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Biomarkers, Genomics, Proteomics, and Gene Regulation

Development of a Malignancy-Associated Proteomic Signature for Diffuse Large B-Cell Lymphoma

Paul B. Romesser,* David H. Perlman,† Douglas V. Faller,* Catherine E. Costello,† Mark E. McComb,† and Gerald V. Denis*

From the Cancer Research Center, and the Center for Biomedical Mass Spectrometry,† Boston University School of Medicine, Boston, Massachusetts*

The extreme pathological diversity of non-Hodgkin's lymphomas has made their accurate histological assessment difficult. New diagnostics and treatment modalities are urgently needed for these lymphomas, particularly in drug development for cancer-specific targets. Previously, we showed that a subset of B cell lymphoma, diffuse large B cell lymphoma, may be characterized by two major, orthogonal axes of gene expression: one set of transcripts that is differentially expressed between resting and proliferating, nonmalignant cells (ie, a "proliferative signature") and another set that is expressed only in proliferating malignant cells (ie, a "cancer signature"). A differential proteomic analysis of B cell proliferative states, similar to previous transcriptional profiling analyses, holds great promise either to reveal novel factors that participate in lymphomagenesis or to define biomarkers of onset or progression. Here, we use a murine model of diffuse large B cell lymphoma to conduct unbiased two-dimensional gel electrophoresis and mass spectrometry-based comparative proteomic analyses of malignant proliferating B cells and tissue-matched, normal resting, or normal proliferating cells. We show that the expression patterns of particular proteins or isoforms across these states fall into eight specific trends that provide a framework to identify malignancy-associated biomarkers and potential drug targets, a signature proteome. Our results support the central hypothesis that clusters of proteins of known function represent a panel of expression markers uniquely associated with malignancy and not normal proliferation. ***(Am J Pathol 2009, 175:25–35; DOI: 10.2353/ajpath.2009.080707)***

Lymphomas, which include non-Hodgkin's lymphoma, are the fifth most common type of diagnosed cancer in males, the sixth most common type in females and the fifth most common cause of cancer mortality in the United States.[1] Current standard chemotherapy/antibody-directed immunotherapy regimens are effective in only 40% of cases.[2] Non-Hodgkin's lymphoma is more common than Hodgkin's lymphoma, with 16,000 new cases diagnosed annually. Moreover, patients with aggressive forms of non-Hodgkin's lymphoma, such as diffuse large B cell lymphoma (DLBCL) with poor scores on the revised International Prognostic Index, have a four year overall survival of 55% when treated with the current standard of care.[3] More effective early diagnostic measures are urgently needed, such as sensitive biomarker assays that exploit proteomic signatures potentially unique to aggressive lymphomas.

Major progress in lymphoma diagnosis and prognosis has been built on recent advances in genome-wide transcriptional profiling methods.[4,5] For example, it has been possible to resolve transcriptional signatures of B cells of different origins and proliferative states (eg, normal germinal center B cells, mitogenically activated B cells, tonsillar B cells, and resting peripheral B cells, all exhibiting unique and resolvable transcriptional signatures), as well as clinical samples of DLBCL malignancies of greater or lesser aggressiveness that are otherwise indistinguishable histologically.[5–7] However, there remains a major challenge in the development of useful biomarkers: the identification of a small, relevant set of functionally important targets from the vast sums of genomic, proteomic, metabolomic or transcriptomic information of uncertain etiological significance that can be gathered from diseased cells or tissues. Biomarkers that display robust prognostic significance or confer insight into disease mecha-

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P.R. and D.H. contributed equally to this work.

