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Original Article

Morphoproteomics identifies constitutive activation of the mTORC2/Akt and NF-κB pathways and expressions of IGF-1R, Sirt1, COX-2, and FASN in peripheral T-cell lymphomas: pathogenetic implications and therapeutic options

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Abstract: Background: Gaining a better understanding of the molecular circuitries and pathways implicated in the malignant growth and biological behavior of T cell lymphomas may identify potential cellular targets with clinical therapeutic potential. The immunohistochemical characterization of key cellular proteins participating in these pathways can provide surrogate markers of biological activity. The mammalian target of rapamycin complex (mTORC) signaling pathway has been implicated in T-cell lymphopoiesis. The mTORC2 pathway involves downstream activation of nuclear factor (NF)-κB and p-Akt (Ser 473). Fatty acid synthase (FASN) and insulin-like growth factor-1 receptor (IGF-1R) are expressed upstream of the mTORC and NF-κB signaling pathways. Cyclooxygenase (COX)-2 products influence these pathways. Our goal was to use morphoproteomics to characterize the expression patterns of the proteins in various peripheral T-cell lymphomas. Design: Ten cases of peripheral T-cell lymphoma (PTCL) were examined for expression of proteins along the mTORC, Akt and NF-κB pathways and affiliated tumorigenic molecules. These included two angioimmunoblastic PTCL, one natural killer/PTCL, one anaplastic large PTCL, and six PTCL not otherwise specified. Immunostaining for phosphorylated (p) mTOR (Ser 2448), p-Akt (Ser 473), p-NF-κBp65 (Ser 536), IGF-1R (Tyr1165/1166), silent mating type information regulation 2 homolog 1 (Sirt1), COX-2 and FASN was performed on paraffin-embedded tissue for each case. Percent expression was scored using bright-field microscopy with high expression designated as more than 50% of the cells with positive stain in the appropriate subcellular compartment. Results: All ten cases demonstrated nuclear staining for p-mTOR (Ser 2448) corresponding to mTORC 2, and all cases showed strong, diffuse nuclear staining for p-NF-κBp65 (Ser 536). All ten also showed nuclear and cytoplasmic staining for p-Akt (Ser 473) and cytoplasmic staining for IGF-1R. High expressions for nuclear Sirt1, and cytoplasmic COX-2 and FASN were detected in 7, 9, and 8 out of 10 cases, respectively. Six out of 10 cases demonstrated high expression of all the mentioned markers. Conclusion: The constitutive activation of mTORC2, NF-κB, p-Akt and the concomitant expression of IGF-1R suggests convergence of these molecular pathways in T-cell lymphoma. The results of this study also suggest that mTORC2 may be a common denominator among this heterogeneous group of lymphomas. Interference of key nodes of this pathway may carry a clinical therapeutic benefit. Agents that may be considered based on existing data include: bortezomib to inhibit NF-κB pathway activation; metformin to inhibit both NF-κB and mTORC2 and histone deacteylase inhibitors to inhibit mTORC2 pathway signaling. Furthermore, panobinostat inhibits Sirt1 pathway when present, and celecoxib inhibits NF-κB pathway activation independent of COX2.

Keywords: Hematopathology, morphoproteomics, T-cell lymphoma, pathways, expression

Introduction

Increasing knowledge of the molecular biology of T cell lymphomas has begun to identify relevant molecular circuitries and pathways implicated in malignant growth and biological behavior of this heterogeneous group of malignant lymphomas [1, 2]. These findings may carry prognostic implications and also assist in identifying potential targets subject to modulation

Table 1. Demographics of ten patients with T-cell lymphoma included in the study

Case	Patient	Source	Diagnosis
1	41 F	Bone marrow	Peripheral T-cell lymphoma, NOS
2	44 M	Small bowel ulcer	Peripheral T-cell lymphoma, NOS
3	38 M	Spleen	Peripheral T-cell lymphoma, NOS
4	85 F	Tonsil	Peripheral T-cell lymphoma, NOS
5	13 F	Mediastinal mass	Peripheral T-cell lymphoma, NOS
6	80 M	Left forehead tumor	Peripheral T-cell lymphoma, NOS
7	72 F	Right axillary lymph node	Angioimmunoblastic TCL
8	63 F	Left neck lymph node	Angioimmunoblastic TCL
9	68 F	Bone marrow	NK/TCL
10	43 M	Mesenteric lymph node	Anaplastic large TCL

with therapeutic intent [3]. The immunohistochemical characterization of key cellular proteins participating in these pathways provides surrogate markers of biological activity and exposes cellular targets with clinical therapeutic potential [4].

