REGULAR ARTICLE

blood advances

Proteomic profiling identifies outcome-predictive markers in patients with peripheral T-cell lymphoma, not otherwise specified

Maja Ludvigsen,[1,2,*] Martin Bjerregård Pedersen,[2,*] Kristina Lystlund Lauridsen,[3] Tim Svenstrup Poulsen,[4] Stephen Jacques Hamilton-Dutoit,[3] Søren Besenbacher,[5] Knud Bendix,[3] Michael Boe Møller,[6] Peter Nørgaard,[4] Francesco d'Amore,[2,†] and Bent Honoré[7,†]

[1]Department of Clinical Medicine, Aarhus University, Aarhus, Denmark; [2]Department of Hematology and [3]Institute of Pathology, Aarhus University Hospital, Aarhus, Denmark; [4]Department of Pathology, Herlev Hospital, Herlev, Denmark; [5]Bioinformatics Research Center, Aarhus University, Aarhus, Denmark; [6]Department of Pathology, Odense University Hospital, Odense, Denmark; and [7]Department of Biomedicine, Aarhus University, Aarhus, Denmark

Key Points

- Proteomics identified differentially expressed proteins in PTCL-NOS, which reflect biological diversity and disturbance of "immunological" pathways.
- High intratumoral ENO1 expression in PTCL-NOS was associated with adverse outcome.

Peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS) constitutes a heterogeneous category of lymphomas, which do not fit into any of the specifically defined T-cell lymphoma entities. Both the pathogenesis and tumor biology in PTCL-NOS are poorly understood. Protein expression in pretherapeutic PTCL-NOS tumors was analyzed by proteomics. Differentially expressed proteins were compared in 3 distinct scenarios: (A) PTCL-NOS tumor tissue (n = 18) vs benign lymphoid tissue (n = 8), (B) clusters defined by principal component analysis (PCA), and (C) tumors from patients with chemosensitive vs refractory PTCL-NOS. Selected differentially expressed proteins identified by proteomics were correlated with clinico-pathological features and outcome in a larger cohort of patients with PTCL-NOS (n = 87) by immunohistochemistry (IHC). Most proteins with altered expression were identified comparing PTCL-NOS vs benign lymphoid tissue. PCA of the protein profile defined 3 distinct clusters. All benign samples clustered together, whereas PTCL-NOS tumors separated into 2 clusters with different patient overall survival rates ($P = .001$). Differentially expressed proteins reflected large biological diversity among PTCL-NOS, particularly associated with alterations of "immunological" pathways. The 2 PTCL-NOS subclusters defined by PCA showed disturbance of "stress-related" and "protein metabolic" pathways. α-Enolase 1 (ENO1) was found differentially expressed in all 3 analyses, and high intratumoral ENO1 expression evaluated by IHC correlated with poor outcome (hazard ratio, 2.09; 95% confidence interval, 1.17-3.73; $P = .013$). High expression of triosephosphate isomerase (TPI1) also showed a tendency to correlate with poor survival ($P = .057$). In conclusion, proteomic profiling of PTCL-NOS provided evidence of markedly altered protein expression and identified ENO1 as a novel potential prognostic marker.

Introduction

Peripheral T-cell lymphoma (PTCL) constitutes a heterogeneous group of cancers derived from mature T cells and natural killer cells accounting for 12% to 15% of all lymphomas.[1] The classification of PTCL is complex, and >20 different PTCL entities are included in the latest WHO classification, defined by clinical, morphological, immunophenotypical, genetic, and molecular characteristics.[2,3] However, 20% to 40% of all PTCLs do not meet the morphological and molecular criteria for any of the specifically defined entities and are, therefore, categorized as PTCL, not otherwise specified (PTCL-NOS).[4] Generally, PTCL-NOS has an aggressive clinical course with poor response to conventional chemotherapy and a

Submitted 16 April 2018; accepted 27 August 2018. DOI 10.1182/bloodadvances.2018019893.

*M.L. and M.B.P. contributed equally to this study and are joint first authors.

†F.d.A. and B.H. contributed equally this study and are joint last authors.

The full-text version of this article contains a data supplement.

dismal outcome, with 5-year overall survival (OS) rates of 30% to 35%.[1,5] Attempts to characterize PTCL-NOS by histomorphological and immunophenotypical features have been proposed but have not yet translated into clinical practice.[2,3,6,7] In recent years, gene expression profiling studies have displayed altered expression of genes in PTCL-NOS related to the structural machinery as well as to cell signaling transduction, proliferation, and apoptosis.[8-10] Gene expression profiling studies have also led to the identification of several candidate genes that allow better distinction from other primary nodal PTCL subtypes, such as angioimmunoblastic T-cell lymphoma, and anaplastic lymphoma kinase-negative anaplastic large cell lymphoma, with important implications in the diagnostic workup of PTCLs.[11-14]

Various factors acting at different levels may influence the final properties of encoded proteins, for example, translational inhibition/activation, posttranslational modifications, and inhibition/activation of the proteins, as well as specific degradation. Consequently, there can be considerable discrepancy between RNA expression levels and the amount of the corresponding protein product.[15] Therefore, proteomics-based approaches to identify protein expression patterns may be useful to provide pathogenetic and prognostic clues in cancer biology.

The aims of our study were to identify: (A) differentially expressed proteins in primary diagnostic tissue samples from patients with PTCL-NOS compared with the protein expression found in nonneoplastic human lymphoid tissue; (B) proteomic similarities and/or differences between the PTCL-NOS tumors based on principal component analysis (PCA); and (C) the protein expression in archival pretreatment tumor biopsies from patients with treatment-sensitive vs treatment-refractory PTCL-NOS patients. Finally, we wanted to evaluate the findings discovered by proteomics in a larger cohort of PTCL-NOS patients by immunohistochemistry (IHC) and correlate the expression with clinicopathological features and outcome. This is the first study to report the use of proteomics to correlate protein expression profiles in PTCL-NOS with treatment responses and clinical outcome.