Address reprint requests to Gerald V. Denis, Ph.D., Cancer Research Center, Boston University School of Medicine, Room K520, 72 East Concord Street, Boston, MA 02118. E-mail: gdenis@bu.edu.

nism are difficult to discern, even by the most sophisticated statistical analyses,[6] within immense data sets derived from analyses of "normal" and diseased tissues.[7]

We have established that one reasonable approach to simplifying the complexity of this task in the context of discovery of biomarkers and potential drug targets in malignancies is to subtract out the largest possible set of normal tissue-derived background signals from diseased tissue signals: in the case of B cell lymphomas, to subtract out signals from all of the nonmalignant B cell proliferative states (such as resting B cells and normal proliferating B cells) from those of malignant B cells, thus enriching for potential disease biomarker signals of functional importance. As a model system for DLBCL, we used our previously developed transgenic murine model based on Eμ-driven, B cell-restricted, constitutive expression of the double bromodomain protein Brd2 (Tg).[8] This DLBCL model is well characterized, stable and exhibits monoclonal expansion of only mature B cells; it offers a highly reproducible system for the determination of genome-wide and proteome-wide biomarkers for lymphoma expansion and progression.[8] The splenic lymphoma's genome-wide transcriptional signature is most similar to the "activated B cell-like" form of human DLBCL,[8,9] the more aggressive subtype, which is associated with a worse prognosis.[3–6] Previously, we compared genome-wide transcriptional expression profiles of independently arising Tg splenic lymphomas with those of nonmalignant proliferating or normal resting primary splenic B cells from a syngeneic, inbred strain of mice, which controlled as much as possible for variation between individuals and stromal microenvironments. Principal component analysis[10] of these signals identified two distinct axes of differential gene expression. One group of genes, which we called the "proliferation signature," was differentially expressed between normal resting primary B cells and mitogenically stimulated B cells. Another group of genes, which we called the "cancer signature," was differentially expressed along an orthogonal axis of gene expression unrelated to normal proliferation. This axis included genes specific to lymphomagenesis, progression and survival, several human orthologs of which are implicated in human lymphomagenesis. Furthermore, we identified seven statistically distinct clusters of gene expression that agreed with cellular function and pathology.[9] Thus, we were able to use normal B cell counterparts in a subtractive fashion to identify genes of functional importance for the malignant B cell that are uniquely relevant to cancer.

Although transcriptional profiling has matured as a diagnostic and prognostic research tool in pathology[11] and has been widely used to characterize malignancies as diverse as renal cell carcinoma,[12] prostate cancer,[13] metastatic bone cancer[14] and breast cancer,[15] protein-based biomarkers may have potentially greater diagnostic power than genetic or transcriptional profiles because they may reflect disease-related alterations to tissue that are invisible to gene-based analyses.[16] Protein expression and activity are subject to additional layers of metabolic regulation over and above the regulation of gene expression.[17] Moreover, disease states are often associated with deregulation of protein expression or function, which may include significant posttranslational modification, truncation, mislocalization within the cell and other variations.[18] Many cancers, in particular, show deregulated activity of growth factor receptor tyrosine kinases, which in turn alter protein phosphorylation patterns within the cell, and which genome-wide transcriptional analysis is unable to capture. Furthermore, although protein biomarkers may originally be identified through tissue- or proximal fluid-based analyses, it is likely that they may be detectable as species shed from the site of disease into blood or other body fluids, which may be obtained non-invasively. Thus, protein biomarkers potentially represent a powerful source of disease diagnostics. Proteomic analysis, therefore, can play an important, non-redundant role in the identification and characterization of potential biomarkers and targets of cancer intervention.[19]

Several studies have used advanced proteomics methods and statistical analyses to attempt to distinguish normal and cancer specimens for lung,[20] ovarian,[21] and breast cancer.[22] Based on our transcriptome results, we framed a similar hypothesis: nonmalignant proliferating B cells and malignant proliferating B cells will share the induction of important proliferation-associated proteins in comparison with non-proliferating controls; however, malignant cells will show additional changes in protein expression, including posttranslational modification, that are unique to their malignant state, over and above those changes associated with normal, mitogen-stimulated proliferation alone. Here, we have investigated this hypothesis, using a differential proteomic approach within our model of subtractive profiling of B cell lymphomas to develop a framework to evaluate potential protein biomarkers that are malignancy-associated. Specifically, we have conducted differential proteomics[23] using subfractionated protein nuclear lysate from resting and proliferating normal B cells and proliferating malignant B cells. We identified the major protein species that differ between these states and show that distinct patterns of expression define a potentially functionally relevant, malignancy-associated protein expression pattern.