Peripheral T-cell lymphomas (PTCL) encompass a heterogeneous group of malignancies that generally portend a poor prognosis. Treatment advances trail behind the increasing number of effective alternatives in B-cell lymphomas [5]. Despite responses to standard or dose-intense regimens the overall survival of patients with T-cell lymphomas remains dismal. For the majority, the standard chemotherapy approach provides short-lived benefit, if any at all [6, 7]. There is ongoing intense research in search of novel therapies in PTCL to include histone deacetylase inhibitors, immunomodulatory agents, proapoptotic small molecules, newer antifolates, proteasome inhibitors, monoclonal antibodies against T-cell antigens (CD30 and CD52), and immunotoxins (*i.e.*, denileukin, diftitox) [8]. In particular, histone deacetylase inhibitors such as panobinostat have proven to be an efficacious treatment for cutaneous T-cell lymphoma (CTCL), and they are routinely used in the treatment of advanced cases [9]. Their use has been well documented in cutaneous T-cell lymphoma, but to the best of our knowledge has not been explored in depth in more systemic T-cell lymphoma. In addition, silent mating type information regulation 2 homolog 1 (Sirt1), a member of the lysine deacetylase Sirtuin family, was recently shown to be strongly expressed in cutaneous T-cell lymphoma and to represent a therapeutic target [10].

Morphoproteomics utilizes bright-field microscopy and immunohistochemistry to characterize the molecular circuitry of tumors by noting the state of activation of various protein analytes [4]. It has proved useful in a diverse number of tumors, including in a patient with relapsed acute lymphoblastic leukemia [11]. The identification of key proteins in the molecular pathways participating in the genesis and growth of T cell lymphomas may uncover potential targets, amenable to therapeutic interventions [8].

Our objectives in this study were twofold: 1) to assess the state of activation of the prosurvival mammalian target of rapamycin complex (mTORC) 2/V-Akt Murine Thymoma Viral Oncogene Homolog 1 (Akt, also known as Protein Kinase B or PKB) and nuclear factor-kappa B (NF-κB) pathways, and the expression levels of potential tumorigenic molecules affiliated with these pathways in PTCL, such as insulin-like growth factor -1 receptor (IGF-1R), Sirt1, cyclooxygenase-2 (COX-2) and fatty acid synthase (FASN); and 2) to identify therapeutic agents which might be appropriate to target such prosurvival and tumorigenic factors should they be identified in PTCL. In short, this could lead to the application of targeted therapies resulting in the downregulation of constitutively activated tumorigenic pathways detected in an individual patient's PTCL, rendering the tumor cells more vulnerable to chemotherapy and allowing for a maintenance therapy to reduce the risk of recurrent disease.

Materials and methods

Approval by the Institutional Review Board (IRB) was obtained for this study. Using morphoproteomics [4], ten cases of peripheral T-cell lymphoma (PTCL) cases were examined for expression of proteins along the mTORC, Akt and NF-κB pathways and affiliated tumorigenic molecules. These cases included two angioimmunoblastic TCL, one natural killer/TCL, one anaplastic large TCL, and six TCL not otherwise specified (see **Table 1**). Immunohistochemical staining for phosphorylated (p) mTOR [Ser