Patients and methods

Patient samples

Pretherapeutic tumor biopsies from 87 patients diagnosed with PTCL-NOS, aged 20 years or older, with adequate clinical information and available formalin-fixed, paraffin-embedded (FFPE) archive tissue samples, were included in a tissue microarray (TMA) as previously described.[16] The patients were diagnosed between 1989 and 2011, and FFPE tissue samples were retrieved from the pathology departments at 3 Danish tertiary referral centers (Aarhus, Herlev, and Odense University Hospitals). Eighteen patients had both pretherapeutic FFPE and fresh frozen tissue collected from the same biopsy (supplemental Table 1). FFPE tissues were collected, processed, and stored using standard procedures. Frozen tissue specimens from 8 healthy adult donors who underwent tonsillectomy for benign hyperplasia were included as a reference material for "normal" nonneoplastic protein expression in lymphoid tissues. Shortly after collection, frozen samples were embedded in OCT (optimal cutting temperature) medium (Tissue-Tek; Sakura), snap-frozen in isopentane, and immersed in liquid nitrogen, before being stored at −80°C until use. Frozen tumor tissues were cut on a cryostat (Microm HM560; Thermo Fisher Scientific Microm International GmbH, Walldorf, Germany), and tissue sections stained with hematoxylin and eosin (H&E) to confirm the presence of appropriate lymphoma tissue and estimate the proportion of tumor in the samples. No unique marker for the tumor cells in the FFPE PTCL-NOS tumors were used, and the estimate of neoplastic cells was obtained from H&E-stained whole slides supplemented by relevant IHC in each case (eg, CD2, CD3, CD4, CD8, CD20, CD21, CD30, EBER, etc.). This was done by an experienced expert hematopathologist (SHD) and was in all cases estimated to be >60%. In order to secure the presence of neoplastic cells in frozen material used for proteomics, a "sandwich-approach" for "capturing the tumor tissue" was used where (1) the top section was validated for the presence of lymphoma on H&E-stained whole section, (2) consecutive sections were used for proteomics, and (3) the final section was validated for the presence of lymphoma on H&E-stained whole section, thus assuming that the slides in between represented sufficient and adequate PTCL tumor tissue.

The study was approved by the Central Denmark Region Committees on Health Research Ethics (1-10-72-392-12) and the Danish Data Protection Agency (1-16-02-26-11) and was conducted in accordance with the Helsinki Declaration.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Tissue for 2D-PAGE was pretreated as previously described.[17-19] Total protein concentration was determined by a noninterfering assay (488250; Calbiochem). The 2D-PAGE procedure was performed as previously reported by our group.[17-19] From each tumor tissue specimen, 100 μg of total protein was loaded. The gels were stained with Sypro-Ruby overnight (Bio-Rad), and the protein spots were visualized by ImageQuant Las4010 luminescent Image Analyzer (GE Healthcare). Gel images were loaded to the PDQuest Advanced 2D Gel Analysis Software Version 8.0 (Bio-Rad) and analyzed in accordance with previously described protocols.[17-19] Subsequently, the gels were silver stained according to the Vorum protocol.[17-20] Differentially expressed proteins, defined as twofold or more (Mann-Whitney U test, $P < .05$), were excised, and in-gel tryptic digestion was performed.[17-19]

Liquid chromatography tandem mass spectrometry (MS/MS)

Sample preparation and mass spectrometry (MS) analysis were performed as previously described.[17-19,21] The identified peptides were used to search the Swiss-Prot Database (versions 2013_03, 2013_06 or 2013_10) using the online version of the Mascot MS/MS Ions Search facility (Matrix Science; http://www.matrixscience.com).[22] For database searches, doubly, triply, and quadruply charged ions with up to 2 missed cleavages, a peptide mass tolerance of 20 ppm, and an MS/MS tolerance of 0.05 Da were allowed. Searches included modification on cysteine with carbamidomethyl. Contaminating peptides, ie, keratins, cingulin, filaggrin-2, dermicidin, desmoglein-1, trypsin, bovine serum albumin, and casein, were disregarded. At least 1 "bold red" peptide, with scores giving a <5% probability that the observed match was a random event, was required in order to qualify as "a protein hit." Information on the peptides corresponding to these hits is given in supplemental Tables 2-4.

