 (100%)		4 (18.2%)	18 (81.8%)		5 (22.7%)	17 (77.3%)	
BM infiltration										
Absent	36 (72%)	20 (55.6%)	16 (44.4%)	0.002§	26 (72.2%)	10 (27.8%)	<0.001§	27 (75%)	9 (25%)	<0.001§
Present	14 (28%)	1 (7.1%)	13 (92.9%)		1 (7.1%)	13 (92.9%)		1 (7.1%)	13 (92.9%)	
Extranodal lesions										
Absent	14 (28%)	12 (85.7%)	2 (14.3%)	<0.001§	13 (92.9%)	1 (7.1%)	0.001§	13 (92.9%)	1 (7.1%)	0.001§
Present	36 (72%)	9 (25%)	27 (75%)		14 (38.9%)	22 (61.1%)		15 (41.7%)	21 (58.3%)	
Histopathological subtype										
NS	14 (28%)	12 (85.7%)	2 (14.3%)	<0.001§	14 (100%)	0 (0%)	<0.001§	14 (100%)	0 (0%)	<0.001§
MC	31 (62%)	8 (28.5%)	23 (74.2%)		10 (32.3%)	21 (67.7%)		11 (35.5%)	20 (64.5%)	
LR	5 (10%)	1 (20%)	4 (80%)		3 (60%)	2 (40%)		3 (60%)	2 (40%)	
Stage										
Stage I	7 (14%)	7 (100%)	0 (0%)	<0.001‡	7 (100%)	0 (0%)	<0.001‡	7 (100%)	0 (0%)	<0.001‡
Stage II	13 (26%)	10 (76.9%)	3 (23.1%)		13 (100%)	0 (0%)		13 (100%)	0 (0%)	
Stage III	12 (24%)	2 (16.7%)	10 (83.3%)		6 (50%)	6 (50%)		6 (50%)	6 (50%)	
Stage IV	18 (36%)	2 (11.1%)	16 (88.9%)		1 (5.6%)	17 (94.4%)		2 (11.1%)	16 (88.9%)	
Prognostic group										
Unfavorable	30 (60%)	4 (13.3%)	26 (86.7%)	<0.001§	7 (23.3%)	23 (76.7%)	<0.001	8 (26.7%)	22 (73.3%)	<0.001§
Favorable	20 (40%)	17 (85%)	3 (15%)		20 (100%)	0 (0%)		20 (100%)	0 (0%)	
Risk group										
Low favorable	16 (32%)	16 (100%)	0 (0%)	<0.001‡	16 (100%)	0 (0%)	<0.001‡	16 (100%)	0 (0%)	<0.001‡
Low unfavorable	3 (6%)	0 (0%)	3 (100%)		3 (100%)	0 (0%)		3 (100%)	0 (0%)	
Intermediate	18 (38%)	5 (27.8%)	13 (72.2%)		8 (44.4%)	10 (55.6%)		9 (50%)	9 (50%)	
High	13 (26%)	0 (0%)	13 (100%)		0 (0%)	13 (100%)		0 (0%)	13 (100%)	

Categorical variables were expressed as number (percentage); continuous variables were expressed as mean ± SD & median (range)
* Mann Whitney U test; § Chi-square test; ‡ Chi-square test for trend; p<0.05 is significant.

Table 1: Correlation between clinicopathological criteria, Sirt1, CD163 & Tryptase expression in our patients.

• The 3-year recurrence free survival rate of our patients was 91.2% for all cases, 95.2% and 84.6% in patients with low and high Sirt1 expression respectively, 100% and 57.1% in patients with low and high tryptase and CD163 expression respectively (p<0.001).