Materials and Methods

Preparation of Murine Normal and Malignant B Cell Extracts

Mouse splenic B cells were isolated by magnetic cell sorting-based magnetic bead separation with anti-CD43 negative selection, as previously described.[9] Normal resting B cells were stimulated *in vitro* with 30 μg/ml *Salmonella typhimurium* lipopolysaccharide for 48 hours. Unless otherwise described, all reagents were from Sigma (St. Louis, MO). Percoll (Amersham Biosciences, Piscataway, NJ) gradient centrifugation was used to enrich for stimulated B cells, while removing cellular debris, apoptotic cells and resting B cells. Percoll gradients of 50%, 60%, 66%, and 70% were prepared from an ice-cold Percoll solution supplemented with Hanks' balanced salt solution (Invitrogen/GIBCO-BRL, Grand Island, NY). The Eμ-*BRD2*-driven large B cell lymphoma, adoptively

transferred between mice, provided a constant source of lymphoma cells for the study.[8] After sublethal irradiation (6 Gy), mice were inoculated by intraperitoneal injection of 10^7 cells. Mice were maintained on antibiotic water (trimethoprim and sulfamethoxazole). Malignant splenic B cells were purified and cell extractions performed as previously described.[24] Tumor cells were obtained from female FVB mice that were 6 to 8 weeks old and normal cells were obtained from syngeneic age-matched female controls housed under the same conditions. Animals were handled humanely in accordance with Federal and institutional requirements; this study was conducted with Boston University Institutional Animal Care and Use Committee authorization.

Two-Dimensional Polyacrylamide Gel Electrophoresis Fractionation of Cell Extracts and Gel Image Analysis

Two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE) separation defined 2D reference maps. B cell-soluble nuclear extracts were prepared and subjected to size exclusion chromatography with Bio-Gel P-60 gel (Bio-Rad). This step enriched for proteins with a molecular mass >60 kd, which significantly improved 2D gel electrophoresis and resulted in more accurate mass spectrometry protein identifications. Proteins were acetone-precipitated and solubilized in DeStreak rehydration solution (Amersham Biosciences). First-dimension isoelectric focusing was performed overnight on a Protean IEF cell (Bio-Rad, Richmond, CA) with pI 3–10 immobilized pH gradients, in accordance with the manufacturer's recommendations, in the presence of 1 mmol/L dithiothreitol. In-gel reduction and alkylation was performed with dithiothreitol and iodoacetamide before running second dimension electrophoresis. Second dimension sodium dodecyl sulfate-PAGE was performed on 10% Tris-HCl gels or on 4 to 12% gradient bis-Tris gels. Gels were washed in water, followed by colloidal Coomassie staining overnight (or until protein spots were sufficiently visualized). Alternatively, gel replicates were fixed and stained with Plus One silver stain (GE HealthCare, Piscataway, NJ).

In-Gel Digestion Methods

Resolved, stained and quantitated protein spots of interest were excised, destained, washed extensively, and digested with trypsin, as previously described.[25] Gel plugs were destained three times with 100 mmol/L ammonium bicarbonate (pH 8.8)/50% acetonitrile (Fisher Scientific, Pittsburgh, PA), and subjected to four rounds of washing with alternating solutions of 100 mmol/L ammonium bicarbonate (pH 8.8), 100 mmol/L ammonium bicarbonate (pH 8.8)/50% acetonitrile and 100% acetonitrile. Trypsin (Trypsin Gold, Promega, Madison, WI) digestion was conducted at 37°C overnight after swelling the gel plugs in digestion solutions containing a 1:10 enzyme-substrate (w/w) ratio (estimating substrate quantity by relative staining intensity of original spot) and 50 mmol/L ammonium bicarbonate (pH 8.8)/5% acetonitrile. Peptides were extracted from the gel pieces twice with alternating solutions of 20 mmol/L ammonium bicarbonate (pH 8.8), 1% trifluoroacetic acid/50% acetonitrile and 100% acetonitrile. Extracts were pooled and subjected to desalting with microreversed phase chromatography (ZipTips, Millipore, Bedford, MA).

