Int J Clin Exp Med 2010; 3(1):55-68
www.ijcem.com /IJCEM201001007

Original Article

Tissue-microarray based immunohistochemical analysis of survival pathways in nodular sclerosing classical Hodgkin lymphoma as compared with Non-Hodgkin's lymphoma

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Received January 18, 2010, accepted January 25, 2010, available online January 30, 2010

Abstract: Neoplastic cells rely on key oncogenic pathways for their gain of proliferative and/or loss of apoptotic potential. Therapy targeted at specific points in these pathways has the potential to eliminate cancer cells by inducing differentiation or apoptosis. Concurrent immunophenotypic evaluation of survival pathways in nodular sclerosing classical Hodgkin lymphoma (cHL-NS) tissues has not previously been undertaken. We took the tissue microarray (TMA)-based approach to retrospectively evaluate the activation state of key oncogenic pathways by immunohistochemistry (IHC) in a series of 6 cases of cHL-NS (with predominantly syncitial areas). For comparison, 2 cases of diffuse large B-cell lymphoma (DLBCL), and 1 case of follicular hyperplasia (FH) were included in the study. Infiltration of T regulatory cells (Tregs) in the tumor microenvironment was assessed by expression of the Foxp3 transcription factor. Differential upregulation of the mitogen-activated protein kinase (MAPK)-extracellular signal related kinase (ERK), signal transducers and activators of transcription (STAT)3, and protein kinase c – alpha (PKC-α) pathways was seen among the cHL cases, whereas nuclear factor – kappa B (NF-κB) and phosphoinositide 3 kinase (PI3 K)-AKT-mammalian target of rapamycin (mTOR) pathways were equally activated in the neoplastic Reed-Sternberg cells of the 6 cHL-NS cases. Marked difference in the morphoproteomic profile was seen amongst the two cases of DLBCL. The amount of Foxp3+ T regulatory cells (Tregs) in the tumor microenvironment was highly variable ranging from 6/hpf to 120/hpf in cHL-NS, and 1/hpf to 82/hpf in DLBCL. In this pilot study, concurrent evaluation of oncogenic pathways in cHL-NS and DLBCL offers powerful insights in the putative therapeutic targets for an individualized approach to diagnosis and therapy.

Keywords: Classical Hodgkin lymphoma, diffuse large B-cell lymphoma, survival pathways, tissue microarray, morphoproteomics

Introduction

The standard approach to therapy of classical Hodgkin lymphoma includes a combination of chemotherapy and involved field radiation. A second tier approach of high dose chemotherapy/transplantation is considered in relapsed and refractory cases. These advances have significantly improved outcome. Despite that, more than 15% of patients die of progressive lymphoma [1] . Relapsed patients could benefit from novel therapeutic agents and regimens. Even patients who do not relapse often experience late treatment effects including secondary cancer, heart failure and hypothyroidism. Thus, new regimens could potentially lead to eliminating radiation or decreasing intensity of chemotherapy.

Knowledge of the activation state of signaling pathways gained from proteomics has the potential to instigate the design of logical combinations of enzyme-targeted agents. Recently, in the last decade, agents specifically targeting tyrosine kinases have successfully allowed a targeted approach to therapy [2].The potential for enzyme targeted therapy relies on activated oncogenic kinases that deregulate apoptotic mechanisms and promote survival in neoplastic cells. The pathogenesis of cHL is highly complex

and thus far, not been attributed to a single transforming pathway. Nevertheless, survival of the neoplastic cells rely on constitutive activation of NF-κB, MAPK-ERK, STAT3, and PI3-AKT -mTOR pathways that trigger anti-apoptotic mechanisms [3-10]. Furthermore, the PKC-α pathway is activated by a number of growth factors including platelet-derived growth factor, which is expressed in cHL, and is an important mechanism supporting proliferation of lymphoma cells [11, 12].

"Morphoproteomics" is a term coined for a direct visualization approach by IHC of proteins at sequential points in signaling pathways in order to depict the protein networks in abnormal cells and uncover potential molecular targets amenable to specific intervention, thereby facilitating personalized therapy [13]. The main advantage of an IHC -based approach of formalin-fixed neoplastic tissue for analysis of signaling pathways is the preservation of the overall tissue architecture and the relationships between the neoplastic and reactive component, thus facilitating assessment of cellular and sub-cellular localization within both components simultaneously. The dynamic range of intensity can be readily appreciated and quantified. Since protein expression levels can predict outcome of molecular-targeted therapy, this type of evaluation has the potential to predict therapy responsiveness based on a) expression levels and b) phosphorylation and/or sub-cellular localization, indicating activation state.

We sought to determine the activation state of known survival pathways in nodular sclerosing classical Hodgkin lymphoma (cHL-NS) with a TMA-based morphoproteomic approach. Six cases of cHL-NS were selected for the study. In order to visualize the dynamic range of protein activation state by IHC, two cases of DLBCL and one case of FH were included for comparison. The cases of DLBCL were: # 7, a vaginal wall mass with CD5-positivity, and #8, nodal Bcl6 + DLBCL, with concomitant Grade 1-2 follicular lymphoma (FL), representing transformation. Specifically for cHL-NS, cases with heavy syncitial areas were selected for adequate representation of the neoplastic Reed-Sternberg (R-S) cells and variants within each core. Tissue expression analysis of phosphorylated forms of STAT-3, NF-κB, mTOR and its downstream p70 S6 Kinase, and MAP family of kinases including p38 MAP Kinase and ERK were performed, in addition to PKC-α. T regulatory cells were identified and enumerated within the inflammatory milieu of R-S cells based on Foxp3 expression. By performing morphoproteomic analysis, we demonstrate universal activation of NF-κB and PI3-AKT-mTOR pathways and differential activation of the MAPK (p38 MAPK and ERK), PKC-α, and STAT3 pathways in morphologically similar cases of cHL-NS. In the context of these findings, we review the pathogenesis of cHL and the rationale for implementing molecular targeted therapy.

