Original Article

Expression of Sirt1 and FoxP3 in classical Hodgkin lymphoma and tumor infiltrating lymphocytes: Implications for immune dysregulation, prognosis and potential therapeutic targeting

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Abstract: Background: Hodgkin Reed-Sternberg (HRS) cells may promote differentiation of CD4+ naïve T cells toward both FoxP3+ T regulatory (Treg) cells and TIA-1+ cytotoxic T lymphocytes (CTL). Previous studies suggest that an overabundance of cytotoxic TIA-1+ cells in relation to FoxP3+ T reg cells portends unfavorable outcomes in classical Hodgkin lymphoma (cHL), raising the possibility that its pathogenesis may be related to immune dysregulation. Sirt1 deacetylates FoxP3 and leads to decreased Treg functionality. Our objective was to compare Sirt1 and FoxP3 expressions in Hodgkin lymphoma infiltrating lymphocytes (HLIL) and confirm Sirt1 expression in HRS cells. Design: Immunohistochemical staining of paraffin-embedded tissue with antibodies to Sirt1, FoxP3, TIA-1, and CD8 was performed. Expression of Sirt1 was assessed in both the HRS cells and in the HLILs in twenty-four cases. Adequate tissue was available in 13 cHL cases to permit the enumeration of FoxP3, TIA-1 and CD8 by giving their percent staining of HLILs. Results: In HLILs, nuclear expression of Sirt1 was 32-88% (mean 67%); FoxP3 expression was 9-40% (mean 23.9%); TIA-1 expression was 15-87% (mean 32%); and CD8 expression was 10-45% (mean 31%). Sirt1 to FoxP3 ratio was 0.96-5.5 (mean 3.2); TIA-1 to FoxP3 ratio was 0.6-5.1 (mean 1.6); CD8 to FoxP3 ratio was 0.43-3.7 (mean 1.5). There was a difference of Sirt1 to FoxP3 ratios between remission and recurrence groups, being significantly higher in the recurrence group (P = 0.005). Sirt1 demonstrated high nuclear expression in the HRS cells of 21 out of 24 (88%) cases analyzed. Conclusion: The relative overexpression of Sirt1 to FoxP3 in HLILs may be considered possible targets for immune modulation. Histone deacetylase inhibitors may increase the efficacy of existing treatment regimens by downregulating SIRT1 gene mRNA/ Sirt1 protein function and together with rapamycin could expand the T regulatory/FoxP3 population and functionality and improve prognosis for remission in cHL. Targeting Sirt1 in the HRS cells may facilitate their ability to promote naïve T cell differentiation toward Treg cells over CTL.

Keywords: Sirt1, FoxP3, Hodgkin lymphoma, immune dysregulation, morphoproteomics

Introduction

Classical Hodgkin lymphoma (cHL) is composed of various non-neoplastic, reactive cells with only a minority of malignant cells (Reed-Sternberg cells and Hodgkin cells) [1, 2]. Existing evidence in most cases supports the notion that these malignant cells in cHL originate from germinal center B cells [1-6]. The etiopathogenesis of cHL is still a subject of considerable investigation and debate. A role for an autoimmune component in the pathogenesis of cHL has been raised and supported by the following associations: 1. a personal or family history of certain autoimmune conditions was strongly associated with the increased risk of Hodgkin lymphoma in a Scandinavian study [7]; 2. The risk of Hodgkin lymphoma developing in systemic lupus erythematosus, an autoimmune disease, is increased [8]; 3. A link was noted between dys-immunity with autoimmune diseases preceding or following the diagnosis...
of Hodgkin lymphoma in 11 children, in one study [9]; and 4. An associated autoimmune disease was reported in 14 of 121 (11.5%) Hodgkin lymphoma patients in a report of a single center experience [10].

In support of immune dysregulation and/or autoimmunity in the pathogenesis of Hodgkin lymphoma, it is noteworthy that the presence of cytotoxic TIA-1+ cells was found to be an unfavorable prognostic factor of event-free survival and disease-free survival in Hodgkin lymphoma [11]. Also, activated cytotoxic T cells expressing granzyme B and associated with CD8+ cells had a shortened progression-free survival time in patients with Hodgkin’s lymphoma [12]. Additionally, a ratio of FoxP3 positive T regulatory cells to activated cytotoxic T lymphocytes (identified by positive granzyme B stain) of 1 or less may predict a poor failure-free survival [13]. Alvarez and co-workers reported that a low infiltration of FoxP3+ cells in conjunction with a high infiltration of TIA-1+ cells may represent biological markers predicting an unfavorable outcome [14]. Conversely, the failure-free survival in cHL statistically increased with a higher number of FoxP3+ tumor infiltrating lymphocytes [14] and in another study, an increased number of tumor-infiltrating FoxP3+ cells over the receiver operating characteristic-determined cut-offs positively influenced overall and failure-free survival in cHL [15].