Mass Spectrometric Data Acquisition

Matrix-assisted laser desorption ionization/time of flight mass spectrometry (MS) of purified peptides was conducted with a Bruker Reflex IV mass spectrometer (Bruker Daltonics, Billerica, MA) using the matrix, 2,5-dihydroxybenzoic acid, and an AnchorChip target (Bruker Daltonics). Mass spectra were internally recalibrated to within 50 ppm mass accuracy using known peptide ions and peak lists were generated using the software MoverZ (Genomic Solutions, Ann Arbor, MI).

Data Analysis

Gel images were captured with a VersaDoc3000 (Bio-Rad) and were processed with Proteomeweaver (Bio-Rad), for generation of aligned and warped gel overlay images for graphical representation. In-depth gel image analyses was performed with SameSpots (Nonlinear Dynamics, Durham, NC), for cross-experiment gel alignment, feature identification, feature volume quantification, expression, principal component analysis and hierarchical clustering analyses. Peak lists were submitted for peptide mass fingerprint database search with Mascot (Matrix Science, Boston, MA). The databases used were SwissProt version 50.3 and NCBInr 20060718, limiting the searches to *Mus musculus* entries, tryptic peptides with up to one missed cleavage, carbamidomethylation of cysteines, variable oxidation of methionines, and an error tolerance within 80 ppm. Under these searching parameters, Mascot scores of >53 for the SwissProt database and scores of >63 for the NCBInr database corresponded to statistically significant ($P \leq 0.05$) protein assignments. Protein functional assignment, Gene Ontology (GO) term annotation and graphical rendering were accomplished with software written in-house that mines protein GO annotations from the public repository available through the *http://uniprot.org* website.

Results

To define biomarkers associated with lymphoid malignancies more effectively, we previously used a Tg model for DLBCL to demonstrate that B cell gene expression may be characterized by both a proliferation signature and an orthogonal cancer signature.[9] Consequently, we hypothesize here that on subtraction of proteomic signatures of normal resting and nonmalignant proliferating B cells from the proteome of malignant B cells, we will be able to categorize a set of unique protein biomarkers that define proliferating malignant B cells, distinct from nonmalignant pro-