Table 2. Immunohistochemical protein markers and antibody specifics

Protein Analyte	Antibody Specifics
p-mTOR (Ser 2448)	Cell Signaling Technology Inc., Danvers, MA. (49F9) monoclonal rabbit antibody
p-AKT (Ser 473)	Cell Signaling Technology Inc., (736E11) monoclonal rabbit antibody
p-NF-κBp65 (Ser 536)	GeneTex Inc. (GTX30886) polyclonal rabbit antibody
IGF-1R (Tyr1165/1166)	GenWay Biotech, Inc polyclonal rabbit antibody
Sirt1	Abcam Sirt1 monoclonal mouse antibody [1F3] ab104833
COX- 2	Leica Biosystems Novocastra™ COX-2 monoclonal mouse antibody
FASN	Cell Signaling Technology Inc., Danvers, MA Fatty Acid Synthase (C20G5) rabbit monoclonal antibody

Table 3. Number of PTCL patient cases demonstrating high expression in the appropriate staining pattern for the protein analytes tested

Protein analytes	Number of cases with High (>50%) PTCL Tumor Cell Expression of Protein Analyte	Pattern of staining
p-mTOR (Ser 2448)	10	Nuclear and Cytoplasmic
p-NF-κB p65 (Ser 536)	10	Nuclear
p-Akt (Ser 473)	10	Nuclear and Cytoplasmic
IGF-1R (Tyr1165/1166)	10	Cytoplasmic
Sirt1	7	Nuclear
COX-2	9	Cytoplasmic
FASN	8	Cytoplasmic
All 7 markers	6 out of 10 PTCL cases	

2448], p-Akt (Ser 473), p-NF-κBp65 (Ser 536), IGF-1R (Tyr1165/1166), Sirt1, COX-2 and FASN was performed on paraffin-embedded tissue for each case (**Table 2**). Percent expression was scored using bright-field microscopy with high expression designated as more than 50% of the cells with positive stain in the appropriate subcellular compartment (**Table 3**). Positive and negative controls were run concomitantly and noted to react appropriately.

Results

All ten cases demonstrated nuclear and cytoplasmic staining for p-Akt (Ser 473) and p-mTOR (Ser 2448) corresponding to mTORC 2 [12-15], and all cases showed strong, diffuse nuclear translocation for p-NF-κBp65 (Ser 536) (see **Table 3** and **Figure 1**). All ten cases also showed cytoplasmic staining for IGF-1R (Tyr1165/1166). In addition, high expression in the tumor cells (>50%) of nuclear Sirt1, and cytoplasmic COX-2 and FASN were detected in PTCL cells in 7, 9 and 8 out of 10 cases, respectively (see **Table 3** and **Figures 2** and **3**). Six out of ten cases demonstrate high expression of all these markers.

Discussion

T cell lymphomas are a challenging group of malignancies with a poor prognosis. Their pathogenesis remains a subject of incomplete understanding and ongoing research. This study attempts to provide further insight into pathogenetic mechanisms that may be driving the development and progression of peripheral T cell lymphomas. We posed the question of whether there are any molecular pathways which may be constitutively activated in PTCL, specifically, the mTORC2/Akt and NF-κB pathways and what other prosurvival pathways may be collaborating with such pathways and contributing to the pathogenesis of PTCL. Our findings in this series of PTCL patients support the constitutive activation of mTORC2/Akt and NF-κB pathways and relatively high and frequent expression of IGF-1R, Sirt1, COX-2 and FASN protein analytes which are correlative and tumorigenic in this setting. Downregulating such pathogenetic, prosurvival and tumorigenic pathways in an individual patient's PTCL could provide therapeutic benefit.

To that end, it is important to note that the mTOR complex is comprised of two distinct complexes. The first, mTORC1 complex, is linked to the regulatory associated protein of mTOR, raptor. The other complex, mTORC2, is coupled to rictor [12]. Moreover, Rosner and Hengstschläger have reported that mTORC1 is in a primarily cytoplasmic distribution, whereas mTORC2 is abundant in both the cytoplasmic and nuclear compartments [12]. Because