Materials and methods

Marker selection

Markers were selected based on the literature of upregulation of the AKT-mTOR-S6K pathway, MAPK, NF-κB, and STAT3 pathways in cHL. In addition, PKC-α was selected based on its importance in oncogenesis in general, and hematopoietic malignancies in particular. Translocation of phospholipase D1 (PLD1) from the secretory granules and lysosomes to the plasma membrane, and colocalization with PKC-α occurs upon antigen stimulation, with cooperatively increased PLD1 activity, also tested in this study. A simplified schematic view of these pathways is shown in **Figure 1.**

Case selection

The retrospectively selected cases were diagnosed from 1995 -2009 at University of Michigan Health System and were 6 cases of cHL-NS with predominantly syncitial areas, 2 cases of DLBCL (#7, CD5+ and #8, Bcl-6+ with concurrent follicular lymphoma, grade 1), and one case of a reactive lymph node (#9) (**Figure 2**). Paraffin blocks were selected on the basis of availability of suitable formalin-fixed paraffin-embedded tissue.

Tissue microarray design

All cHL cases were reviewed and the areas richest in the neoplastic R-S cells and variants were marked on the slide and subsequently in the paraffin blocks. From each case, two - 2 mm-diameter cores from different areas were selected to construct duplicate TMA blocks. One core from each case was represented, except from Case #5 from which two cores were represented. Thus, 2 TMA blocks were constructed,

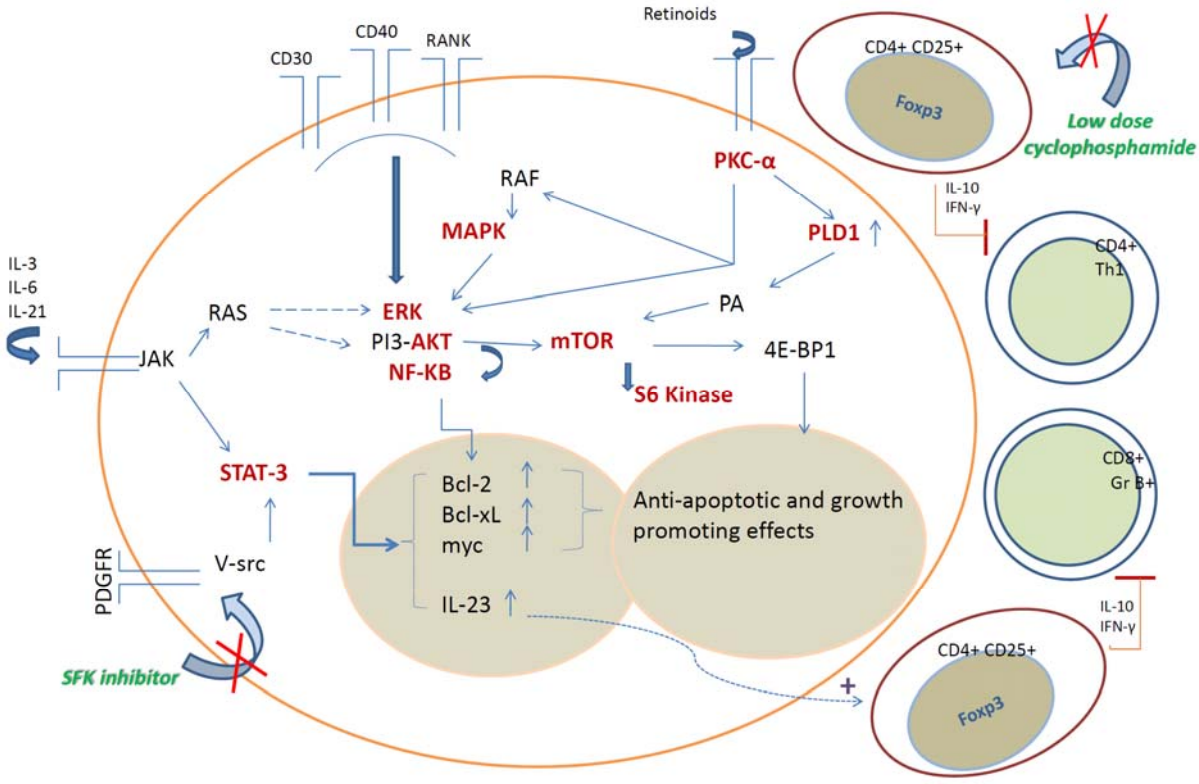

Figure 1. Survival pathways with their cross-talk and immune regulatory mechanisms are shown in this pictorial representation of the Reed-Sternberg (R-S) cell. CD30, CD40, and receptor activator of NF-κB (RANK) receptors on R-S cells are activated by autocrine and paracrine loops leading to activation of PI3K and phosphorylation of AKT, promoting growth and survival. IL-21 activates STAT3 and upregulates STAT3 target genes, triggering anti-apoptotic mechanisms, and inducing trafficking of CCR4 and CCR6- expressing T regulatory cells towards the neoplastic cells. Phosphorylated STAT3 upregulates IL-23 and induces Foxp3 expression in Tregs expressing the IL-23 receptor. These immune regulatory cells modulate the anti-tumor immunity by secretion of IL-10 and IFN-γ, both of which block the adaptive and effector immune response. PKC-α activation is associated with increased immune modulation and reduced apoptosis by Bcl-2 overexpression in hematologic malignancies. Inhibition of AKT, NF-κB, STAT3, PKC-α and MAPK-ERK pathways induce cell cycle arrest and apoptosis. The use of an SFK inhibitor has the potential to modulate the immune escape mechanisms by blocking the STAT pathway.

each containing 10 cores from the 9 total cases. Except for Case #3 which only had a few R-S cells upon deeper sectioning, abundant R-S cells were represented in the cores **(Figure 3).**