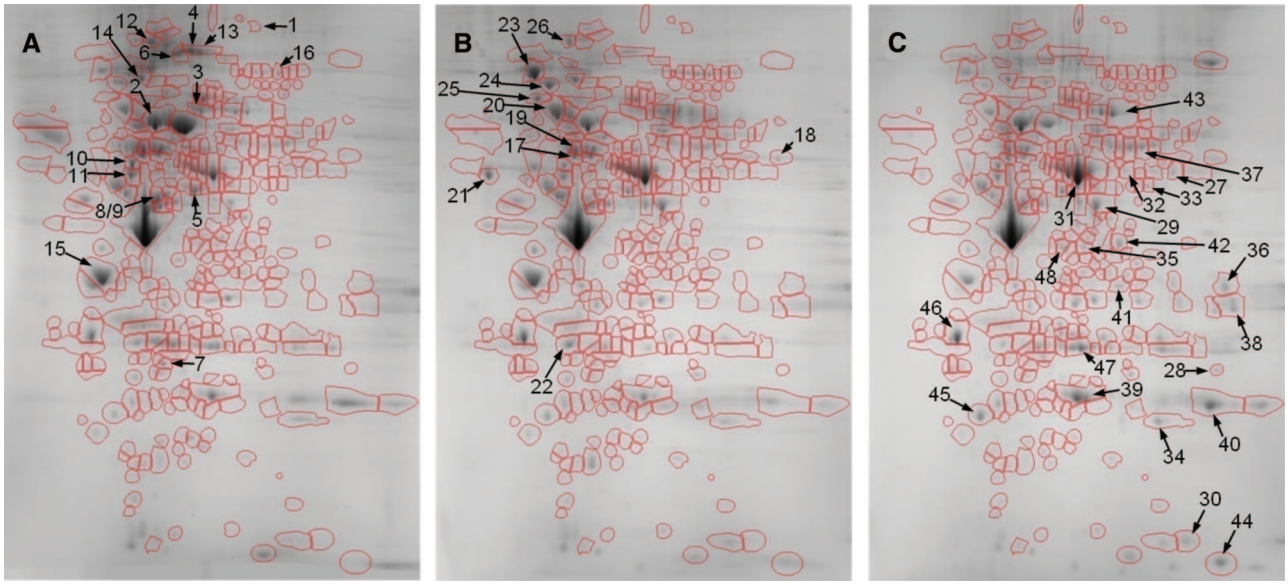

Figure 1. Example of 2D reference maps of resting, proliferating, and lymphoma B cell proteomes. Nuclear extract was isolated from spontaneous Tg B cell lymphomas, syngeneic resting splenic B cells, and B cells mitogenically stimulated to proliferate in culture. Lysate was subjected to desalting by size exclusion chromatography and then to 2D gel electrophoresis over the pI range of 3–10 and the molecular mass range of approximately 10 to 250 kd, followed by Coomassie staining. Gel images were warped, aligned and analyzed for changes in protein expression allowing for equivalent spot features defined across all data sets. **A:** Resting B cells. **B:** Normal, mitogenically stimulated, proliferating B cells. **C:** Proliferating lymphoma B cells. The gel region of approximately pI 4–9 (from **left** to **right**) and molecular mass 250 to 15 kd (from **top** to **bottom**) are shown, with spot features outlined and numbered according to the protein assignments reported in Table 1.

liferating cells. We have explored this hypothesis by undertaking a 2D-PAGE and mass spectrometric-based proteomic analysis of our Tg DLBCL, in relation to syngeneic, normal B cells, both resting and proliferating.

Two-dimensional proteomic reference maps were produced for each of the three B cell proliferative states: normal resting B cells (Figure 1A), nonmalignant proliferating B cells (Figure 1B) and malignant B cells (Figure 1C). Differential image analysis allowed the comparison of the 2D reference map for the lymphoma state both to that of the resting state and to that of the nonmalignant proliferating state (Figure 2, A and B). Above the background of similarity, particular protein spot features were seen to change robustly between the three states.

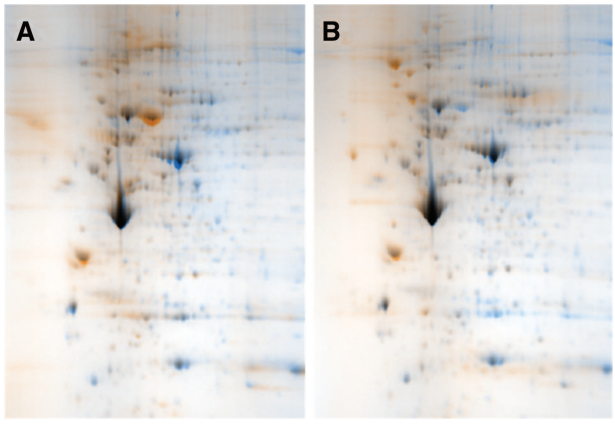

Figure 2. Comparative proteomic analysis of B cell proliferative states. Differential image analysis was conducted using warped and aligned 2D proteome reference maps for lymphoma B cells in comparison with normal resting and mitogenically stimulated, proliferating B cells. Overlapping areas of protein expression appear black while non-overlapping areas that are unique to each state are shown in color. **A:** Lymphoma B cells (blue) versus normal resting B cells (orange). **B:** Lymphoma B cells (blue) versus normal, mitogenically stimulated, proliferating B cells (orange).

We identified major proteins that underwent dramatic expression changes between B cell proliferative states through the use of in-gel trypsin digestion, matrix-assisted laser desorption ionization/time of flight MS and peptide mass fingerprint analyses (Table 1). Relative expression levels (as indicated in Table 1) of this set of proteins across all of the states showed that they could be assigned to three distinct groups: those that were up-regulated in the resting state with respect to the other two states (Table 1, first grouping), those that were up-regulated in the nonmalignant proliferative state with respect to the other two states (Table 1, second grouping), and those that were up-regulated in the lymphoma state with respect to the other two states (Table 1, third grouping). GO functional assignments (within the GO supercategory of Biological Process) highlight functional differences between the major up-regulated proteins in each B cell proliferative state (Figure 3, A–C).