If one accepts, for the sake of argument, this premise of an autoimmune component or immune dysregulation in the pathogenesis of Hodgkin disease, then expanding the T regulatory cells and their functionality would be critical in effecting a positive outcome for patients with this disease. Furthermore, identifying pathways and proteins that reduce the functionality of the T regulatory cells would be important in assessing the resistance signature to conventional therapies, in correlating the clinical response to agents that reduce such inhibitory pathways and in designing future therapies intended to target proliferation while restoring proper immune regulation. Conceivably, an increase in the numbers of functional FoxP3 T regulatory cells could be accomplished by inhibition of Sirt1 (silent mating type information regulation 2 homolog 1), a member of the histone deacetylase Sirtuin family which is known to downregulate FoxP3 [16-20]. To expand on this, FoxP3 expression and stabilization via acetylation is necessary for regulatory T cell differentiation and functionality and Sirt1, as an NAD+ histone deacetylase can lead to destabilization. Inhibition of Sirt1 strengthens the suppressive action of Tregs [16-20]. Panobinostat is a pan-deacetylase inhibitor which was shown by Lemoine et al. to have activity against Hodgkin lymphoma derived cell lines, inducing cell death as well as inhibiting the Sirt1 pathway [21]. Similarly, the combinatorial use of niacinamide and vorinostat, as sirtuin and pan-class I/II deacetylase (DAC) inhibitors, has been shown to result in a response rate of 24% in relapsed or refractory lymphoma patients with an additional 57% reportedly achieving stable disease [22].

Morphoproteomics is a technique which uses bright-field microscopy and immunohistochemistry to study and understand the molecular circuitry of tumors and their microenvironment by observing the expression and when applicable, activation of various protein analytes and their correlative expressions in order to define the biology of pathologic processes [23, 24]. Our study was designed to attempt to gain further understanding of the pathologic mechanisms which may be driving classical Hodgkin lymphoma. We sought to confirm that the Hodgkin Reed-Sternberg cells express Sirt1 [25]. In addition, we proposed to analyze the ratios of Sirt1/FoxP3, CD8/FoxP3 and TIA-1/FoxP3 in the Hodgkin lymphoma infiltrating lymphocytes (HLIL) to characterize the T lymphocyte milieu, which may be contributing to tumorigenesis and prognosis. A better understanding of the tumoral microenvironment, particularly immunologically active T lymphocytes may allow favorable interventions via promotion of growth or biological activity of subsets of T cells that leads to immune balance and that may be essential to the successful remission of Hodgkin disease.

Materials and methods

Approval by the Institutional Review Board (IRB) was obtained for this study. Twenty-four cases of cHL were retrospectively examined between 2008 and 2011. All patients were treated with standard frontline chemotherapy as per national guidelines and clinically assessed for response by standard imaging and laboratory evaluations at regular intervals. Clinical follow-
Sirt1 and FoxP3 expression in classical Hodgkin lymphoma

Table 1. Immunohistochemical protein markers and antibody specifics

<table>
<thead>
<tr>
<th>Protein Analyte</th>
<th>Antibody Specifics</th>
<th>Represents</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8</td>
<td>Dako monoclonal mouse IgG1 anti-human CD8 clone C8/144B</td>
<td>Cytotoxic T cells</td>
</tr>
<tr>
<td>TIA-1</td>
<td>Abcam monoclonal mouse IgG1 anti-TIA1</td>
<td>Cytotoxic T cell marker</td>
</tr>
<tr>
<td>FoxP3</td>
<td>Abcam monoclonal mouse IgG1 [236A/E7]</td>
<td>T regulatory cells</td>
</tr>
<tr>
<td>Sirt1</td>
<td>Abcam Sirt1 monoclonal rabbit antibody [E104] ab32441</td>
<td>NAD+ histone deacetylase</td>
</tr>
</tbody>
</table>

Figure 1. Sirt1 highly expressed in the nuclei of Hodgkin Reed-Sternberg cells by immunohistochemistry (A and B, arrows HR-S) and variably in the tumor infiltrating lymphocytes (B) from two cases of cHL in our study, contrast with negative control in (C) (original magnification, ×400 A ×600 B and C).