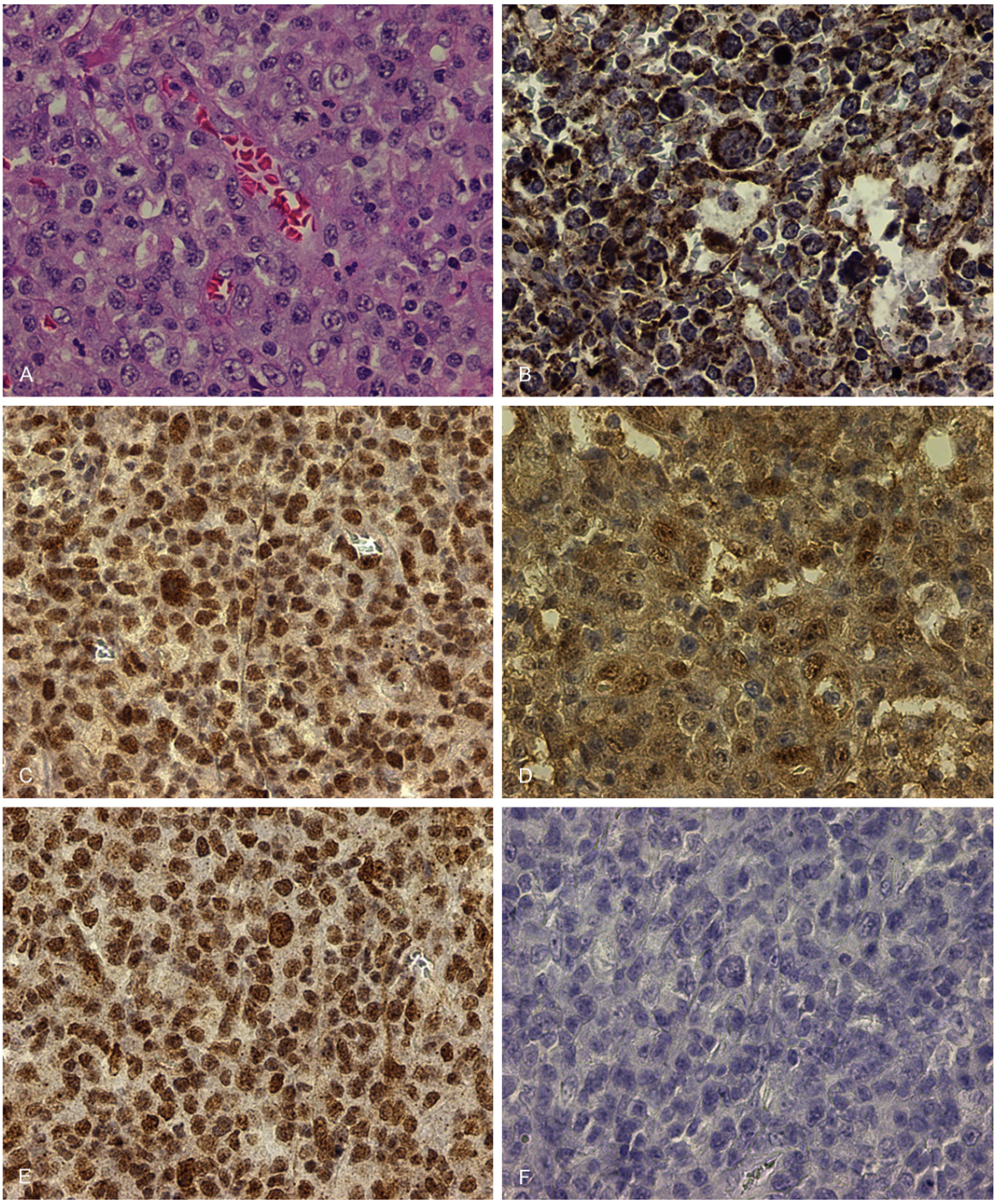

Figure 1. Microanatomic and morphoproteomic analysis of Peripheral T Cell Lymphoma (PTCL): hematoxylin-eosin (H&E, Frame A); and representative images of expression levels and signal intensity of the protein analytes to include: IGF-1R (Tyr1165/1166, Frame B); p-mTOR (Ser 2448, Frame C) and p-Akt (Ser 473, Frame D) in both nuclear and cytoplasmic compartments; and p-NF-κBp65 (Ser 536, Frame E) evidencing nuclear translocation of this analyte. Contrast with negative control (Frame F). (Original magnifications, ×400).