• The 3-year overall survival (OS) rate of our patients was 75.8% for all cases, 58.2% in patients with high Sirt1 expression (p=0.008), 46.6% in patients with high CD163 expression and 44.1% in patients with high tryptase expression (p<0.001).
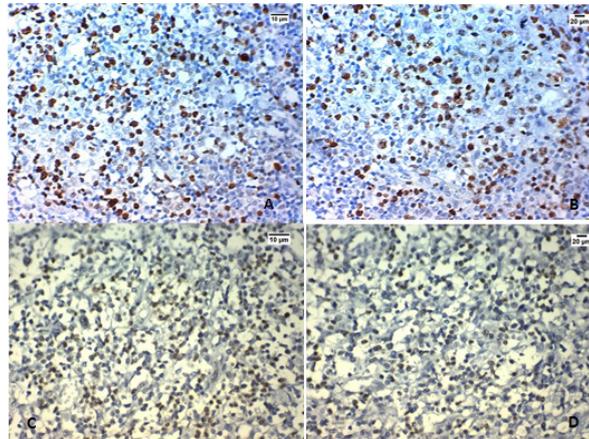
Figure 1: Immunohistochemical staining of Sirt1 in classical Hodjkin lymphoma (cHL): (A & B) High expression in the nucleus of cHL cells x 400 (C & D); Low expression in the nucleus of cHL cells x 400.
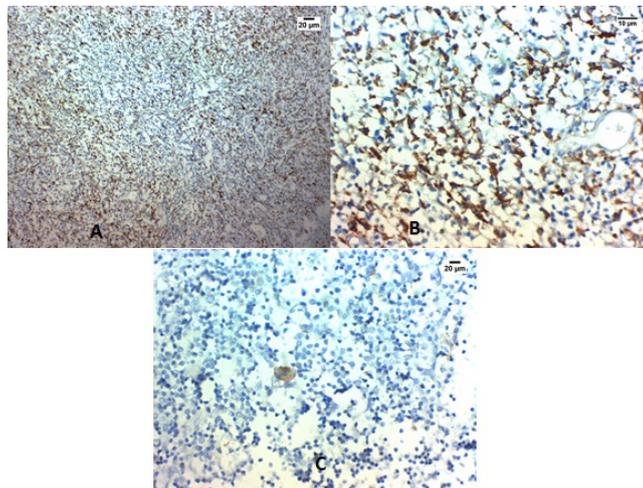
Figure 2: Immunohistochemical expression of CD163 in macrophages that infiltrate classical Hodgkin lymphoma (A) High expression of CD163 in cHL x 100 (B & C) High expression of CD163 in cHL x 400, low expression of CD163 in cHL x 400.
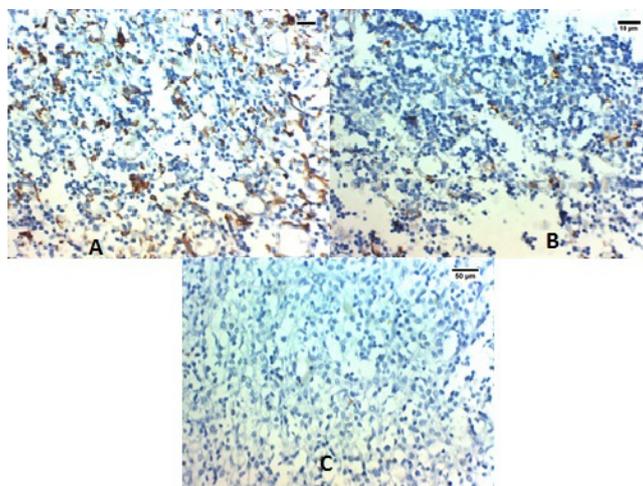

Figure 3: Immunohistochemical expression of Tryptase in mast cells that infiltrate classical Hodgkin lymphoma (A) High expression of Tryptase in cHL x 400 (B & C) low expression of Tryptase in cHL x 400, negative expression of Tryptase in cHL x 400.