Results

Sirt1, TIA-1 and CD8 expressions and correlation with FoxP3

Sirt1 demonstrated high nuclear expression in 21 out of 24 (88%) classical Hodgkin lymphoma...
ma cases analyzed (Figure 1). Three cases did not show high expression in the HRS cells. In addition, Sirt1 was also variably positive in the background tumor infiltrating lymphocytes with 16 of the 24 cases (67%) showing nuclear expression of Sirt1 in 50% or more cells (Figure 1).

The ratio between Sirt1 and FoxP3 positive lymphocytes was examined in thirteen cases. The nuclear expression of Sirt1 in HLILs was 32-88% (mean 67%), while FoxP3 expression was 9-40% (mean 23.9%) (Figure 2). The Sirt1 to Foxp3 ratio ranged from 0.96-5.5 (mean = 3.2). TIA-1 expression was 15-87% (mean = 32%). The TIA-1 to FoxP3 ratio ranged from 0.6-5.1 (mean = 1.6). The expression of CD8 was 10-50% (mean = 31%). The CD8 to FoxP3 ratio ranged from 0.43-3.7 (mean = 1.5) (Table 2 and Figure 2).

Figure 2. HLIL expression of (A) FoxP3, labeled as 9% staining in case 7, (B) FoxP3, labeled as 40% in case 11, (C) TIA-1, 15% in Case 4, (D) TIA-1, 87% Case 10, (E) Sirt1, 50% in Case 6, (F) Sirt1, 88% Sirt1 in Case 10. Images for CD8 not depicted (original magnification, ×400).
Sirt1 and FoxP3 expression in classical Hodgkin lymphoma

There were 21 cases of Hodgkin lymphoma which showed increased expression of Sirt1 within the HRS cells. Statistical analysis showed a significant difference between the mean percent expression within the HRS cells when compared to the mean percent expression within the HLILs (78.3±6.70 versus 59.7±6.52; P = 0.03) (Table 3). The correlation coefficient obtained by linear regression was r = 0.39.

Sirt1 to FoxP3 and cytotoxic T lymphocyte to FoxP3 ratios and clinical outcomes

In the ten cases with follow-up data, seven patients obtained remission whereas three patients had recurrence at the time of the analysis. Statistical analysis revealed a significant difference (P < 0.005) of Sirt1:FoxP3 ratio between the remission and recurrence groups. There was also a trend towards significance in the CD8:FoxP3 ratio (P = 0.052) (Table 4).

Discussion

In this study, we documented the expression patterns of Sirt1 in HRS of cHL. We also sought to investigate the tumor milieu in the form of HLILs by determining the ratio of cytotoxic T lymphocytes to T regulatory lymphocytes. Any upregulated proteins in the tumor cells themselves may provide supplemental targets with therapeutic intent, and a better understanding of the environment in which the tumor is growing may provide greater insight into the pathophysiology of the disease. In addition, modifying this environment could suppress tumor growth.

Our finding of high nuclear expression of Sirt1 in HRS cells in 88% of the cases of cHL reaffirms the earlier report of Sirt1 expression in the Hodgkin Reed-Sternberg cells [25]. In this context, the observation that Reed-Sternberg cells, in an in vitro cell line study, have been shown to promote bidirectional differentiation of CD4+ naïve T cells toward both T regulatory and cytotoxic T cells [27] is relevant to the Sirt1 expression in HRS and in the HLIL. We found a statistical difference between the percent

Table 2. Thirteen cases of classical Hodgkin lymphoma with the percentage expression of FoxP3, TIA-1, CD8 and Sirt1 in HLILs and the ratios of CD8, TIA-1 and Sirt1 to FoxP3