mTOR phosphorylated at serine 2448 binds to both raptor and rictor [13], our finding of nuclear and cytoplasmic compartmentalization of p-mTOR (Ser 2448) in all cases and in this context, supports a role for mTORC2 in PTCL. This is reinforced, in a correlative fashion, by the concurrent finding in all of our study cases of nuclear and cytoplasmic p-Akt (Ser 473), a

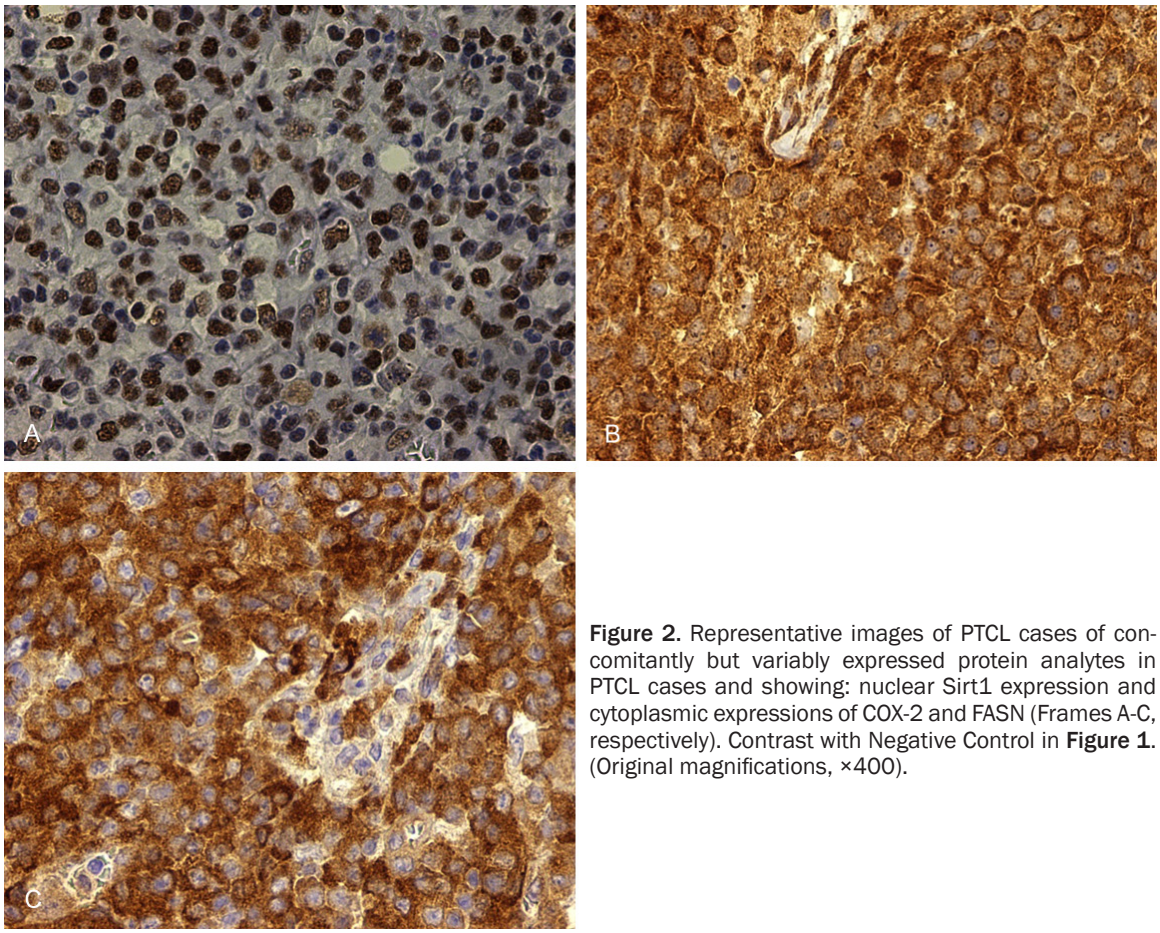

Figure 2. Representative images of PTCL cases of concomitantly but variably expressed protein analytes in PTCL cases and showing: nuclear Sirt1 expression and cytoplasmic expressions of COX-2 and FASN (Frames A-C, respectively). Contrast with Negative Control in **Figure 1**. (Original magnifications, ×400).