Characteristics	All (N=50)	Response				p-value	Response		p-value
		PD (N=6)	SD (N=6)	PR (N=4)	CR (N=34)		NR (N=24)	OAR (N=38)	
ESR									
<50 mm/h	34 (68%)	0 (0%)	0 (0%)	2 (5.9%)	32 (94.1%)	<0.001§	0 (0%)	34 (100%)	<0.001§
>50 mm/h	16 (32%)	6 (37.5%)	6 (37.5%)	2 (12.5%)	2 (12.5%)		12 (75%)	4 (25%)	
Albumin									
<4 g/dl	30 (60%)	6 (20%)	6 (20%)	4 (13.3%)	14 (46.7%)	0.001§	12 (40%)	18 (60%)	0.001§
>4 g/dl	20 (40%)	0 (0%)	0 (0%)	0 (0%)	20 (100%)		0 (0%)	20 (100%)	
Hemoglobin									
<10 g/dl	30 (60%)	6 (20%)	6 (20%)	4 (13.3%)	14 (46.7%)	0.001§	12 (40%)	18 (60%)	0.001§
>10 g/dl	20 (40%)	0 (0%)	0 (0%)	0 (0%)	20 (100%)		0 (0%)	20 (100%)	
WBCs									
<15 x 10³/mm³	30 (60%)	6 (20%)	6 (20%)	4 (13.3%)	14 (46.7%)	0.001§	12 (40%)	18 (60%)	0.001§
>15 x 10³/mm³	20 (40%)	0 (0%)	0 (0%)	0 (0%)	20 (100%)		0 (0%)	20 (100%)	
Stage									
Stage I	7 (14%)	0 (0%)	0 (0%)	0 (0%)	7 (100%)	<0.001‡	0 (0%)	7 (100%)	0.002‡
Stage II	13 (26%)	0 (0%)	0 (0%)	0 (0%)	13 (100%)		0 (0%)	13 (100%)	
Stage III	12 (24%)	1 (8.3%)	3 (25%)	1 (8.3%)	7 (58.3%)		4 (33.3%)	8 (66.7%)	
Stage IV	18 (36%)	5 (27.8%)	3 (16.7%)	3 (16.7%)	7 (39.9%)		8 (44.4%)	10 (55.6%)	
Prognostic group									
Unfavorable	30 (60%)	6 (20%)	6 (20%)	4 (13.3%)	14 (46.7%)	0.001§	12 (40%)	18 (60%)	0.001§
Favorable	20 (40%)	0 (0%)	0 (0%)	0 (0%)	20 (100%)		0 (0%)	20 (100%)	
Risk group									
Low favorable	16 (32%)	0 (0%)	0 (0%)	0 (0%)	16 (100%)	<0.001‡	0 (0%)	16 (100%)	<0.001‡
Low unfavorable	3 (6%)	0 (0%)	0 (0%)	0 (0%)	3 (100%)		0 (0%)	3 (100%)	
Intermediate	18 (38%)	0 (0%)	0 (0%)	3 (16.7%)	15 (83.3%)		0 (0%)	18 (100%)	
High	13 (26%)	6 (46.2%)	6 (46.2%)	1 (7.7%)	0 (0%)		12 (92.3%)	1 (7.7%)	
<u>Sirt1</u>									
Low	21 (42%)	0 (0%)	0 (0%)	0 (0%)	21 (100%)	0.001§	0 (0%)	21 (100%)	0.001§
High	29 (58%)	6 (20.7%)	6 (20.7%)	4 (13.8%)	13 (44.8%)		12 (41.4%)	17 (58.6%)	
CD163									
Low	27 (54%)	0 (0%)	0 (0%)	0 (0%)	27 (100%)	<0.001§	0 (0%)	27 (100%)	<0.001
High	23 (46%)	6 (20.7%)	6 (20.7%)	4 (13.8%)	7 (30.4%)		12 (52.2%)	11 (47.8%)	
Tryptase									
Low	28 (56%)	0 (0%)	0 (0%)	1 (3.6%)	27 (96.4%)	<0.001§	0 (0%)	28 (100%)	<0.001§
High	22 (44%)	6 (27.3%)	6 (27.3%)	3 (13.6%)	7 (31.8%)		12 (54.5%)	10 (45.5%)	

Categorical variables were expressed as number (percentage); continuous variables were expressed as mean ± SD & median (range).
* Mann Whitney U test; Ø Kraskall Wallis test for more than two groups; § Chi-square test; ‡ Chi-square test for trend; p<0.05 is significant.