Immunohistochemistry

TMA blocks were sectioned at a thickness of 3-4 μm. Sections were dried for 16 hours at 48C before being dewaxed in xylene and rehydrated through a graded ethanol series and washed with phosphate-buffered saline. Antigen retrieval was performed by heat treatment in a pressure cooker for 5 min at 119C and 15psi in 10 mM citrate buffer (pH 6.5). Endogenous peroxidase was blocked before staining the sections. Immunohistochemical staining was performed with antibodies directed towards phosphorylated AKT (Ser 473)NF-κB p65 (Ser 536), mTOR (Ser 2448), p70 S6 Kinase (Thr 389), STAT3 (Tyr 705), p38 MAP Kinase (Thr 180/Tyr182), ERK ½ (Thr202/Tyr204); and C-terminus of PKC-α (**Table 1**) by overnight incubation in the primary antibody. Immunodetection was performed with the VectaStain ABC-Peroxidase kit (Vector Laboratories, Cat no. PK-6101) detection system for p-NF-κB p65, p-mTOR, p-p38 MAP Kinase and p-ERK; and the PowerVision+ Poly-HRP IHC detection system (Leica Biosystems Newcastle Ltd., Cat no. PV-6107) employing diaminobenzidine chromogen as the substrate. Sections were counterstained

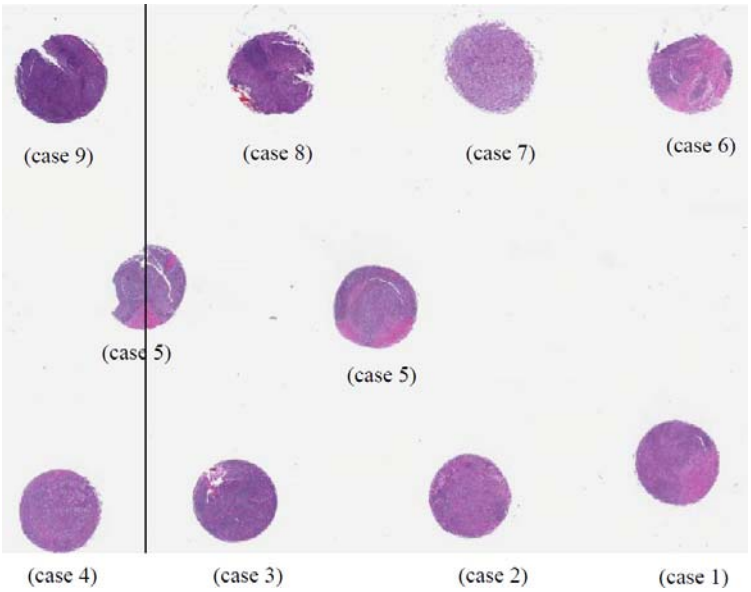

Figure 2. Outlay of the tissue microarray constructed with 9 (2mm) cylindrical cores from representative paraffin-embedded tissue blocks. *H&E at original magnification.*

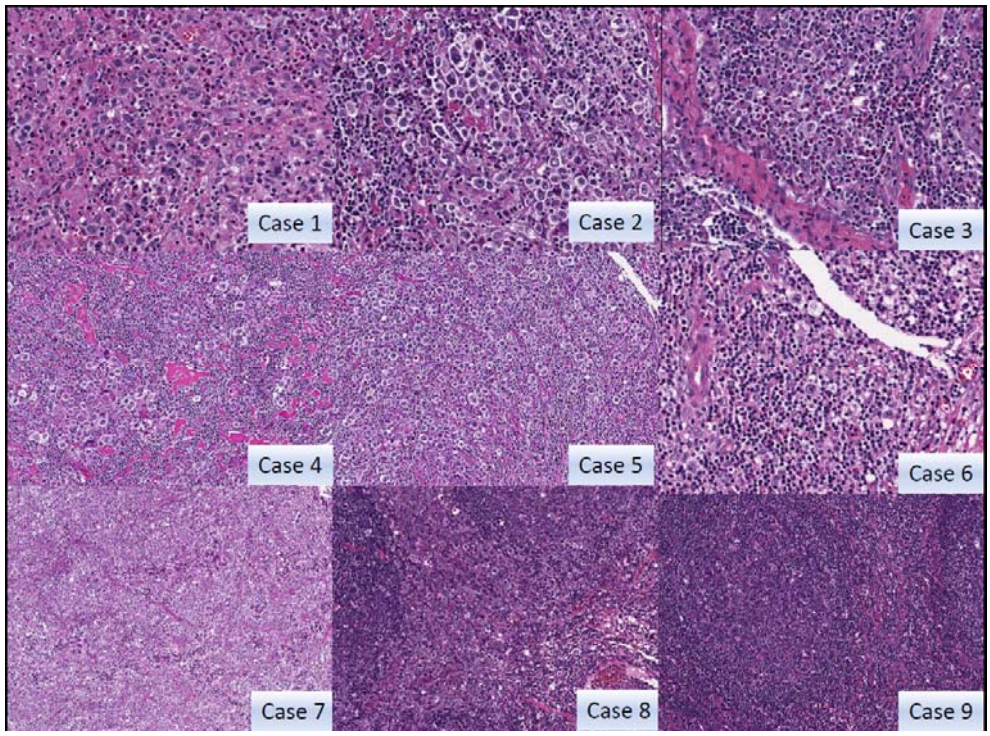

Figure 3. Histologic representation of the 9 cases included in this study. 1-6) nodular sclerosing classical Hodgkin lymphoma with predominantly syncitial areas, 7) diffuse large B-cell lymphoma with CD5-positivity, 8) diffuse large B-cell lymphoma with Bcl-6 positivity and concomitant Grade 1-2 follicular lymphoma, 9) reactive lymph node with follicular hyperplasia. *H&E x 40X.*

with hematoxylin. The staining of the TMA sections was independently evaluated by two pathologists (J.D. and R.E.B.). The intensity of staining was recorded as positive (+ to ++++) or

Table 1. List of antigenic epitopes and sources of antibodies used for morphoproteomic analysis of the TMA