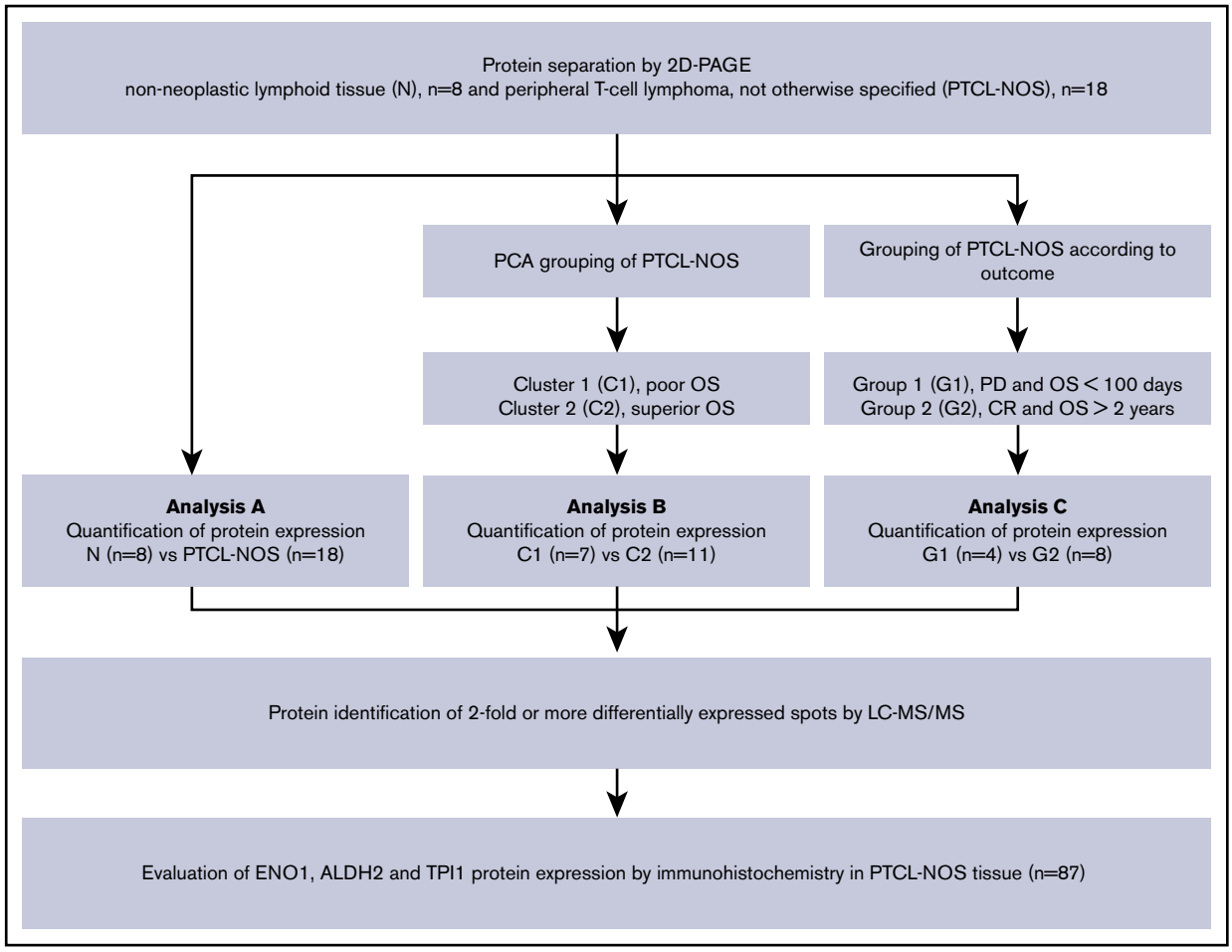

Figure 1. Study design and flowchart of the analyses. Initially proteins in all samples were separated using 2D-PAGE. PTCL-NOS cases were dichotomized according to PCA and clinical outcome in C1 vs C2 and G1 vs G2, respectively. Differential protein expressions were compared in: analysis A, PTCL-NOS (n = 18) vs N (n = 8); analysis B, PCA defined clusters C1 (n = 7) vs C2 (n = 11); and analysis C, PTCL-NOS patients with primary refractory disease (G1) with survival <100 days (n = 4) vs PTCL-NOS patients with chemosensitive disease and survival >2 years (G2) (n = 8). In the latter analysis, 6 cases were omitted for failing to fulfill the defined criteria of treatment response. Spots with ≥twofold differential expression were excised, and protein identifications were performed by liquid chromatography tandem mass spectrometry (LC-MS/MS). ENO1, TPI1, and ALDH2 protein expression was assessed in a larger cohort of PTCL-NOS patients (n = 87) by IHC and digitally quantified using digital image analysis on TMA cores. ALDH2, aldehyde dehydrogenase 2, mitochondrial; CR, complete remission; N, nonneoplastic tissue; TPI1, triosephosphate isomerase.

Bioinformatic analysis

PCA and hierarchical clustering were performed using Cluster 3.0 software on log-transformed data without missing values.[23] Average linkage was used as the clustering method and Spearman rank correlation as the similarity metric. The data were visualized with Java Treeview software.[24] Pathway enrichment analysis was performed using the Reactome Pathway Database.[25,26] Lists with UniProt identifications are shown in supplemental Table 5.

Digital quantification of ENO1, TPI1, and ALDH2

Immunohistochemical staining was performed on 4-μm FFPE tissue TMA sections with the Ventana Benchmark XT automated staining system (Ventana Medical Systems). Stains for differentially expressed proteins identified by proteomics were performed for α-enolase 1 (ENO1) (ab155955, Abcam, dilution 1:50), TPI1 (GTX104618, GeneTex, dilution 1:1000), and ALDH2 (ab108306, Abcam, dilution 1:50). Images of stained TMA slides were captured and digitalized at a magnification of ×40 using the Hamamatsu Nanozoomer 2.0HT scanner (Hamamatsu Photonics). Protein expression was determined by IHC; expression levels were digitally quantified using VIS (Visiopharm Integrator System 2017.2, Visiopharm) as previously described.[27] An area fraction (AF) of IHC positivity (ie, both tumor and nontumor cells together) was computed as the IHC stained area normalized to the total TMA core area and interpreted as the protein expression by IHC for each patient sample.[28] The median AF was used as cutoff for the IHC samples to dichotomize the results.

Statistical analysis

Differences between groups of categorical and continuous variables were tested using Fisher's exact test, Wilcoxon rank-sum test, and Kruskal-Wallis equality-of-populations rank test as appropriate. Treatment outcome was described by OS defined as the time interval from diagnosis to last follow-up or death from any cause.[29] Survival curves were generated according to the

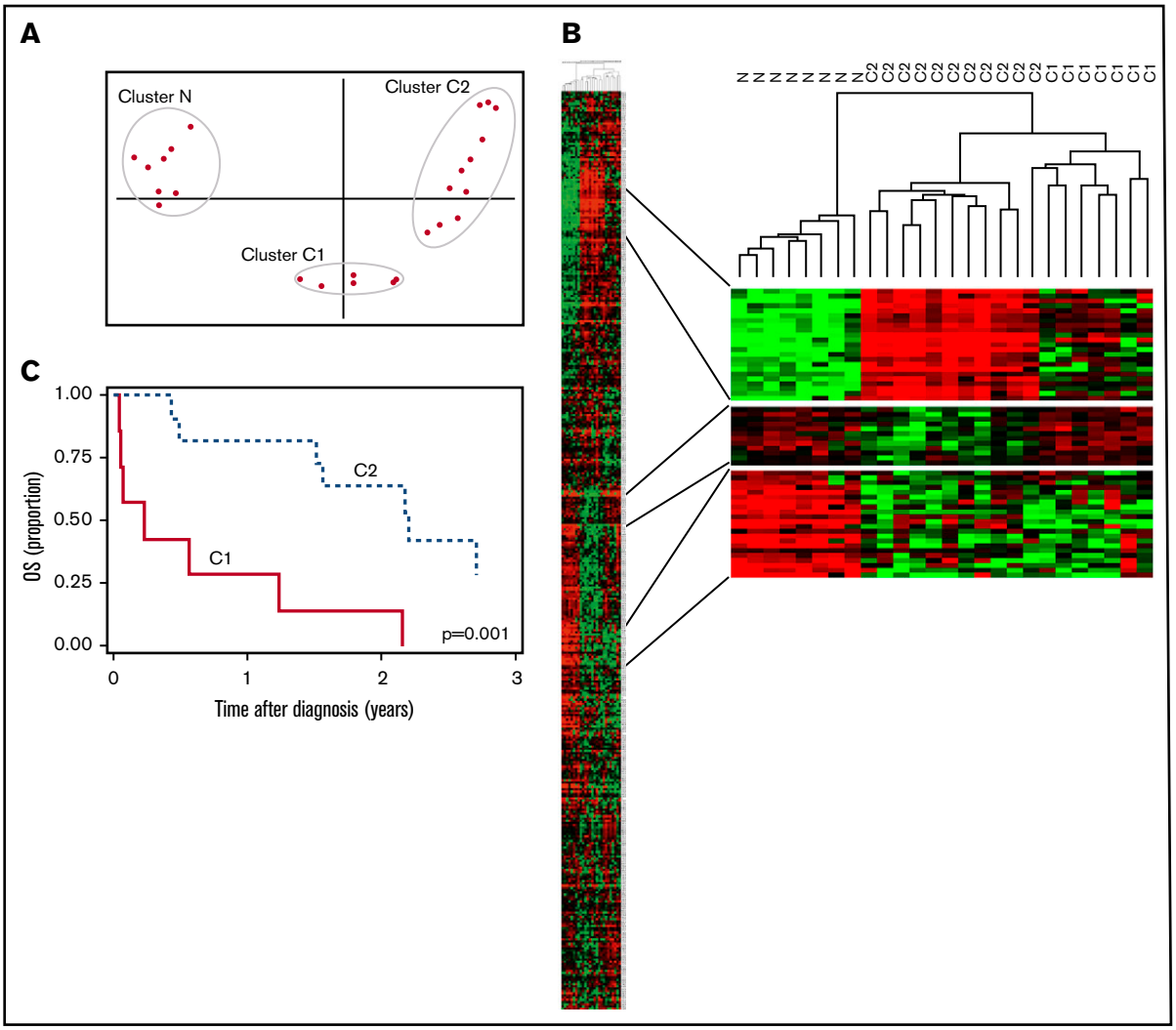

Figure 2. PCA cluster analysis. (A) PCA analysis showing distinct separation of PTCL-NOS tumors from nonneoplastic lymphoid reference tissue and additional clustering within PTCL-NOS in distinct groups (ie, C1 and C2). (B) Hierachical clustering showing distinct protein profiles between N, C1, and C2. With this analysis, 1 of the C2 patients clustered with the C1 group. Green denotes downregulation, and red denotes upregulation. (C) OS of PTCL-NOS patients according to groups (C1 and C2) identified from cluster analysis. N, nonneoplastic lymphoid tissue.