Clustering of spot features into groups of relevant expression trends was performed to create a useful framework for the improved identification of potential biomarkers. We conducted differential analysis on the individual gel spot features for each of the B cell proliferative states. Raw data were rendered as vectors in three dimensions, comprising the X, Y, Z coordinates of resting state intensity, proliferating state intensity and lymphoma state intensity. Relative expression differences between states for each feature are depicted (Figure 4), calculated by determining the components of each vector that were perpendicular to a vector of equivalent expression across all states.

Table 1. Assignments for the Major Proteins That Differ among B Cell Proliferative States, as Determined by Mass Spectrometry and Mass Fingerprint Analyses

Protein no.	Protein name	Accession	Score	Expect	R	P	L
Normal resting							
1	Eukaryotic translation initiation factor 3	IF3A_MOUSE	82	7.80E-05	+	−	−
2	Heat shock cognate 71 kd protein (Heat shock 70 kd protein 8)	HSP7C_MOUSE	263	6.20E-23	+++	+	+
3	Ig μ chain C region	MUC_MOUSE	109	1.60E-07	++	+	−
4	Leukemia inhibitory factor precursor (LIF)	LIF_MOUSE	59	1.40E-02	+	v−	−
5	Heterogeneous nuclear ribonucleoprotein H (hnRNP H)	HNRH1_MOUSE	157	2.50E-12	+++	++	+
6	Matrin-3	MATR3_MOUSE	71	1.00E-03	+++	−	+
7	Prohibitin (B cell receptor-associated protein 32)	PHB_MOUSE	193	6.20E-16	+	−	−
8+9	Protein DEK + Actin-like protein 6a	DEK_MOUSE+ ACL6A_MOUSE	113	5.70E-08	+++	+	++
10	Vimentin	VIME_MOUSE	272	7.80E-24	+++	++	++
11	Tubulin α6	TBA2_MOUSE	147	2.50E-11	++	+	+
12	Splicing factor 3b	74191506*	156	2.70E-11	++	−	−
13	Heterogeneous nuclear ribonucleoprotein U	17390825*	131	8.50E-09	+++	+	+
14	Transitional endoplasmic reticulum ATPase 1	TERA_MOUSE	120	1.20E-08	+	+	−
15	Nucleophosmin (NPM)	NPM_MOUSE	108	2.00E-07	++	++	+
16	Elongation factor 2 (EF-2)	EF2_MOUSE	140	1.20E-10	+	+	−
Normal, mitogenically stimulated, proliferating							
17	Splicing factor 3A	SF3A3_MOUSE	62	8.80E-03	+	+++	+
18	Pyruvate kinase isozyme M2	KPYM_MOUSE	130	1.20E-09	−	+	−