Protein	Species	P-site/Epitope	Dilution	Source
p-AKT	Rabbit	Ser 473	1:50	Cell Signaling
p-44/42 MAPK (ERK1/2)	Rabbit	Thr202/Tyr204	1:100	Cell Signaling
p-38 MAP kinase	Rabbit	Thr180/Tyr182	1:500	Cell Signaling
p-p70 S6 kinase	Mouse	Thr 389	1:500	Cell Signaling
p-NF Kappa B p65	Rabbit	Ser536	1:500	Signalway Antibody
p-STAT3	Mouse	Tyr 705	1:50	Santa Cruz Biotechnology
p- mTOR	Rabbit	Ser2448	1:200	Cell Signaling
PKC-α	Mouse	C terminus	1:50	Santa Cruz Biotechnology
PC PLD1	Mouse	aa 1-160	1:100	Santa Cruz Biotechnology
Fox-P3	Mouse	fusion protein	1:100	abcam

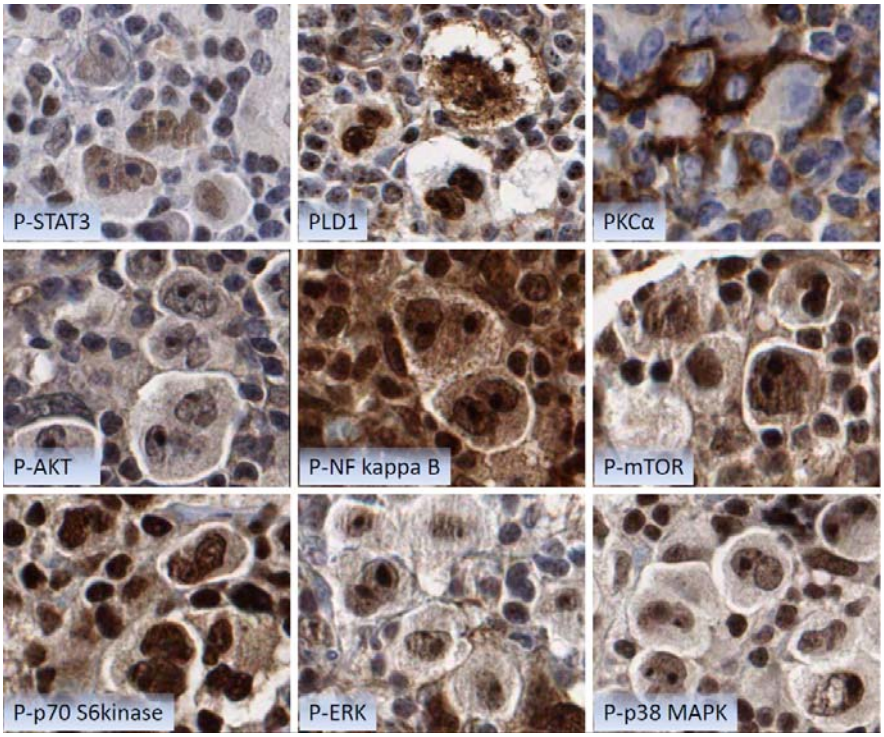

Figure 4. Tissue expression profile of a representative case of classical Hodgkin lymphoma is shown here. The choice of markers was based on current literature of constitutively activate pathways in classical Hodgkin lymphoma (NF-κB, PI3-AKT-mTOR, MAPK-ERK, STAT-3) and PKC-α which promotes growth in hematologic malignancies. Nuclear p-STAT3, p-NF-κB, p-ERK, p-p38 MAPK is evidence of activation of these pathways in majority of cHL cases evaluated in this study. Phosphorylated ERK is differentially expressed in the neoplastic cells of classical Hodgkin lymphoma. Intense membranous PKC-α was noted in the majority of cHL cases with PLD1 co-localization. *Original magnification x 400X.*

negative (-). Only nuclear expression of the epitopes (except forPKC-α and p-m-TOR) in the neoplastic cells was considered positive indicating the activated form of the signal transduction molecules. PKC-α was considered positive if detected in the plasmalemmal aspect. P-mTOR was considered to represent mTORC2 complex, if nuclear.

Table 2. Intensity scoring of nuclear and membranous expression of phosphospecific proteins and PKC-α, respectively, and enumeration of the Foxp3+ Tregs infiltrate per 40x high power field

Age, Case	48y M, #1	25y F, #2	31y M, #3	34y M, #4	49y F, #5	16y M, #6	68y M, #7	47y F, #8	61y M, #9
Location	Scalene LAD	Recur-rence in the lung	Bilateral cervical LAD	Medias-tinal mass	Medias-tinal & cervical LAD	Medias-tinal & cervical LAD	R cervical lymph node	Anterior vaginal wall mass	L axillary lymph node
Diagnosis	cHL-NS	cHL-NS	cHL-NS	cHL-NS	cHL-NS	cHL-NS	Bcl6+DLBCL & Gr 1-2 FL	CD5+ DLBCL	FH (GC/MZ/IF)¶
p-AKT	+++	+++	+++	++	++	++	-	-	-/-/-
p-mTOR	++++	+	++++	+++	++	+	+++	++	++/++/++
p-p70-S6Kinase	+++	+++	+++	+++	+++	++	+++	++	++/++/++
p-NF KB	++++	+	++	+++	++	+++	++	++++	+/+/+
p-ERK	++++	++	+	++	++	++	+	-/+	-/-/+ (IDC)
p-p38 MAPK	++++	++	+	+++	++	++	++	+	-/-/++
p-STAT-3	+	+	++++	+++	++	-/+	+++	-	-/-/var
Foxp3 (# cells/hpf)	6	50	75	120	70	110	4 (FL) - 40 (DLBCL) var** (- to +++)	0.5	0.5/2/30
PKC-alpha	+++	+++	+	++++	-	+		-	-/-/++

¶Germinal center (GC), mantle and marginal zones (MZ), and interfollicular (IF) compartments in a reactive lymph node with follicular hyperplasia (FH)