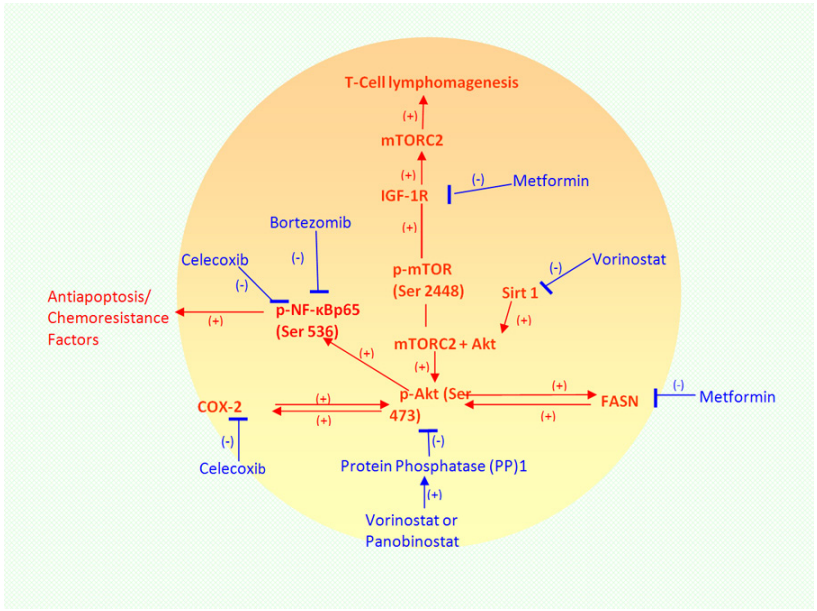

Figure 3. Schematic summary of signal transduction pathways in PTCL incorporating potentially pathogenetic and interactive prosurvival pathways responsible for tumor growth, recurrence and chemoresistance (red lettering (⟶) and (+)). Opportunities for therapeutic intervention targeting such pathways include: vorinostat or panobinostat, celecoxib, metformin and bortezomib (Blue lettering (⟝) and (-)) (See references 12-38).

putative downstream product of mTORC2 signaling [14, 15]. From a pathogenetic standpoint, it is noteworthy that the mTOR signaling pathway has been implicated in T-cell lymphopoiesis [16]. Specifically, an inactivating mutation of the *RICTOR* gene in mice reduced the cellularity of the thymus by dramatically decreasing the proliferation of immature thymocytes [16]. As previously noted, the protein product of the *RICTOR* gene is a key component of the mTOR complex 2, which implies a critical role for the latter in T-cell lymphocytogenesis [12, 16], and by extension, in PTCL lymphomagenesis. Thus, the mTO-

RC2 pathway represents a potential therapeutic target in PTCL.

Additionally, the observations of COX-2 expression in 9 out of 10 of our cases, Sirt1 in 7 out of 10 cases and FASN in 8 out of the 10 cases support a central role of the mTORC2/Akt pathway in PTCL. Data mining of the National Library of Medicine's MEDLINE Database provides for their interaction with the Akt and NF-κB pathways in that: 1) COX-2 has been reported to be an upregulator of Akt [17] and Akt signaling in turn, induces COX-2 expression [18]; 2) Sirt1, reportedly deacetylates Akt and thereby, promotes phosphatidylinositol (3,4,5)-triphosphate binding to Akt and participates in its activation [19]; 3) IGF-1R is a tyrosine kinase receptor which, once it has bound to its ligand, stimulates the PI3K-AKT/mTOR [20] and 4) Collaboratively, phospho-Akt has been reported to induce the expression of FASN and conversely, FASN has been reported to increase phospho-Akt [21]. Finally, downstream signaling from constitutively activated Akt results in the activation of the NF-κB pathway [22] and Akt upregulates a subset of NF-κB-dependent genes for T cell activation [23].