19	Heterogeneous nuclear ribonucleoprotein K	HNRPK_MOUSE	164	4.90E-13	−	++	+
20	78 kd glucose-regulated protein precursor (GRP 78)	GRP78_MOUSE	256	3.10E-22	+	+++	−
21	Calreticulin	CALR_MOUSE	118	2.00E-08	−	+	−
22	Chloride intracellular channel protein 1	CLIC1_MOUSE	158	2.00E-12	−	+	−
23	Endoplasmin (Heat shock protein 90 kd β member 1)	ENPL_MOUSE	257	2.50E-22	+	++	−
24	Hsp 90- β (HSP 84)	HS90B_MOUSE	151	9.90E-12	+	++	−
25	Hematopoietic cell specific Lyn substrate 1	HCLS1_MOUSE	142	7.80E-11	−	+	−
26	30 hypoxia up-regulated 1 homologue	74192146*	151	8.50E-11	−	+++	+
Proliferating lymphoma							
27	Adenylyl cyclase-associated protein 1 (CAP 1)	CAP1_MOUSE	93	6.80E-06	−	−	+
28	Aldose reductase	ALDR_MOUSE	63	6.80E-03	−	−	+
29	Alpha enolase	ENOA_MOUSE	252	7.80E-22	−	+	+++
30	Cofilin 1	COF1_MOUSE	62	8.40E-03	−	−	+
31	Coronin 1A	COR1A_MOUSE	213	6.20E-18	+	+	++
32	Dihydrolipoyl dehydrogenase, mitochondrial	DLDH_MOUSE	87	2.80E-05	−	−	+
33	Glutamate dehydrogenase 1, mitochondrial	DHE3_MOUSE	63	5.40E-03	−	−	+
34	Ran GTP-binding nuclear protein	RAN_MOUSE	123	6.20E-09	−	−	+
35	Heterogeneous nuclear ribonucleoprotein A/B	ROAA_MOUSE	65	3.80E-03	−	−	+
36	Heterogeneous nuclear (hnRNP A3) ribonucleoprotein A3	ROA3_MOUSE	101	9.90E-07	−	−	+
37	Heterogeneous nuclear (hnRNP L) ribonucleoprotein L	HNRPL_MOUSE	89	1.70E-05	−	+	+++
38	Heterogeneous nuclear ribonucleoproteins A2/B1 (hnRNP A2 / hnRNP B1)	ROA2_MOUSE	127	2.50E-09	−	−	+
39	High mobility group protein B1	HMGB1_MOUSE	61	1.10E-02	−	−	+
40	High mobility group protein B2	HMGB2_MOUSE	91	1.00E-05	−	−	+
41	LIM and SH3 domain protein 1	LASP1_MOUSE	65	3.90E-03	−	−	+
42	Macrophage capping protein (Myc basic motif homolog 1)	CAPG_MOUSE	83	6.20E-05	−	−	+
43	Moesin	MOES_MOUSE	244	4.90E-21	−	+	+++
44	Peptidyl-prolyl *cis-trans* isomerase A (PPIA)	PPIA_MOUSE	96	3.00E-06	−	−	+
45	Rho GDP-dissociation inhibitor 2 (Rho GDI 2)	GDIS_MOUSE	108	2.00E-07	+	+	+++
46	Tropomyosin α−3 chain (Tropomyosin-3)	TPM3_MOUSE	117	2.50E-08	+	++	+++
47	UTP-glucose-1-phosphate uridylyltransferase 2	UGPA2_MOUSE	76	2.50E-04	++	+	+++
48	Serpin b1a	74354376*	141	8.50E-10	−	+	+++