Table 2: Correlation between clinicopathological criteria, Sirt1, CD163, Tryptase expression and response to treatment in our patients.

High Sirt1, tryptase and CD163 expression was significantly positively correlated with poor response to therapy and high incidence of relapse after treatment and poor 3-year overall survival rate (OS) (p<0.001) (Tables 2-4 and Figure 4A-F).

Discussion

In our study we found that Sirt1 was overexpressed in the nuclei of both Reed-Sternberg (RS) cells and the background of tumor infiltrating lymphocytes in 58% cases of cHL and in the remaining 42% of cases there were no lymphocytes expressing Sirt1 at all. Sirt1 expression was associated with poor clinic-pathological parameters and poor prognosis, and our results were the same like Quesada et al., and Frazzi et al. [27,28]. Our results were explained that one of the Sirt1 de-acetylation targets is FoxP3 that is a transcription factor which is essential for T regulatory lymphocytes (Tregs) differentiation and function. So, Sirt1 de-acetylation of FoxP3 could result in FoxP3 destabilization and increased its degradation that decreased Tregs activity, those tregs stimulate the immunity against cancer and had

onco-suppressive role, so, inhibition of Sirt1 increases the suppressive action of Tregs cells [29,30]. Moreover, Sirt1 inhibition in cHL allows RS cells to promote CD4+ naïve T cells differentiation toward Tregs as RS could promote differentiation of CD4+ naïve T cells toward both Tregs and cytotoxic T cells that are both increase their onco-suppressive role [31]. All these results are in line with ours that Sirt-1 over expression in cHL is a marker of poor prognosis and its inhibition was a novel therapeutic target to improve patient outcome and also histone deacetylase inhibitors (such as vorinostat [SAHA]) could increase the efficacy of the present therapeutic agents by down regulating Sirt1 and decreasing Sirt1 deacetylase activity [32,33], which could decrease the inhibitory effects on Tregs and restore the immune balance in cHL microenvironment. We found that cHL patients with Sirt1 over expression had higher incidence of recurrence after successful therapy which suggest the possibility that immune dys-regulation played an essential role in the cHL pathogenesis and could decrease Tregs resulting in imbalance between them and cytotoxic T-cells. This was in agreeing with immune dysregulation