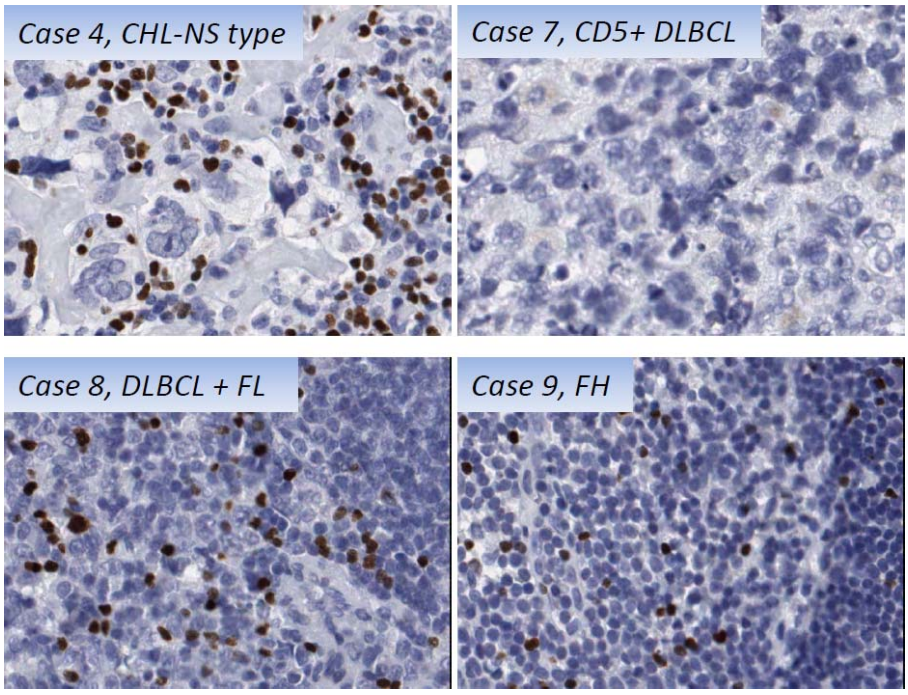

Figure 5. Infiltration of T regulatory cells within the tumor microenvironment is shown here by immunostaining for Foxp3 transcription factor. Enumeration of the number of Foxp3+ cells demonstrates variability amongst the classical Hodgkin lymphoma cases (see Table 2), and distinct difference amongst the two cases of DLBCL with the GC-derived lymphoma (#8) surrounded by greater number of T regulatory cells than the ABC-derived lymphoma (#7) and the reactive lymph node (#9). *Original magnification x 200X.*

Results and discussion

Signaling pathways that are crucial for growth and survival of cHL-NS, and are putative targets for molecular targeted therapy were evaluated by morphoproteomics on a TMA comprised of a series of 6 cHL-NS cases, 2 DLBCL cases, and one reactive lymph node. This analysis was undertaken to gain insight in potential novel therapeutic approaches for cHL-NS. The complete morphoproteomic profile of a prototype case of cHL (#5) is shown in **Figure 4**. Scoring based on intensity of antibody uptake is summarized in **Table 2**.

When expression of analytes was compared amongst the 6 cHL-NS cases, remarkable similarity was noted with universal expression of phosphorylated AKT, mTOR, p70 S6 kinase, and NF kappa B. However, p-STAT3, p-ERK, and PKC -α were differentially expressed. Nuclear p-STAT3 was detected in the vast majority of the R -S cells in all but one case (#6). Its expression was largely absent in the neoplastic follicles of #8 and reactive germinal centers of #9. While nuclear p-ERK was expressed in all 6 cHL-NS cases, variable staining of p–ERK was seen in the R-S cells within each case. Expression of PKC-α was highly variable from intense cytoplasmic and plasmalemmal reactivity in #4 to undetectable in #5. The infiltration of Foxp3+ cells was also highly variable in cHL-NS cases ranging from 6/hpf to 120/hpf (**Figure 5**). The number of Foxp3+ cells correlated with nuclear p-STAT3 expression in the R-S cells.

The two cases of DLBCL demonstrated remarkable differences in their morphoproteomic profile and also in comparison with cHL (**Figure 6**). Due to CD5-positivity and lack of Bcl-6 in case 7, we considered this case to be most likely a ABC-derived DLBCL. Based on Bcl-6-positivity and concurrent grade 1 follicular lymphoma, we considered case 8 most likely a GC-derived DLBCL. More intense nuclear p-NF-κB was noted in #7 than in #8. Weak expression of p-ERK and p-p38 MAPK was noted in #8, but both were dim to absent in #7. Membranous localization of PKC- α in a subset of cells with dim co-localization of PLD1 was found in #8, but not in #7. On the contrary, nuclear p-STAT3 expression was bright in #8 and virtually absent in #7. Finally, the Tregs enumerated based on Foxp3 expression were significantly fewer in the tumor microenvironment of #7 than in #8. These re-