Kaplan-Meier method, and associations of factors with OS were estimated using Kaplan-Meier curves, log-rank test, and Cox proportional hazards models. For survival analysis on digitally quantified specimens, 2 groups were considered as defined from the median AF for the specific IHC staining (ie, high $\geq$ median AF vs low expression $<$ median AF). Treatment responses were defined as complete remission, partial remission, progressive disease (PD), or no change.[30] Significant differences were defined as $P < .05$. All statistical analyses were performed using STATA IC 11 (StataCorp, College Station).

Results

Protein expressions in pretherapeutic samples were assessed by proteomics and compared in 3 analyses: (A) PTCL-NOS (n = 18) vs nonneoplastic lymphoid tissue (n = 8); (B) groups defined by unsupervised PCA clustering analysis, C1 (n = 7) vs C2 (n = 11); and (C) groups according to clinical outcome, that is, PTCL-NOS patients with primary refractory disease and survival $<$100 days (G1) (n = 4) vs PTCL-NOS patients with chemosensitive disease and survival of $>$2 years (G2) (n = 8) (see flowchart in Figure 1).

Differentially expressed proteins identified between nonneoplastic lymphoid tissue and PTCL-NOS (analysis A)

In total, 92 protein spots in the 2D gels were identified as differentially expressed in analysis A (supplemental Figure 1A; supplemental Table 2). Of these, 54 protein spots were down-regulated and 38 upregulated in tumor specimens. In 79 out of the 92 spots, the MS analysis identified specific proteins. Single protein identification was obtained in 50 spots on the 2D gels covering 32 different proteins (supplemental Table 2). Bioinformatic analyses revealed disturbance of immunological pathways, for example, neutrophil degranulation, gene and protein expression by JAK-STAT signaling after interleukin-12 (IL-12) stimulation, and IL-12 family

signaling (supplemental Table 6). Particularly upregulated proteins in the tumors included various actin fragments (eight- to 87-fold), ENO1 fragment (eightfold), and transgelin (20-fold).

Differentially expressed proteins identified between groups defined by unsupervised clustering (analysis B)

PCA of all matched spots showed distinct clustering in nonneoplastic and lymphoma specimens. Among the PTCL-NOS tumors, PCA revealed 2 distinct clusters (C1 and C2) encompassing 7 and 11 cases, respectively (Figure 2A). Unsupervised hierarchical clustering showed an essentially similar result, with separation of the nonneoplastic cluster from 2 tumor clusters encompassing 8 and 10 cases, respectively (Figure 2B). Despite clinico-pathological features and risk factors being evenly distributed between patients in the C1 and C2 groups (supplemental Table 7), a significant difference in OS was demonstrated between the patients in the 2 clusters ($P = .001$; Figure 2C). Comparison of intratumoral protein expression between C1 and C2 identified 57 differentially expressed spots (44 upregulated in C1 and 13 in C2). Single protein identification was found in 27 spots (supplemental Figure 1B; supplemental Table 3). Pathways found to be disturbed were primarily related to cellular stress and protein metabolism, including regulation of HSF1-mediated heat shock response, prefoldin mediated transfer of substrate to CCT/TriC, and cellular response to heat stress, and protein expression by JAK-STAT signaling after IL-12 stimulation (supplemental Table 8). Prominent upregulated proteins in C1 included aldehyde dehydrogenase, mitochondrial (ALDH2), and T-complex protein 1 subunit ε, whereas in C2 upregulation of, for example, ENO1 fragment and triose-phosphate isomerase (TPI1) were identified.

Differentially expressed proteins between outcome-defined PTCL-NOS groups (analysis C)

In analysis C, we compared protein expression in tumors from PTCL-NOS patients with primary refractory disease (ie, PD and survival <100 days [G1]), with that from tumors in patients who showed initial chemosensitivity (ie, complete remission and survival >2 years [G2]). Thirty-seven differentially expressed protein spots were identified, 32 upregulated in G1 and 5 upregulated in G2 (supplemental Figure 1C; supplemental Table 4). Out of these 37 spots, 21 yielded single protein identifications. Similar to analysis B, bioinformatic pathway enrichment analysis identified disturbance of pathways primarily related to cellular stress, that is, regulation of HSF1 heat shock response and cellular response to stress (supplemental Table 9). Upregulation of proteins in the G1 included proteins such as ALDH2 and coronin 1A, whereas both full-length ENO1, as well as ENO1 fragment, and TPI1 were upregulated in G2.

Expression of ENO1, ALDH2, and TPI1 estimated by IHC in a larger cohort

We selected some of the differentially expressed proteins (ie, ENO1, ALDH2, and TPI1) as putative prognostic markers to be tested in a larger cohort of 87 PTCL-NOS patients by IHC, with correlation to clinico-pathological features. When comparing quantitative protein expression results from 2D-PAGE with those from IHC, it is important to recognize that 2D-PAGE is able to separate proteins into various proteoforms each migrating in different spots.[31] Thus, quantitative changes of a spot identified in 2D-PAGE, that is, of 1 proteoform, could be different from that observed in the combined set of all proteoforms. Antibodies used for IHC generally monitor the proteoforms as a whole, rather than a single proteoform. In our proteomic analysis, ENO1 was significantly increased (eightfold) in the PTCL-NOS group compared with the nonlymphoma tissue, as well as in the subgroups C2 (twofold) and G2 (twofold) (spot 3407 and spot 4405; supplemental Tables 2-4). When analyzed by IHC and quantified by digital image analysis in the larger PTCL-NOS cohort, 73 cases (83%) had sufficient tissue for analysis. ENO1 expression was generally high in all PTCL-NOS samples with a median AF of 86% (range 15% to 97%). A crude comparison of outcome according to ENO1 expression showed that patients with high intratumoral ENO1 expression (ie, ≥median expression) had significantly poorer outcomes (5-year OS, 15%; 95% confidence interval [CI], 5% to 30%) compared with those with low expression (5-year OS, 45%; 95% CI, 27% to 58%; $P = .009$) (Figure 3A-C). This finding was also evident after limiting the analysis to PTCL-NOS patients that received curatively intended treatment (n = 68; hazard ratio, 2.09; 95% CI, 1.17-3.73; $P = .013$). No differences in baseline clinico-pathological features were found between the 2 ENO1 groups (Table 1). Independent risk factors for adverse outcome in the PTCL-NOS cohort were IPI ($P < .001$) as well as the IPI parameters age >60 years ($P < .001$) and performance status ≥2 ($P = .010$). In a multivariate analysis adjusted for these parameters, high ENO1 expression remained as an independent prognostic factor for outcome (hazard ratio, 1.88; 95% CI, 1.01-3.5; $P = .046$). The proteomic analysis revealed that a proteoform of ALDH2 was downregulated in PTCL-NOS compared with nonneoplastic tissues (sixfold) as well as downregulated in C1 compared with C2 (ninefold) and in G1 compared with G2 (sevenfold) (spot 3407; supplemental Tables 2-4). Quantitative ALDH2 expression by IHC showed a median AF of 17% (range 1% to 63%). Although low ALDH2 correlated with improved outcome when confined to the 18 patients enrolled for proteomics ($P = .029$, data not shown), this finding was not confirmed in the larger cohort with quantification of immunohistochemical staining (Figure 3D-F). TIP1 was identified as upregulated in the proteomics analysis in both nonmalignant lymphoid tissue, C2 and G2, suggesting a correlation with improved outcome (spot 7202; supplemental Tables 2-4). TIP1 showed a wide expression range, estimated to span from 10% to 99%, with a median AF of 77%. Patients with high TIP1 expression tended to have an adverse outcome in the larger PTCL-NOS cohort ($P = .057$; Table 1; Figure 3G-I).