Characteristics	All (N=34)	Relapse No (N=20)	Relapse No (N=14)	p-value	Median RFS	1 year RFS (%)	3 year RFS (%)	5 year RFS (%)	HR (95%CI)	p-value
All patients					NR	100%	91.2%	56.5%		
Initial site										
Cervical	14 (41.2%)	9 (64.3%)	5 (35.7%)		NR	100%	85.7%	62.9%	----	
SC	3 (8.8%)	1 (33.3%)	2 (66.7%)		45 month	100%	100%	33.3%		
Axilla	8 (23.5%)	4 (50%)	4 (50%)	0.474§	NR	100%	100%	50%		0.242†
Mediastinum	8 (23.5%)	6 (75%)	2 (25%)		NR	100%	100%	75%		
Inguinal	1 (2.9%)	0 (0%)	1 (100%)		42 month	100%	100%	0%		
Bulky LN										
Absent	23 (67.6%)	18 (78.3%)	5 (21.7%)	0.002§	NR	100%	100%	75.3%	6.931	<0.001†
Present	11 (32.4%)	2 (18.2%)	9 (81.8%)		42 month	100%	72.7%	18.2%	(1.898-25.304)	
Bulky Med. LN										
Absent	32 (94.1%)	19 (59.4%)	13 (40.6%)	1.000§	NR	100%	90.6%	56.8%	1.399	0.737†
Present	2 (5.9%)	1 (50%)	1 (50%)		NR	100%	100%	50%	(0.132-14.785)	
Number of nodal sites										
One site	7 (20.6%)	7 (100%)	0 (0%)		NR	100%	100%	100%	----	
Two sites	10 (29.4%)	9 (90%)	1 (10%)		NR	100%	100%	85.7%		
Three sites	3 (8.8%)	0 (0%)	3 (100%)	0.001‡	45 month	100%	100%	0%		<0.001†
Four sites	4 (11.8%)	2 (50%)	2 (50%)		52 month	100%	100%	37.5%		
Five sites	3 (8.8%)	1 (33.3%)	2 (66.7%)		45 month	100%	100%	33.3%		
>Five sites	7 (20.6%)	1 (14.3%)	6 (85.7%)		40 month	100%	57.1%	14.3%		
BM infiltration										
Absent	33 (38.2%)	19 (57.6%)	14 (42.4%)	1.000§	NR	100%	90.9%	55.1%	0.000	0.438†
Present	1 (2.9%)	1 (100%)	0 (0%)		NR	100%	100%	100%		
Extranodal lesions										
Absent	13 (38.2%)	12 (92.3%)	1 (7.7%)	0.002§	NR	100%	100%	87.5%	11.043	0.002†
Present	21 (61.8%)	8 (38.1%)	13 (61.9%)		45 month	100%	85.7%	37.5%	(3.860-31.595)	
Histopathological subtype										
NS	14 (41.2%)	12 (85.7%)	2 (14.3%)		NR	100%	100%	86.7%	----	
MC	16 (47.1%)	8 (50%)	8 (50%)	0.005§	52 month	100%	81.2%	48.2%		0.028†
LR	4 (11.8%)	0 (0%)	4 (100%)		45 month	100%	100%	0%		
Stage										
Stage I	7 (20.6%)	7 (100%)	0 (0%)		NR	100%	100%	100%	----	
Stage II	13 (38.2)	9 (69.2%)	4 (30.8%)	0.001‡	NR	100%	100%	65.9%		<0.001†
Stage III	7 (20.6%)	3 (42.9%)	4 (57.1%)		52 month	100%	100%	38.1%		
Stage IV	7 (20.6%)	1 (14.3%)	6 (85.7%)		40 month	100%	57.1%	14.3%		
Prognostic group										
Unfavorable	14 (41.2%)	4 (28.6%)	10 (71.4%)	0.003§	45 month	100%	78.6%	26.8%	0.181	<0.001†
Favorable	20 (58.8%)	16 (80%)	4 (20%)		NR	100%	100%	77.9%	(0.058-0.561)	
Sirt1										
Low	21 (61.8%)	18 (85.7%)	3 (14.3%)	<0.001§	NR	100%	95.2%	82.7%	9.590	<0.001†
High	13 (38.2%)	2 (15.4%)	11 (84.6%)		45 month	100%	84.6%	-----	(2.973-30.935)	
CD163										
Low	27 (79.4%)	20 (74.1%)	7 (25.9%)	0.001§	NR	100%	100%	71.2%	11.545	<0.001†
High	7 (20.6%)	0 (0%)	7 (100%)		40 month	100%	57.1%	-----	(1.669-79.866)	
Tryptase										
Low	27 (79.4%)	20 (74.1%)	7 (25.9%)	0.001§	NR	100%	100%	71.2%	11.545	<0.001†
High	7 (20.5%)	0 (0%)	7 (100%)		40 month	100%	57.1%	-----	(1.669-79.866)	

Categorical variables were expressed as number (percentage); continuous variables were expressed as mean ± SD & median (range).
▪ Mann Whitney U test; § Chi-square test; ‡ Chi-square test for trend; † Log rank test; NR denote not reached yet; HR Hazards Ratio; 95%CI: 95% confidence interval; p<0.05 is significant.

Table 3: Correlation between clinicopathological criteria, Sirt1, CD163, Tryptase expression and relapse of our patients.