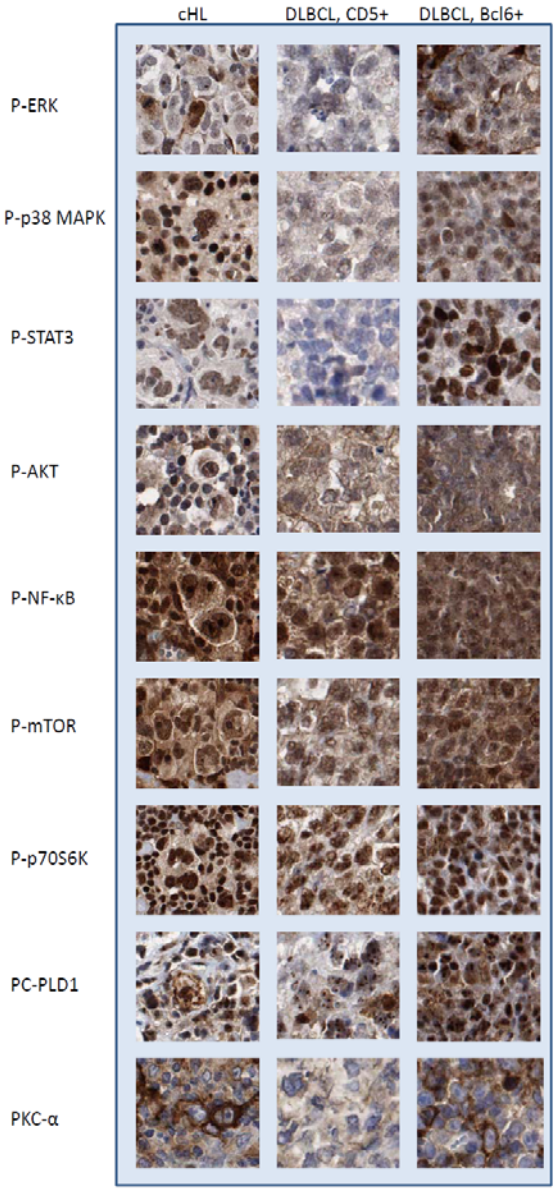

Figure 6. Tissue expression profile of proteins within key survival pathways is shown with comparison amongst a representative cHL case and the two cases of DLBCL. While p-NF-κB, p-mTOR, and p-p70 S6K were nuclear in all three examples, nuclear p-AKT was lacking in the two cases of DLBCL as opposed to cHL which showed nuclear localization of p-AKT (suggesting mTORC1 complex is operative in DLBCL, and mTORC2 complex in cHL). PKC-α was intensely reactive in cHL and Bcl6+ DLBCL, but not in CD5+ DLBCL. The Bcl-6+ DLBCL (#8) differed from the CD5+ DLBCL (#7) included in the study in its higher p-p38 MAPK, p-ERK, p-STAT3 and PKC-α reactivity; and lower p-NF- κB expression. *Original magnification x 200X.*

sults are consistent with the favorable prognosis associated with higher Tregs in the microenvironment of GC-derived DLBCL [14]. Also, the lower Foxp3+ Tregs in the ABC-derived lymphoma correlates with lower p-STAT3 expression.

The PI3-AKT-mTOR pathway in cHL-NS

The PI3-AKT-mTOR pathway was analyzed by probing for p-AKT (Ser 473), p-mTOR (Ser 2448), and p-p70 S6K (Thr 389), a marker for effective receptor tyrosine kinase signaling through the PI3-AKT-mTOR pathway. In all cHL-NS cases, p-AKT was localized in the nucleus and Golgi area of the R-S cells. Phosphorylated mTOR and p70 S6Kinase were nuclear as well in all 6 cases. Both granulocytes and a subset of lymphocytes within the reactive infiltrate of cHL-NS lacked nuclear p-AKT. The two cases of DLBCL lacked nuclear localization of AKT in the lymphoma cells, but showed dim cytoplasmic reactivity. Similarly, only cytoplasmic reactivity was detected in the lymphocytes of the reactive lymph node. The expession of p-mTOR and p-p70 S6K was nuclear in cHL-NS as well as in DLBCL. Besides the PI3-AKT pathway, the ERK pathway can contribute to the phosphorylation of p70- S6K, and the activation of p70-S6K independent of AKT.

Because of the requirement of the mTORC2 complex for phosphorylation of AKT at S473, our IHC data showing nuclear p-AKT suggests that mTORC2 is operative in cHL-NS. A higher concentration of rapamycin would be necessary to suppress AKT phosphorylation. This could be attributed to the higher affinity of mTORC2 than mTORC1 for PA and thus lower susceptibility to dissociate with rapamycin treatment [15]. It is important to note that Th2 cells downregulate the AKT-mTOR pathway as part of the TGF-β-induced differentiation towards Foxp3+ Tregs [16]. Thus, A potential effect of mTORC1 inhibition by rapamycin is inhibition of the PI3-AKT-mTOR pathway and thus, expansion of Tregs which normally downregulate AKT during Foxp3 expression. Rapamycin, targeting the Akt-mTOR signaling, induces differentiation of Tregs, and Foxp3 expression is enhanced by HDAC inhibitors [17]. Thus the use of an mTOR inhibitor would further suppress the anti-tumor immunity in cHL by enhancing the Tregs.

Because of the immunosuppressive role of Tregs in cHL, a therapeutic strategy such as rapamycin or perhaps also HDAC inhibitors (as epigenetic modification of the Foxp3 promoter is considered to be an important mechanism by which the PI3K-AKT-mTOR signaling antagonizes Fox-P3 expression) is likely to be of less significance. Instead, an approach to inhibit the Tregs would have the desirable effect of enhancing the anti-tumor immunity. Selective inhibition of Foxp3 in Tregs can be achieved by low dose cyclophosphamide and could be considered a reasonable therapeutic adjunct in cHL.

The NF-Kappa B pathway

The NF-κB pathway was active in all 6 cases of cHL-NS as demonstrated by intense nuclear reactivity for phosphorylated NF-κB . This pathway was also active in the two cases of DLBCL, however a higher intensity of expression was noted in the CD5+ DLBCL than in the Bcl-6+ DLBCL representing FL transformation. These data are consistent with the role of Bcl-6 as an NF-κB regulator [18]. The ubiquitous role of the NF-κB pathway is suggested by the expression of NF-κB in the reactive lymph node.

The NF-κB pathway is important in proliferation and apoptosis resistance in cHL as demonstrated by identification of numerous genomic defects of target genes affecting key regulators of this pathway [19-21]. Its constitutively active state in cHL is attributed, in part, to the persistently active IκK complex [9]. Constitutively active NF-κB upregulates STAT5a and synergistically regulates cell cycle progression by activating D cyclins, and inhibiting apoptosis by stimulating *Bcl-xL* expression [3, 22-24]. The target genes of NF-κB contributing to the tumor microenvironment include CCR7, a chemokine receptor implicated in the characteristic homing of cHL cells to lymphoid compartments; ICAM-1 and CD86, both of which influence preferential influx of Th2-type T cells and suppression of Th1 -type immune response in cHL [25]; and TNF-α which induces eotaxin in fibroblasts facilitating recruitment of T cells and eosinophils [26, 27]. Additionally, IL-6 and GM-CSF stimulate plasma cells, Th2 cells, and eosinophils within the reactive cellular milieu of Hodgkin lymphoma [28]. Inhibiting the NF-κB pathway induces apoptosis in cHL cell lines [10].