Discussion

PTCL-NOS constitutes the most ill-defined PTCL subtype, which includes all mature T-cell malignancies not fulfilling the diagnostic criteria of any of the specifically defined other PTCL entities.[2,3] Consequently, the underlying biology of this group of tumors is expectedly rather heterogeneous. In this study, we present the first report of the use of proteomics-based analyses to describe the PTLC-NOS-associated proteome. Bioinformatic analyses identified "immunological" pathways as being particularly perturbed. Based on proteomic profiles, 2 distinct subgroups were identified by PCA among the PTCL-NOS tumors. These groups showed marked alterations in "stress related" and "protein metabolic" pathways and differed significantly in their clinical outcome. Several novel putative prognostic protein markers were identified when comparing these groups, 3 of which were evaluated by IHC in tumors from a larger

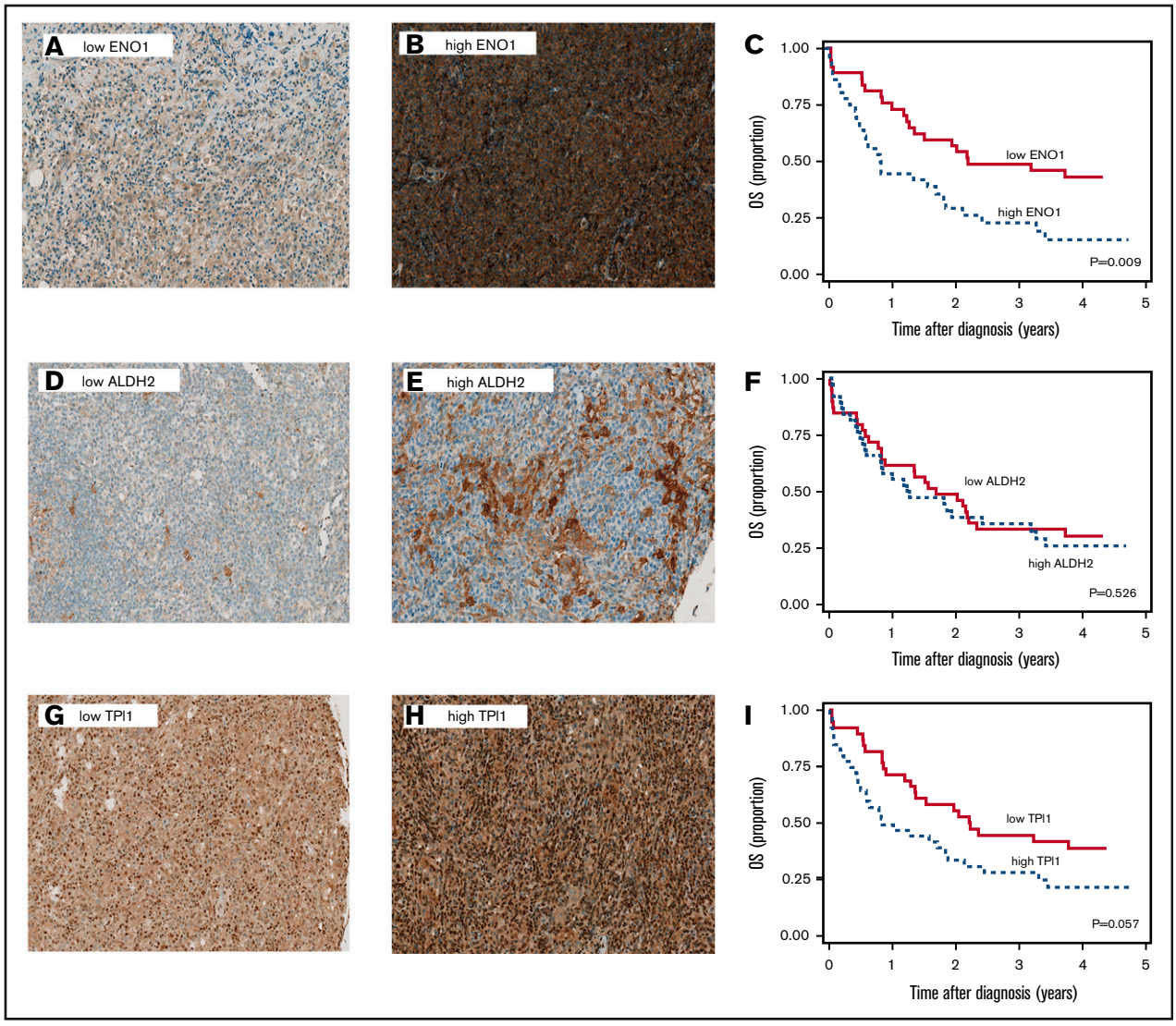

Figure 3. Immunohistochemical evaluation of putative predictive markers and outcome in PTCL-NOS patients. Representative images showing high and low expression of ENO1 (A-B), ALDH2 (D-E), and TPI1 (G-H) by IHC are shown in PTCL-NOS tissue. OS curves for the patients according to marker expression (C,F,I). Original magnification ×10.

cohort of patients with PTCL-NOS. High ENO1 expression was found to be significantly associated with poor outcome in PTCL-NOS patients, and high TPI1 expression tended to show a similar effect.