Characteristics	All (N=50)	Death		p-value	Median OS	Overall Survival (OS)			HR (95%CI)	p-value
		No (N=34)	Yes (N=16)			1 year OS (%)	3 year OS (%)	5 year OS (%)		
All patients					NR	96%	75.8%	63.9%		
Spleen involvement										
Absent	28 (56%)	24 (85.7%)	4 (14.3%)	0.002§	NR	100%	96.4%	80.8%	6.091	<0.001†
Present	22 (44%)	10 (45.5%)	12 (54.5%)		36 month	90.9%	49.6%	44.6%	(2.148-17.270)	
Histopathological subtype										
NS	14 (28%)	11 (78.6%)	3 (21.4%)	0.429§	NR	100%	100%	74%	-----	0.216†
MC	31 (62%)	19 (61.3%)	12 (38.7%)		NR	93.5%	64%	60.5%		
LR	5 (10%)	4 (80%)	1 (20%)		NR	100%	80%	80%		
Stage										
Stage I	7 (14%)	6 (85.7%)	1 (14.3%)	0.054‡	NR	100%	100%	80%	-----	0.056†
Stage II	13 (26%)	11 (84.6%)	2 (15.4%)		NR	100%	100%	80.8%		
Stage III	12 (24%)	7 (58.3%)	5 (41.7%)		NR	100%	66.7%	58.3%		
Stage IV	18 (36%)	10 (55.6%)	8 (44.4%)		NR	88.9%	55.6%	55.6%		
Prognostic group										
Unfavorable	30 (60%)	17 (56.7%)	13 (43.3%)	0.035§	NR	93.3%	59.4%	55.7%	0.226	0.009†
Favorable	20 (40%)	17 (85%)	3 (15%)		NR	100%	100%	81.2%	(0.085-0.604)	
Risk group										
Low favorable	16 (32%)	15 (93.8%)	1 (6.3%)	<0.001‡	NR	100%	100%	90.9%	-----	<0.001†
Low unfavorable	3 (6%)	1 (33.3%)	2 (66.7%)		50 month	100%	100%	0%		
Intermediate	18 (38%)	18 (100%)	0 (0%)		NR	100%	100%	100%		
High	13 (26%)	0 (0%)	13 (100%)		25 month	84.6%	7.6%	0%		
Sirt1										
Low	21 (42%)	20 (95.2%)	1 (4.8%)	<0.001§	NR	100%	100%	92.9%	17.085	<0.001†
High	29 (58%)	14 (48.3%)	15 (51.7%)		48 month	93.1%	58.2%	-----	(6.399-45.617)	
CD163										
Low	27 (54%)	24 (88.9%)	3 (11.1%)	0.001§	NR	100%	100%	85.8%	9.232	<0.001†
High	23 (46%)	10 (43.5%)	13 (56.5%)		36 month	91.3%	46.6%	-----	(3.227-26.407)	
Tryptase										
Low	28 (56%)	25 (89.3%)	3 (10.7%)	<0.001§	NR	100%	100%	86.4%	10.508	<0.001†
High	22 (44%)	9 (40.9%)	13 (59.1%)		36 month	90.9%	44.1%	-----	(3.576-30.873)	

Categorical variables were expressed as number (percentage); continuous variables were expressed as mean ± SD & median (range).
· Mann Whitney U test; § Chi-square test; ‡ Chi-square test for trend; † Log rank test; NR denote not reached yet; HR Hazards Ratio; 95%CI: 95% confidence interval; p<0.05 is significant.

Table 4: Correlation between clinicopathological criteria, Sirt1, CD163, Tryptase expression and survival of our patients.