In vitro studies show that inhibition of this pathway downregulates the anti-apoptotic molecule,

c-FLIP, thus inducing apoptosis [29]. It has demonstrated therapeutic benefit in combination with IGEV chemotherapy in a single case report; however its efficacy is limited in combination with either dexamethasone or gemcitabine [30-32]. Thus, its relevance as a therapeutic adjunct in cHL in a molecular targeted approach to therapy remains to be proven in larger studies.

The MAPK pathway

Variable upregulation of the MAPK pathway was seen in all cases of cHL with equivalence in p-ERK and p-p38 MAPK results. The percentage of R-S cells that expressed nuclear p-ERK in the cHL-NS cases was highly variable. In one cHL-NS case (#4), intense nuclear expression, significantly greater than in R-S cells, was seen in the endothelial and the interdigitating dendritic cells (IDC). The intensity of p38 MAPK positivity correlated with that of p-ERK.

The Ras-Raf-MAPK-ERK signaling pathway is implicated in the pathogenesis of cHL. Overlapping functions of the TNF family of receptors, CD30, CD40, and receptor activator of nuclear factor –KB (RANK) expressed on cHL cells, include activation of both NF-κB and ERK signaling pathways. CD30 triggers constitutive activation of the MAPK pathway by ligand-independent receptor aggregation and is considered one of the common B7-H1/CD274 regulatory pathways in cHL [33], being crucial in the expression of B7-H1 whose expression facilitates immune escape by inhibiting the activity of anti-tumor T cells. In addition, the ERK pathway mediates resistance to APO2L/TRAIL-induced apoptotic signaling. Besides its role in TNF-α-mediated survival and proliferation in malignant lymphomas, p38 MAPK facilitates the transcriptional activation function of NF-κB [34]. Furthermore, the induced Jun B activates the CD30 promoter [35]. Inhibition of the phosphorylated state of ERK has preferential anti-proliferative and pro-apoptotic activity in cHL cell lines over normal lymphocytes [6].

Inhibition of both NF-κB and MAPK-ERK pathways of convergence potentiates the activity of chemotherapy and induces apoptosis in cell lines [10]. Thus, a treatment strategy designed to inhibit both pathways in cHL is desirable [6, 36-39]. Sorafenib, designed to target *Raf* kinase, also has activity against VEGF-R2, expressed in R-S cells [40].

The PKC-α pathway

The PKC-α pathway is activated by a number of growth factors including platelet-derived growth factor (PDGF), vascular endothelial growth factor, epidermal growth factor, and fibroblast growth factor. Its downregulation leads to perforin dependent apoptosis [11, 41] . Membranous localization of PKC-α was detected in 5/6 cHL-NS cases, but the intensity of staining was variable, further supporting the concept that of heterogeneity in the protein expression profile exhibited by morphologically similar cases of cHL-NS. Signaling through the PDGFR-α receptor in cHL-NS has previously been reported [12]. In contrast to malignant lymphomas, cells within the reactive germinal centers were largely negative for PKC- α. Many lymphocytes within the interfollicular area were positive; however, only rare cells within the mantle and marginal zones were positive with plasmalemmal expression (fewer than 1%). Unlike in cHL-NS, co-localization of PLD1 was not seen in lymphocytes of the reactive lymph node. The relevance of this pathway as a target for therapy remains to be explored further in cHL-NS.

The JAK-STAT pathway

The STAT family of proteins has an important role in tumor progression and immune evasion. Nuclear accumulation of p- STAT3 was visualized in 5/6 cases of cHL-NS included in this study. Expression of p-STAT3 was detected in a subset of lymphocytes within the reactive infiltrate.

Constitutive STAT activation is linked to the IL-6 receptor complex expressed by R-S cells [5, 42-44]. Binding of IL-6 to its receptor activates JAK which in turn phosphorylates STAT3 leading to its dimerization, nuclear translocation, and activation of target genes. Furthermore, the synergy of NF-KB and STAT3 pathways is demonstrated by IL-6 (target gene of NF-κB) mediated interaction of STAT3 (unphosphorylated) with the p65 subunit of NF-κB, thus inhibiting the binding of IκB and freeing the NF-KB molecule to translocate to the nucleus [45]. Besides IL-6 family of cytokines, STAT3 is activated by several other extra- and intracellular stimuli including IL-2, IL-10, and other γ chain cytokines such as IL-7, IL-9 and IL-15 for paracrine and autocrine effects. JAK-independent constitutive activation of STAT3 is attributed to dysregulated upstream

signaling pathways including the *Src* family of kinases (SFKs) [46, 47]. STAT3 pathway is also activated by PDGF, whose signal transducer PDGFRA is expressed in cHL cells [12, 48]. Besides its proliferative effects, STAT3 promotes tumor angiogenesis through VEGF induction, acting downstream of VEGFR2 [49, 50], which is expressed by cHL cells [40].

STAT3 activation induces a pro-carcinogenic cytokine, IL-23, by direct transcriptional activation. IL-23 in turn activates STAT3 transcription factor in Tregs expressing IL-23R; consequently, resulting in upregulation of Foxp3 and the immunosuppressive cytokine, IL-10. Thus, Stat3 promotes IL-23-mediated Treg differentiation and inhibits antitumor immunity [51].

Thus SFKs are putative therapeutic targets due to their role in activating STAT3 and inducing growth promoting and immune escape effects in cHL. There is remarkable variability in nuclear p-STAT3 intensity in our series of cHL-NS. Due to the variability in tissue expression levels, it is possible that the response to an inhibitor of the STAT3 pathway is variable.