Several studies have proposed stratification of PTCL-NOS into different tumor subsets based on cell of origin, phenotypic traits, molecular characteristics, and outcome; however, these lymphomas remain the most frequently diagnosed PTCL entity worldwide.[1,12,32-36] Specific subgrouping is a huge challenge and possibly is not achievable in all cases because of the innate heterogeneity of this lymphoma subgroup.[37] Until now, large-scale studies in PTCL-NOS have primarily focused on alterations at the genetic level.[12,32-36] Nevertheless, the protein product of a certain gene may be influenced and altered at several levels in the pre- and posttranscriptional processes in cells, and this may not necessarily be truly reflected by the RNA expression.[38] Because proteins are the functional end product of gene expression, we posit that it may be an important addition to the investigations at the DNA/RNA level to search for potential novel tumor biomarkers at the protein level as a hypothesis-generating methodology. Archival frozen tumor tissue specimens where analyzed by a 2D-PAGE proteomic approach, which has been shown to be both feasible and reproducible in lymphomas.[17-19,39,40]

PCA of the proteomic data showed that the PTCL-NOS tumors clustered in 2 distinct groups that shared some common traits in their protein expression profile. To some extent, this clustering mirrored the predefined grouping according to clinical outcome and chemosensitivity; that is, the 4 cases with the most dismal outcome (PD and OS <100 days) were included in the same cluster. ENO1 was among the proteins identified as showing marked differential expression in these groups. This protein, encoded by the ENO1 gene on the long arm of chromosome 1, plays a major role as a glycolytic enzyme in cellular energy metabolism and is expressed in most tissues.[41] In addition to its glycolytic function, ENO1 has been found to be deregulated in various types of cancer.[42-44] An

Table 1. Clinical parameters of patients with PTCL-NOS and their distribution according to ENO1 and TPI1 expression by IHC

Clinical feature	ENO1 "low" (n = 37)	ENO1 "high" (n = 36)	*P*	TPI1 "low" (n = 38)	TPI1 "high" (n = 39)	*P*
Age, y			.34			.27
Median	58	63		56	62	
Range	20-92	25-82		20-92	20-92	
Sex			.16			1.00
Female	20	13		18	18	
Male	17	23		20	21	
Performance status			.41			.71
0-1	30	25		35	33	
2-4	7	10		3	5	
Missing	0	1		0	1	
Elevated LDH			.09			.15
Yes	17	24		19	25	
No	18	10		18	11	
Missing	2	2		1	3	
Stage (Ann Arbor)			.61			.39
I-II	8	9		7	11	
III-IV	27	23		29	24	
Missing	2	4		2	4	
Extranodal sites			.56			1.00
≤1	30	26		30	30	
>1	15	5		8	9	
Missing	2	5		0	0	
IPI			.53			.92
Low	10	6		10	7	
Low-intermediate	11	6		10	8	
High-intermediate	7	8		8	9	
High	4	6		5	5	
Missing	5	10		5	10	
Treatment (first line)			1.00			1.00
Curative intent*	34	34		36	36	
Other†	3	2		2	3	
Consolidation with HDT/ASCT			.52			1.00
Yes	4	6		5	6	
No	33	30		33	33	
Outcome			.01			.57
5-y OS, %	43	15		38	20	
95% CI	27-58	5-30		23-53	10-35	

ASCT, autologous stem cell transplantation; CHOP, cyclophosphamide, hydrocydaurubicin, vincristine, prednisone; HDT, high-dose therapy; IPI, International Prognostic Index; LDH, lactate dehydrogenase.

*CHOP/CHOP-like and/or radiotherapy in low-stage disease.

†Palliative treatment or unspecified treatment.

oncogenic role for ENO1 has been proposed in carcinomas, based on the observation that high ENO1 expression was reported to be associated with poor outcome and high metastatic potential.[42,45] Recently, in lymphomas, ENO1 has also been reported to promote tumor cell proliferation and alterations of the intercellular signaling phosphatidylinositol 3-kinase/Akt pathway, as well as to mediate drug resistance.[46] In line with this, PTCL-NOS tumors with the highest ENO1 IHC expression levels showed striking chemoresistance. ENO1 expression was generally very high in all the lymphoma specimens and showed eightfold greater differential protein expression compared with benign lymphoid tissues. This may be because of an increased level of glycolysis in lymphomas, which is an acknowledged hallmark of cancer.[47] In addition, we found upregulation of a proteoform of TPI1, another enzyme involved in the glycolytic machinery. High TPI1 expression, as defined by IHC, showed a tendency to correlate with poor

survival, although this was not statistically significant in our material. As with ENO1, TPI1 has also emerged among the differentially expressed proteins in proteomic-based studies of carcinomas, where it has been proposed associated with chemotherapeutic resistance.[48,49] However, both ENO1 and TPI1 are involved in other intracellular metabolic pathways and not solely confined to glycolysis.[41,50] Thus, other mechanisms may also contribute to the altered expression found in our analysis. The oxidative enzyme ALDH2 was also found to be markedly altered in our proteomic profiling analysis. We chose this for further investigation because blocking of ALDH activity by the drug disulfiram has been shown to increase cytotoxic activity in hematological malignancies.[51] Although high ALDH2 expression correlated with poor survival of the patients included in the proteomic analysis, this finding could not be validated by IHC in a larger patient cohort.

The patterns of protein expression we found in PTCL-NOS underline the complexity and heterogeneity of these tumors. Given that PTCL-NOS is currently used as a diagnosis of exclusion for T-cell lymphomas, their apparent high degree of biological heterogeneity is not surprising. This probably makes it less realistic to distinctly categorize all of these tumors into definable subcategories of PTCL. In this study, we have used quantification based on 2 different methodologies, 2D-PAGE and antibody-based IHC staining. An advantage of 2D-PAGE is that proteoforms are separated according to molecular mass and isoelectric points, enabling quantification of a specific proteoform. In comparison, IHC antibody-based quantification will be dependent on the sum of the expression patterns of all proteoforms that contain the specific target epitope used. This may explain the apparent discrepancy between the 2D-PAGE upregulation of ENO1 and TPI1 found in patient groups C2 and G2 with better outcome, while IHC revealed high expression to be associated with poor prognosis. Thus, a few proteoforms may be upregulated as visualized with 2D-PAGE while the combined set of proteoforms as monitored with IHC may be downregulated. A disadvantage of 2D-PAGE is that it is a rather laborious technique and is often only possible to perform on smaller cohorts. In our study, sample size was limited by the availability of tumors with archived fresh-frozen tissue from patients with a minimum follow-up of 5 years. The relatively small patient sample size achieved in our proteomic cohort indicates a major challenge to be faced by future proteomic-based studies of PTCLs, a challenge that may undermine the applicability of protein profiling in these tumors. Another aspect of heterogeneity in the protein expression is that both proteomic and IHC analyses are based on whole-tumor sections (ie, tissue containing a mixture of both neoplastic and nonneoplastic bystander cells). Although estimates of neoplastic cell content were made for each case studied, the cells of the tumor microenvironment also contribute to the protein profile. In future studies, it will be important to distinguish between the contributions from the neoplastic cells relative to the signals from the tumor microenvironment.