theory that played a role in cHL pathogenesis. Proofs of theory of the role of inhibition of Sirt1 pathway and Tregs stimulation to restore immune balance in cHL are recently proved [34,35]. In our study we demonstrate that, in patients with cHL, the recurrence of the disease after successful therapy can be correlated with Sirt-l expression so it carries prognostic significances, so Histone deacetylase inhibitors can increase the efficacy of pre-existing drugs by inhibiting *Sirt1* gene mRNA/Sirt1 protein function, which subsequently improve prognosis and decrese remission in cHL [29,30,32-34]. Targeting Sirt1 in the RS cells also may facilitate their ability to promote naïve T cell differentiation toward Treg cells over CTL [31]. Further examination of this pathway and its relationship to T cell microenvironment and immune dysregulation in cHL is warranted, as it carries therapeutic implications for improved patient out-comes. Another explanation of how Sirt-1 overexpression increasing cHL progression is that it could deacetylate p53 causing its inactivation so decreasing growth arrest or apoptosis of malignant cells and hence increasing oncogenic functions [36,37]. Similar to our results in cHL Sirt1 over expression is associated with poor prognosis in malignancies of many organs as stomach and liver [38,39]. Recently, many studies had focused on the role of the microenvironment of cHL in prediction of treatment outcome. In our study, we evaluated the presence of 2 types of benign reactive inflammatory cells in the microenvironment of cHL that are the macrophages and mast cells detecting their presence and number by CD163 & Tryptase expression respectively then we correlating their number with clinicopathological and prognostic status. Patients with high counts of mast cells evidenced by tryptase over expression and macrophages evidenced by CD163 overexpression were found to have more advanced stages (stage III–IV, *vs.* I–II), poor clinicopathological criteria, high incidence of disease relapse, poor response to therapy and poor survival. Survival analyses were performed reveal significant differences between the groups. Our results are like Englund et al., who proved similar results to ours but in pediatric HL [40], also the study by Kamper et al. that had supported the value of evaluating tumor-infiltrating macrophages in pretreatment samples for prediction of patient outcome in cHL [41]. Our study confirms results of previous studies about the prognostic role of tumor-infiltrating macrophages in cHL and we used the macrophage marker CD163 that was found to be more accurate than CD68. Klein et al., Koh et al., Tudor et al., found similar results to us in cHL [42-44]. On the contrary from us Kayal et al., results showed no significant difference between macrophages infiltration, clinicopathological parameters or cHL prognosis [45].
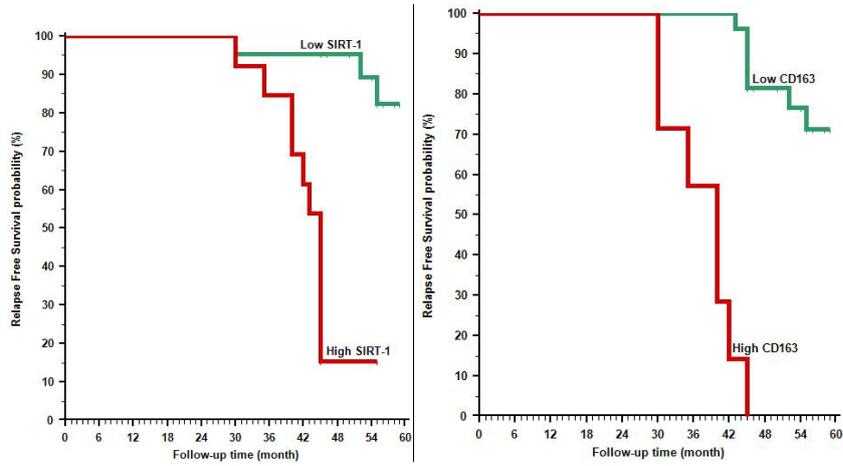
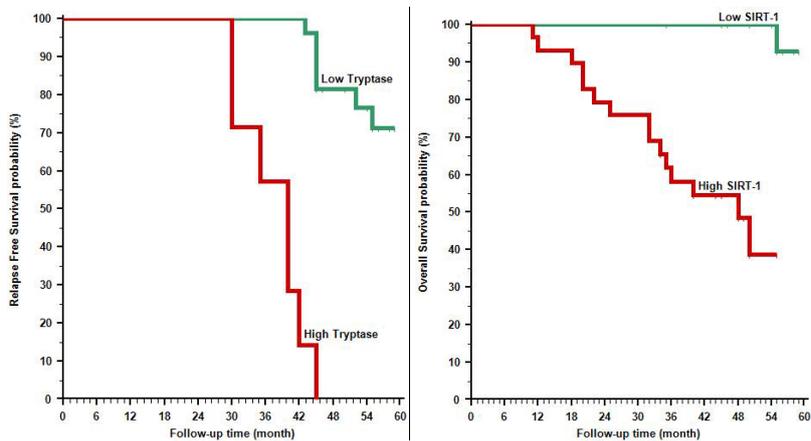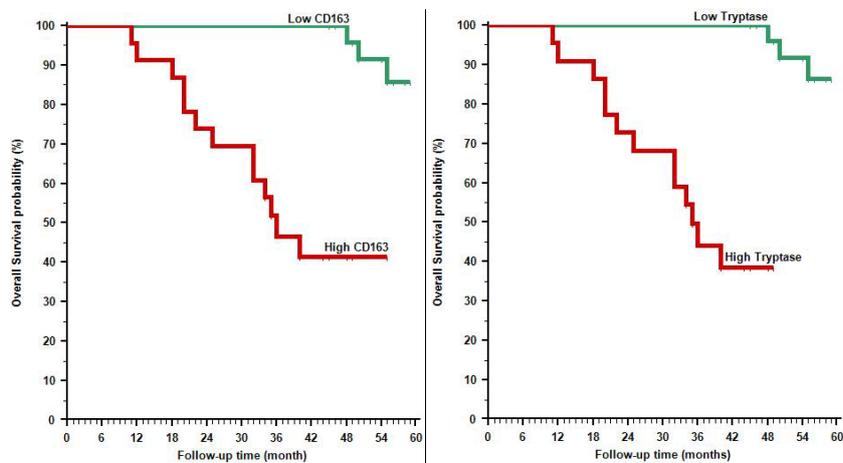**Figure 4:** Kaplan Meir plot of Relapse Free Survival (Left panel) and Overall Survival (Right panel) of Hodgkin's lymphoma patients: (A) & (D) stratified by SIRT-1 expression; (B) & (E) stratified by CD163 expression; (C) & (F) stratified by Tryptase expression.