<table>
<thead>
<tr>
<th>Case</th>
<th>FoxP3</th>
<th>TIA</th>
<th>CD8</th>
<th>Sirt 1 in HLIL</th>
<th>CD8/FoxP3</th>
<th>TIA-1/FoxP3</th>
<th>Sirt1/FoxP3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>30</td>
<td>20</td>
<td>33</td>
<td>57</td>
<td>1.1</td>
<td>0.66</td>
<td>1.9</td>
</tr>
<tr>
<td>Case 2</td>
<td>23</td>
<td>45</td>
<td>10</td>
<td>82</td>
<td>0.43</td>
<td>1.95</td>
<td>3.56</td>
</tr>
<tr>
<td>Case 3</td>
<td>33</td>
<td>40</td>
<td>25</td>
<td>32</td>
<td>0.75</td>
<td>1.21</td>
<td>0.96</td>
</tr>
<tr>
<td>Case 4</td>
<td>25</td>
<td>15</td>
<td>40</td>
<td>72</td>
<td>1.6</td>
<td>0.6</td>
<td>2.88</td>
</tr>
<tr>
<td>Case 5</td>
<td>27</td>
<td>30</td>
<td>50</td>
<td>70</td>
<td>1.85</td>
<td>1.11</td>
<td>2.59</td>
</tr>
<tr>
<td>Case 6</td>
<td>30</td>
<td>18</td>
<td>18</td>
<td>50</td>
<td>0.6</td>
<td>0.6</td>
<td>1.66</td>
</tr>
<tr>
<td>Case 7</td>
<td>9</td>
<td>23</td>
<td>33</td>
<td>50</td>
<td>3.66</td>
<td>2.55</td>
<td>5.55</td>
</tr>
<tr>
<td>Case 8</td>
<td>15</td>
<td>35</td>
<td>40</td>
<td>65</td>
<td>2.66</td>
<td>2.33</td>
<td>4.33</td>
</tr>
<tr>
<td>Case 9</td>
<td>18</td>
<td>33</td>
<td>45</td>
<td>65</td>
<td>2.5</td>
<td>1.83</td>
<td>3.61</td>
</tr>
<tr>
<td>Case 10</td>
<td>17</td>
<td>87</td>
<td>27</td>
<td>88</td>
<td>1.58</td>
<td>5.11</td>
<td>5.17</td>
</tr>
<tr>
<td>Case 11</td>
<td>40</td>
<td>25</td>
<td>20</td>
<td>82</td>
<td>0.5</td>
<td>0.62</td>
<td>2.05</td>
</tr>
<tr>
<td>Case 12</td>
<td>27</td>
<td>33</td>
<td>40</td>
<td>77</td>
<td>1.48</td>
<td>1.22</td>
<td>2.85</td>
</tr>
<tr>
<td>Case 13</td>
<td>17</td>
<td>15</td>
<td>25</td>
<td>82</td>
<td>1.48</td>
<td>0.88</td>
<td>4.82</td>
</tr>
<tr>
<td>AVERAGE</td>
<td>23.92</td>
<td>32.23</td>
<td>31.23</td>
<td>67.08</td>
<td>1.55</td>
<td>1.59</td>
<td>3.23</td>
</tr>
</tbody>
</table>

Table 3. Comparison of the mean and standard error of the mean for percent expression of Sirt1 in Hodgkin Reed-Sternberg (HRS) cells versus Hodgkin lymphoma infiltrating lymphocytes (HLIL)

<table>
<thead>
<tr>
<th></th>
<th>Monoclonal Sirt1 in HRS Cells</th>
<th>Monoclonal Sirt1 in HLILs*</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>78.3±6.70</td>
<td>59.7±6.52</td>
</tr>
<tr>
<td>p value</td>
<td>0.03</td>
<td></td>
</tr>
</tbody>
</table>

*Average of percentage of nuclear expression of Sirt1 in HLIL from two independent observers (AQ and BA).

Sirt1 expression in Hodgkin Reed-Sternberg cells versus Hodgkin lymphoma infiltrating lymphocytes

In the ten cases with follow-up data, seven patients obtained remission whereas three patients had recurrence at the time of the analysis. Statistical analysis revealed a significant difference (P < 0.005) of Sirt1:FoxP3 ratio between the remission and recurrence groups. There was also a trend towards significance in the CD8:FoxP3 ratio (P = 0.052) (Table 4).

Discussion

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expression of Sirt1 in the HRS cells as compared to that in the HLILs. Interestingly, however, there was a mild positive correlation between the two. While the HRS appears to express Sirt1 to a significantly greater extent than the surrounding HLILs, the expression of each seems to increase together.