In conclusion, this is the first study to describe the use of proteomic profiling to identify differentially expressed proteins in PTCL-NOS tumor tissues and to relate these to clinical outcome. Among upregulated proteins, expression of ENO1 was notable. High intratumoral expression of this marker was correlated with poor outcome in a larger series of patients with newly diagnosed PTCL-NOS. This finding should be studied further in larger independent cohorts of patients with PTCL.

Acknowledgments

The authors thank Inge Kjærgaard Nielsen, Mona Britt Hansen, Jeanette Georgsen, Line Offenbach Jacobsen, and Tine Meyer for expert technical assistance.

This work was supported by grants from Aarhus University, the Danish Cancer Research Foundation, Gangsted Foundation, the Max and Inger Wørzner Foundation, Aarhus University Research Foundation, Meta and Håkon Baggers Foundation, King Chr. X Foundation, the Harboe Foundation, Manufacturer Einar Willumsens Memorial Scholarship, the John and Birthe Meyer Foundation, MEMBRANE Centre Aarhus University, the Karen Elise Jensen Foundation, the A.P. Møller Foundation for the Advancement of Medical Science, the Foundation of Eva and Henry Frænkel, and the Mimi & Victor Larsens Fund.

Authorship

Contribution: M.L. set up the investigation, performed the analyses, interpreted the data, and wrote the manuscript; M.B.P. set up the investigation, gathered clinical parameters of the patients, performed the statistical analyses, interpreted the data, and wrote the manuscript; K.L.L. performed the analyses, critically revised the manuscript, and approved the final report; K.B., S.J.H.-D., M.B.M., and P.N. identified and located the patient tissue, revised the biopsies and IHC stainings, critically revised the manuscript, and approved the final report; T.S.P. and P.N. performed the T-cell receptor rearrangement analyses, critically revised the manuscript, and approved the final report; S.B. contributed to the PCA and hierarchical clustering, critically revised the manuscript, and approved the final report; F.d.A. contributed to setup of the investigation, interpretation of the data, and approval of the final report; and B.H. contributed to setup of the investigation, performing the MS analyses, interpretation of the data, writing the manuscript, and approval of the final report.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

ORCID profiles: M.L., 0000-0001-5089-3271; S.J.H.-D., 0000-0003-2158-3885; M.B.M., 0000-0003-2041-3630.

Correspondence: Maja Ludvigsen, Department of Clinical Medicine, Aarhus University, Tage-Hansens Gade 2, Entrance 4A 2. Floor, DK-8000 Aarhus, Denmark; e-mail: majlud@rm.dk.

References

1. Vose J, Armitage J, Weisenburger D; International T-Cell Lymphoma Project. International peripheral T-cell and natural killer/T-cell lymphoma study: pathology findings and clinical outcomes. *J Clin Oncol*. 2008;26(25):4124-4130.

2. Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood*. 2016;127(20):2375-2390.

3. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J. WHO Classification of Tumours of the Haematopoietic and Lymphoid Tissues. Lyon, France: IARC Press; 2008.
4. de Leval L, Gaulard P. Pathology and biology of peripheral T-cell lymphomas. *Histopathology*. 2011;58(1):49-68.
5. d'Amore F, Relander T, Lauritzsen GF, et al. Up-front autologous stem-cell transplantation in peripheral T-cell lymphoma: NLG-T-01. *J Clin Oncol*. 2012; 30(25):3093-3099.
6. Geissinger E, Odenwald T, Lee SS, et al. Nodal peripheral T-cell lymphomas and, in particular, their lymphoepithelioid (Lennert's) variant are often derived from CD8(+) cytotoxic T-cells. *Virchows Arch*. 2004;445(4):334-343.
7. Rüdiger T, Ichinohasama R, Ott MM, et al. Peripheral T-cell lymphoma with distinct perifollicular growth pattern: a distinct subtype of T-cell lymphoma? *Am J Surg Pathol*. 2000;24(1):117-122.
8. Martínez-Delgado B, Cuadros M, Honrado E, et al. Differential expression of NF-kappaB pathway genes among peripheral T-cell lymphomas. *Leukemia*. 2005;19(12):2254-2263.
9. Cuadros M, Dave SS, Jaffe ES, et al. Identification of a proliferation signature related to survival in nodal peripheral T-cell lymphomas. *J Clin Oncol*. 2007; 25(22):3321-3329.
10. Ballester B, Ramuz O, Gisselbrecht C, et al. Gene expression profiling identifies molecular subgroups among nodal peripheral T-cell lymphomas. *Oncogene*. 2006;25(10):1560-1570.
11. de Leval L, Rickman DS, Thielen C, et al. The gene expression profile of nodal peripheral T-cell lymphoma demonstrates a molecular link between angioimmunoblastic T-cell lymphoma (AITL) and follicular helper T (TFH) cells. *Blood*. 2007;109(11):4952-4963.
12. Iqbal J, Weisenburger DD, Greiner TC, et al; International Peripheral T-Cell Lymphoma Project. Molecular signatures to improve diagnosis in peripheral T-cell lymphoma and prognostication in angioimmunoblastic T-cell lymphoma. *Blood*. 2010;115(5):1026-1036.
13. Agnelli L, Mereu E, Pellegrino E, et al; European T-Cell Lymphoma Study Group. Identification of a 3-gene model as a powerful diagnostic tool for the recognition of ALK-negative anaplastic large-cell lymphoma. *Blood*. 2012;120(6):1274-1281.
14. Piccaluga PP, Fuligni F, De Leo A, et al. Molecular profiling improves classification and prognostication of nodal peripheral T-cell lymphomas: results of a phase III diagnostic accuracy study. *J Clin Oncol*. 2013;31(24):3019-3025.
15. Chen G, Gharib TG, Huang CC, et al. Discordant protein and mRNA expression in lung adenocarcinomas. *Mol Cell Proteomics*. 2002;1(4):304-313.
16. Pedersen MB, Hamilton-Dutoit SJ, Bendix K, et al. *DUSP22* and *TP63* rearrangements predict outcome of ALK-negative anaplastic large cell lymphoma: a Danish cohort study. *Blood*. 2017;130(4):554-557.
17. Kamper P, Ludvigsen M, Bendix K, et al. Proteomic analysis identifies galectin-1 as a predictive biomarker for relapsed/refractory disease in classical Hodgkin lymphoma. *Blood*. 2011;117(24):6638-6649.
18. Ludvigsen M, Kamper P, Hamilton-Dutroit SJ, et al. Relationship of intratumoural protein expression patterns to age and Epstein-Barr virus status in classical Hodgkin lymphoma. *Eur J Haematol*. 2015;95(2):137-149.
19. Ludvigsen M, Madsen C, Kamper P, et al. Histologically transformed follicular lymphoma exhibits protein profiles different from both non-transformed follicular and de novo diffuse large B-cell lymphoma. *Blood Cancer J*. 2015;5(3):e293.
20. Mortz E, Krogh TN, Vorum H, Görg A. Improved silver staining protocols for high sensitivity protein identification using matrix-assisted laser desorption/ ionization-time of flight analysis. *Proteomics*. 2001;1(11):1359-1363.
21. Honoré B, Buus S, Claësson MH. Identification of differentially expressed proteins in spontaneous thymic lymphomas from knockout mice with deletion of p53. *Proteome Sci*. 2008;6:18.
22. Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis*. 1999;20(18):3551-3567.
23. de Hoon MJ, Imoto S, Nolan J, Miyano S. Open source clustering software. *Bioinformatics*. 2004;20(9):1453-1454.
24. Saldanha AJ. Java Treeview--extensible visualization of microarray data. *Bioinformatics*. 2004;20(17):3246-3248.
25. Fabregat A, Sidiropoulos K, Garapati P, et al. The Reactome pathway Knowledgebase. *Nucleic Acids Res*. 2016;44(D1):D481-D487.
26. Croft D, Mundo AF, Haw R, et al. The Reactome pathway Knowledgebase. *Nucleic Acids Res*. 2014;42(D1):D472-D477.
27. Pedersen MB, Danielsen AV, Hamilton-Dutoit SJ, et al. High intratumoral macrophage content is an adverse prognostic feature in anaplastic large cell lymphoma. *Histopathology*. 2014;65(4):490-500.
28. Pedersen MB, Riber-Hansen R, Nielsen PS, et al. Digital pathology for the validation of tissue microarrays in peripheral T-cell lymphomas. *Appl Immunohistochem Mol Morphol*. 2014;22(8):577-584.
29. Cheson BD, Pfistner B, Juweid ME, et al; International Harmonization Project on Lymphoma. Revised response criteria for malignant lymphoma. *J Clin Oncol*. 2007;25(5):579-586.
30. Cheson BD, Fisher RI, Barrington SF, et al; United Kingdom National Cancer Research Institute. Recommendations for initial evaluation, staging, and response assessment of Hodgkin and non-Hodgkin lymphoma: the Lugano classification. *J Clin Oncol*. 2014;32(27):3059-3067.
31. Smith LM, Kelleher NL; Consortium for Top Down Proteomics. Proteoform: a single term describing protein complexity. *Nat Methods*. 2013;10(3): 186-187.
32. Asano N, Suzuki R, Kagami Y, et al. Clinicopathologic and prognostic significance of cytotoxic molecule expression in nodal peripheral T-cell lymphoma, unspecified. *Am J Surg Pathol*. 2005;29(10):1284-1293.