Similar to our results Barros et al. proved that progression-free survival rate (PFS) was lower in cases with high numbers of CD163+ macrophages [46]. But, Gupta et al. presented that there was no association between survival rates in macrophages evaluated with either CD163 or CD68 [47]. These conflicting data could be due to different number of patients used, variable follow up period, different clones of antibodies used or different method of evaluation.

There are many questions regarding the exact mechanisms of how RS cells can attract the macrophages, how macrophages affect RS cell growth and how RS cells could accquire immune isolation by such interaction. Recently, there are many discovered molecules that had been incriminated in macrophage chemotaxis and signaling in cHL pathobiology, e.g. CSF1R, MIF, fractalkine and CD74 [48], some of these molecules could be promising targets for discovering novel drug therapies that target the macrophages directly or by causing disturbances in the signaling pathways between RS cells and the surrounding tumor microenvironment.

We found that high number of mast cell infiltration in cHL as evidenced by tryptase overexpression was correlated to advanced stage and the presence of B-symptoms that was similar to Molin et al. who found that high mast cell infiltration had found to be related to advanced disease and poor relapse-free survival in adults [49]. Andersen et al. detect the relation between high mast cell count and poor outcome of patients similar to our results but that was only restricted to the mixed cellularity subtype [20]. In our results higher incidence of disease remission after successive therapy was higher in patients with higher mast cells count that was explained by RS cells stimulation by mast cells through CD30-CD30L interaction [49].

Conclusion

We evaluated the clinicopathological and prognostic roles of the microenvironment in cHL and found a significant relations between Sirt1 expression which was related to Tregs differentiation and oncosuppressive role, macrophages as evidenced by CD163 expression, mast cells as evidenced by tryptase expression, poor clinicopathological parameters, advanced stage and poor patient prognosis, which may help to detect novel therapeutic targets to improve cHL outcome mainly in advanced and relapsed patients. Because of the conflicting results between our study and different previous studies we recommend that a large scale study on large number of patients with different subtypes to prove our results and detect the possible use of targeted therapies against the inflammatory infiltrate in the cellular microenvironment of HL.

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