Role of Foxp3+ T regulatory cells in cHL-NS

The tumor microenvironment in cHL is comprised of a variety of cells with dysfunctional effector and adaptive immunity. The lymphoma cells evade the host immune response, in part by secretion of chemokines such as thymus and activation related chemokine (TARC/CCL17) and macrophage-derived chemokine (MDC/CCL22) which attract chemokine receptor 4 (CCR4)-expressing Tregs. Inhibition of CCR4 has the ability to overcome the immunosuppressive effect of Tregs [52]. Another mode of immune suppression is through aberrant expression of IL-21, a Th2 cytokine which stimulates production of macrophage-inflammatory protein (MIP)-3α, a potent chemoattractant of Tregs by binding to the CCR6 receptor [53]. Tregs secrete IL-10 and TGF-β, factors which inhibit anti-tumor immunity.

The number of Tregs varied widely amongst the cHL-NS cases in our study. A number of studies have attributed prognostic relevance to enumeration of Tregs. One study of 87 cases of cHL showed a high ratio of Foxp3+ Tregs to overall Th2 lymphocytes associated with shortened disease free survival in cHL [54]. While a low Foxp3+ response compared to an overall Th2 response is favorable; relative to the granzyme B+ cytototoxic T-cell (CTL) response, a low Foxp3+ Treg response is unfavorable [55]. Thus, an exaggerated CTL response is associated with a worse outcome despite a low Treg infiltrate. In part, this is due to the anti-granzyme B serine protease inhibitor, PI9, produced by the R-S cells, thus evading granzyme B -induced apoptosis [56]. It is suggested that a similar mechanism could modulate chemoresistance. Nevertheless, the prognostic impact of the number of Foxp3+ Tregs in the tumor microenvironment is not entirely clear as one study incorporating 156 cases of cHL-NS showed greater numbers of Foxp3+ Tregs associated with a better failure-free and overall survival outcome [14].

Because of the role of Foxp3+ Tregs in immune suppression, a therapeutic intervention inhibiting these cells holds potential. Downregulation of Foxp3 transcription can be achieved with low dose cyclophosphamide, thus abrogating the suppressive effects of Tregs, weakening the regulatory mechanisms and strengthening the innate immune response [57]. The use of cyclophosphamide prior to the current standard regimen in cHL had marked subjective and objective response [58]. In renal cell carcinoma, sunitinib, an SFK inhibitor, has been shown to reduce the number of Treg cells with concurrent reduction in myeloid derived suppressor cells, thus modulating anti-tumor immunity [59]. The role of sunitinib in STAT3 inhibition as well as downregulation of Foxp3 supports its use in cHL therapy.

Signaling pathways in DLBCL

Marked differences were noted in the phosphoproteome of the two cases of DLBCL recapitulating ABC-type (Case 7, CD5-positivity correlates with ABC-type of origin) and GC-type (Case 8, based on concomitant low grade follicular lymphoma and Bcl-6 positivity in the large cells) cell of origin. Our observation of the active p38 MAPK and ERK pathways in case 8 of transformed DLBCL is congruent with prior studies showing constitutively increased expression of p38 MAPK with transcriptional deregulation of RAS-pathway related genes in FL transformation [60]. While its expression was also negative in the neoplastic germinal centers of case #8, it was positive in the DLBCL component of #8,

and in the lymphoma cells of #7 (CD5+ DLBCL), suggesting the role of the MAPK pathway in transformation. The STAT-3 and NF-κB pathways work synergistically in ABC-type DLBCL with the former activated as a result of IL-6 growth factor (one of the NF-κB target genes) stimulation. Furthermore, the NF-κB pathway plays a pivotal role in the survival of DLBCL cell lines of the ABC-type, but not the GC-type with preferential inhibition of ABC-type DLBCL cell lines by IκB kinase small molecule inhibitor [61, 62]. In our study, the CD5+ DLBCL (#7) showed high NF-κB activity, but no STAT-3 activity; whereas Bcl6+ DLBCL (# 8) showed activation of both NF-κB and STAT3, although NF-κB nuclear intensity much lower compared to #7. These data are consistent with the fact that CD5+ DLBCL tend to recapitulate ABC-type DLBCL which highly relies on the NF-κB pathway for its survival [63]. However, we do not see concurrent upregulation of p-STAT3. Furthermore, the pattern of p-STAT3 expression in the Bcl6+ DLBCL is insightful. It is virtually absent in the neoplastic follicles of FL (as also in the follicles of reactive FH), but variably expressed in the large cell component within the interfollicular area, suggesting the possible role of STAT3 pathway in transformation. The data is consistent with the fact that dasatinib, a SFK inhibitor, has shown efficacy in inducing growth arrest [64]. Thus, as in cHL, an SFK inhibitor is a potential new therapy in DLBCL. Future studies correlating the response to an SFK inhibitor and p-STAT3 activity would provide further insight on the potential of blocking lymphomagenesis by targeting this pathway.

Concluding remarks

Multiple signal transduction cascades are often coupled to protein tyrosine kinase receptors with a high level of cross-talks between distinct pathways. Thus, alternate compensatory mechanisms are often created to overcome therapeutic activity of targeted agents. In order to implement enzyme-targeted therapy, knowledge of the activation status of these survival pathways is likely to be important in establishing response to therapy in each case. Future research correlating the levels of activity with treatment response will be helpful in risk stratification and prediction of therapeutic outcome. Furthermore, due to the key role of immune suppression in classical Hodgkin lymphoma, a therapeutic adjunct targeting the immune suppressive mechanisms may be beneficial.

Acknowledgements

We appreciate the technical support of Ron Craig at University of Michigan Health System for digitizing the TMA data slides. This work was funded by the endowment monies provided by the Harvey S. Rosenberg Endowed Chair in Pathology and Laboratory Medicine dedicated to the morphoproteomic initiative.

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