33. Lemonnier F, Couronné L, Parrens M, et al. Recurrent TET2 mutations in peripheral T-cell lymphomas correlate with TFH-like features and adverse clinical parameters. *Blood*. 2012;120(7):1466-1469.

34. Agostinelli C, Hartmann S, Klapper W, et al. Peripheral T cell lymphomas with follicular T helper phenotype: a new basket or a distinct entity? Revising Karl Lennert's personal archive. *Histopathology*. 2011;59(4):679-691.

35. Nakagawa M, Nakagawa-Oshiro A, Karnan S, et al. Array comparative genomic hybridization analysis of PTCL-U reveals a distinct subgroup with genetic alterations similar to lymphoma-type adult T-cell leukemia/lymphoma. *Clin Cancer Res*. 2009;15(1):30-38.

36. Ishida T, Inagaki H, Utsunomiya A, et al. CXC chemokine receptor 3 and CC chemokine receptor 4 expression in T-cell and NK-cell lymphomas with special reference to clinicopathological significance for peripheral T-cell lymphoma, unspecified. *Clin Cancer Res*. 2004;10(16):5494-5500.

37. Weisenburger DD, Savage KJ, Harris NL, et al; International Peripheral T-cell Lymphoma Project. Peripheral T-cell lymphoma, not otherwise specified: a report of 340 cases from the International Peripheral T-cell Lymphoma Project. *Blood*. 2011;117(12):3402-3408.

38. Wu L, Candille SI, Choi Y, et al. Variation and genetic control of protein abundance in humans. *Nature*. 2013;499(7456):79-82.

39. Antonucci F, Chilosi M, Parolini C, Hamdan M, Astner H, Righetti PG. Two-dimensional molecular profiling of mantle cell lymphoma. *Electrophoresis*. 2003;24(14):2376-2385.

40. Antonucci F, Chilosi M, Santacatterina M, Herbert B, Righetti PG. Proteomics and immunomapping of reactive lymph-node and lymphoma. *Electrophoresis*. 2002;23(2):356-362.

41. Diaz-Ramos A, Roig-Borrellas A, Garcia-Melero A, Lopez-Alemany R. alpha-Enolase, a multifunctional protein: its role on pathophysiological situations. *J Biomed Biotechnol*. 2012;2012:156795.

42. Yoshida A, Okamoto N, Tozawa-Ono A, et al. Proteomic analysis of differential protein expression by brain metastases of gynecological malignancies. *Hum Cell*. 2013;26(2):56-66.

43. Hamaguchi T, Iizuka N, Tsunedomi R, et al. Glycolysis module activated by hypoxia-inducible factor 1alpha is related to the aggressive phenotype of hepatocellular carcinoma. *Int J Oncol*. 2008;33(4):725-731.

44. Chang GC, Liu KJ, Hsieh CL, et al. Identification of alpha-enolase as an autoantigen in lung cancer: its overexpression is associated with clinical outcomes. *Clin Cancer Res*. 2006;12(19):5746-5754.

45. Wu W, Tang X, Hu W, Lotan R, Hong WK, Mao L. Identification and validation of metastasis-associated proteins in head and neck cancer cell lines by two-dimensional electrophoresis and mass spectrometry. *Clin Exp Metastasis*. 2002;19(4):319-326.

46. Zhu X, Miao X, Wu Y, et al. ENO1 promotes tumor proliferation and cell adhesion mediated drug resistance (CAM-DR) in Non-Hodgkin's lymphomas. *Exp Cell Res*. 2015;335(2):216-223.

47. Shaw RJ. Glucose metabolism and cancer. *Curr Opin Cell Biol*. 2006;18(6):598-608.

48. Liu Y, Liu H, Han B, Zhang JT. Identification of 14-3-3sigma as a contributor to drug resistance in human breast cancer cells using functional proteomic analysis. *Cancer Res*. 2006;66(6):3248-3255.

49. Maxwell SA, Cherry EM, Bayless KJ. Akt, 14-3-3ζ, and vimentin mediate a drug-resistant invasive phenotype in diffuse large B-cell lymphoma. *Leuk Lymphoma*. 2011;52(5):849-864.

50. Tajes M, Guivernau B, Ramos-Fernández E, et al. The pathophysiology of triose phosphate isomerase dysfunction in Alzheimer's disease. *Histol Histopathol*. 2013;28(1):43-51.

51. Conticello C, Martinetti D, Adamo L, et al. Disulfiram, an old drug with new potential therapeutic uses for human hematological malignancies. *Int J Cancer*. 2012;131(9):2197-2203.