With regard to the expression of these analytes and pathways in PTCL and their clinical implications, a recent study by Cai and co-authors suggested that positive p-Akt expression has been linked to a worse prognosis and shorter overall survival of the patients. They found p-Akt to be positive in about half of their cases and those patients died nearly 40 months earlier than the half with p-Akt negative expression [24]. Similarly, Odqvist and colleagues [25] reported that the NF-κB pathway was activated in a subset of PTCLs, which were associated with poor overall survival. They also showed that the blocking of both classical and alternative NF-κB activation led to the reduced expression of several prosurvival and antiapoptotic proteins. COX-2 expression has been shown to predict an aggressive histology in non-Hodgkin lymphomas including PTCL [26].

In summary, using morphoproteomic analysis we have identified potentially tumorigenic and prosurvival mTORC2/Akt and NF-κB pathways and a likely collaborative influence of the co-expressed Sirt1, COX-2 and FASN protein analytes in the majority of PTCL. These have pathogenetic and prognostic implications and represent targetable therapeutic options (see **Figure 3**; *vide supra*). The therapeutic possibilities include the use of vorinostat or panobinostat, celecoxib, metformin and bortezomib. Vorinostat has application in this context by virtue of: 1) Its ability to dephosphorylate Akt on serine 473 by increasing the activity of protein phosphatase 1 (PP1) [27, 28]; and 2) Its ability to downregulate *SIRT1*mRNA [29] and to reduce Sirt1 deacetylase activity [30]. Panobinostat acts by a similar mechanism with protein phosphatase 1 involvement in the dephosphorylation of Akt [31]. Parenthetically, panobinostat and other histone deacetylase inhibitors have been proven efficacious and are routinely used in the treatment of advanced cutaneous T-cell lymphoma (CTCL) [9]. Celecoxib, a selective COX-2 inhibitor, in preclinical studies has been shown to decrease p-Akt protein expression in head and neck squamous cell carcinoma [32] and to inhibit the NF-κB pathway leading to apoptosis in human glioblastoma cells [33]. Metformin, also in a preclinical study has been shown to decrease the stimulative effect of a high-energy diet on colon carcinoma growth in vivo associated with the attenuation of the phosphorylation of Akt and decreased expression of FASN [34]. Bortezomib inhibits the NF-κB pathway via inhibition of proteasome degradation [35]. In addition, bortezomib, in preclinical studies has been shown to induce apoptosis and growth suppression in human medulloblastoma cells in association with a suppression of NF-κB signaling and reduction in phosphorylation of Akt [36]. In prostate cancer cells it also caused dephosphorylation of phospho-Akt [37]. Bortezomib should work collaboratively with celecoxib in inhibiting the nuclear translocation of p-NF-κBp65 (Ser 536) [38]. A Phase II study using CHOP-bortezomib (CHOP-B) in T-cell lymphomas showed up to 87% response rate and 73% complete remissions [39]. Despite their favorable clinical results, they were unable to show NF-κB pathway activation due to the use of a non-phosphorylated NF-κB antibody. However, a significant number of patients experienced cell mediated opportunistic infections commonly associated with inhibition of the NF-κB pathway. These proposed targeted agents are schematically depicted in **Figure 3**.

Finally, we have previously reported on a patient with AITL who showed a remarkable durable response using a combination of conventional

CHOP and bortezomib. We utilized an abridged panel of immunohistochemical markers composed of p-mTOR (Ser 2448), p-Akt (Ser 473) p-NF-κBp65 (Ser 536), COX2, Bcl-2, FASN, and Sirt1. All seven markers were highly expressed, indicating expressing constitutive activation of the mTORC2/NF-κB pathway in this patient who responded remarkably well to an NF-κB antagonist [40]. The data from Kim and colleagues [39] and our results offer promise for future applications and therapeutic strategies utilizing the methodology presented here.

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Disclosure of conflict of interest

None.

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