As previously stated, one of the deacetylation targets of Sirt1 is known to be FoxP3, a transcription factor pivotal in the differentiation and function of T regulatory lymphocytes. Sirt1 deacetylation results in destabilization and enhanced degradation of FoxP3 promoting T regulatory cell decline and/or underactivity and inhibition of Sirt1 increases the suppressive action of Tregulatory cells [16-20]. Thus, Sirt1 inhibition may also allow HRS cells to promote the differentiation of CD4+ naïve T cells toward Tregs.

We suggest the possibility that immune dysregulation may be playing a larger role in the pathogenesis of Hodgkin lymphoma than previously thought. The patients who suffered a recurrence in this study had a statistically significant increase in the ratio of Sirt1 to FoxP3 as compared to the patients who were in remission. The skewed ratio implies a decrease in T-regulatory cells with a resultant imbalance relative to cytotoxic T-cells. In the ten cases with follow-up data, statistical analysis revealed a significant difference (P < 0.005) of Sirt1 to FoxP3 ratio between the remission and recurrence groups. There was also a trend towards significance in the CD8 to FoxP3 ratio (P = 0.052). An analogy drawn from the commonality of germinal center B-cell origins of cHL and germinal center-like diffuse large B-cell and follicular lymphomas is the correlation of high numbers of intratumoral FoxP3+ regulatory T cells and improved survival in both [1-6, 15].

The overexpression of Sirt1 in RS and the high mean ratios of Sirt1 and TIA-1 to FoxP3 in the HLILs may be amenable to modulation to influence clinical outcomes. Conceivably, histone deacetylase inhibitors (such as vorinostat [SAHA]) may increase the efficacy of existing treatment regimens by downregulating Sirt1 gene mRNA/Sirt1 protein function and reducing Sirt1 deacetylase activity [28-30]. A decrease in Sirt1-related deacetylation of FoxP3 could reduce the negative modulation of the T regulatory (FoxP3+) cell population. Should immune dysregulation and/or auto-immunity play a contributing role in the pathogenesis of Hodgkin lymphoma, then the inhibition of the Sirt1 pathway could provide additional benefit by upregulating the expression of FoxP3. In turn, this would block the inhibitory modulation of T regulatory cells and potentially restore immune balance in the tumoral microenvironment.

Proofs of concept of the possible role of inhibiting Sirt1 pathway and at the same time expanding the Tregulatory cells to restore immune balance in cHL is contained in the recent report of a case of multiply recurrent Hodgkin lymphoma that experienced complete clinical remission with vorinostat and sirolimus. Morphoproteomics and biomedical analytics, with a focus on the respective roles of vorinostat and sirolimus in targeting Sirt1 and expanding the T regulatory cells and downregulating CD8 cells provided correlates of the response signature in this case [31]. A follow-up study with 28 heavily pretreated refractory cHL patients treated with vorinostat and sirolimus showed a complete response in 32% and a partial response in 25% [32].

In summary, this study demonstrates that, in a limited number of patients with cHL, the remission and relapse status can be correlated with the ratio of Sirt1 and FoxP3 expression and therefore, carries prognostic implications. Our findings also provide a basis to relate the apparent clinical significance of the relative overexpression of Sirt1 vis-à-vis FoxP3 cells in the HLIL with dysregulation of the T cell ratios in the tumoral microenvironment and dys-immunity in cHL. Histone deacetylase inhibitors may increase the efficacy of existing treatment regimens by downregulating SIRT1 gene mRNA/Sirt1 protein function and together with rapamycin could expand the T regulatory/FoxP3 population and functionality and improve prognosis for remission in cHL [16-20, 28-31]. Targeting

Table 4. Statistical analysis of ratios comparing remission to recurrence groups

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>CD8/FoxP3</th>
<th>TIA-1/FoxP3</th>
<th>Sirt1/FoxP3</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Remission</td>
<td>7</td>
<td>1.26±0.31</td>
<td>1.14±0.23</td>
<td>2.45±0.41</td>
<td>0.052</td>
</tr>
<tr>
<td>Recurrence</td>
<td>3</td>
<td>2.6±0.77</td>
<td>1.92±0.64</td>
<td>4.9±0.43</td>
<td>0.129</td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.005</td>
</tr>
</tbody>
</table>
Sirt1 and FoxP3 expression in classical Hodgkin lymphoma

Sirt1 in the HRS cells also may facilitate their ability to promote naïve T cell differentiation toward Treg cells over CTL [27]. 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 outcomes.

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Disclosure of conflict of interest

None.

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References