Major protein spots that differed in staining intensity among the three states were excised, destained, digested with trypsin, and subjected to matrix-assisted laser desorption ionization/time of flight MS and peptide mass fingerprint analyses, as described in *Materials and Methods*. Protein numbers correspond to spot features as labeled in Figure 1. Protein names and database accession numbers are derived from the SwissProt database, except where marked with an **asterisk**, in which case they are from the NCBInr database. Mascot scores and corresponding expect values indicate the high degree of significance for each assignment. Protein expression levels, normalized to total protein, are shown qualitatively (as verified across staining types and replicate gels) across B cell proliferative states. −, +, ++, and +++ indicate 0–25%, 25–50%, 50–75%, and 75–100% normalized expression levels, respectively. R, normal resting B cells; P, normal mitogenically stimulated proliferating B cells; L, malignant proliferating lymphoma B cells.

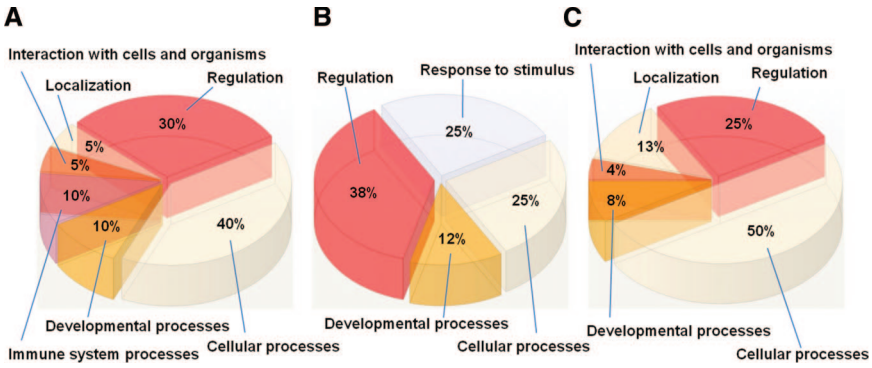

Figure 3. Biological Process GO functional annotation of proteins dramatically up-regulated in each B cell proliferative state. Proteins assigned by matrix-assisted laser desorption ionization/time of flight MS and peptide mass fingerprint were annotated with GO functional tags and graphed to show the relative contributions of each functional assignment within the protein groups. Shown is the GO supercategory of Biological Process. **A:** Proteins up-regulated in normal resting B cells. **B:** Proteins up-regulated in normal, mitogenically stimulated, proliferating B cells. **C:** Proteins up-regulated in proliferating lymphoma B cells.

These relative expression differences were then subjected to principal component analysis and hierarchical clustering. Clusters were grouped according to expression changes, as shown in Figure 5. At a relatedness distance metric of 0.5 out of 2.0 on the dendrogram (Figure 5A), granularity became sufficient that functionally relevant expression trends were apparent in the clusters (Figure 5B; members of each expression cluster that were identified are shown in Table 2). Clusters i and vii contain proteins (or protein isoforms) the behavior of which is similar between the normal proliferative and lymphoma states, but different from the resting state (ie, a proliferation-associated signature), wherein i contains proteins that are up-regulated with respect to resting, while vii contains proteins that are down-regulated with respect to resting. Clusters iii, iv, and vi contain proteins the expression behavior of which is similar between the resting and the lymphoma, but different from the normal proliferative state, wherein iii and iv contain proteins that are down-regulated with respect to the proliferative state, while vi contains proteins that are up-regulated with respect to the proliferative state. Clusters ii, v, and viii contain proteins the expression behavior of which for the lymphoma state is different from both the resting and the nonmalignant proliferative states. This may be regarded as a malignancy-associated protein expression pattern. Cluster viii contains proteins that are down-regulated in lymphoma B cells by comparison with the other two states. Clusters ii and v contain proteins that are up-regulated in lymphoma cells with respect to the other states. Thus, the expression clusters ii, v, and viii represent a source of malignancy-associated biomarkers. The significance of the result lies in the identification of proteins of known function that are uniquely associated with malignancy, and not with normal proliferation.

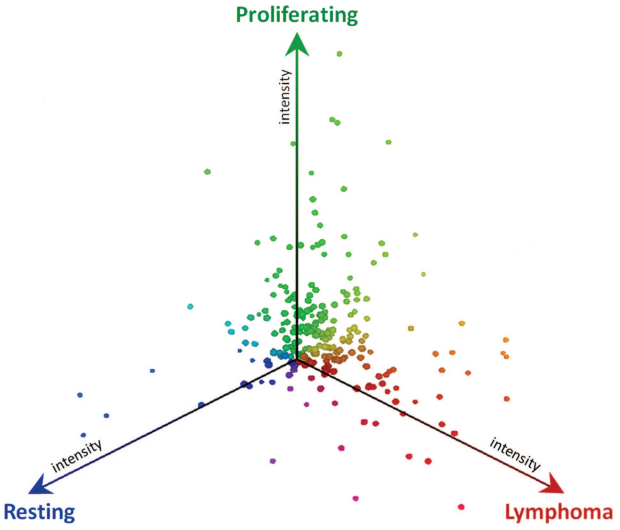

Figure 4. Expression difference profiling of protein spots between proliferative states. Gel spot feature intensity was quantified for each of the B cell proliferative states (Figure 1). Relative differences in expression levels between proliferative states were calculated and are shown plotted on a difference vector diagram, in which each spot feature is represented as a point with coordinates in the three intensity difference axes of resting, proliferating and lymphoma (reflecting its expression level difference for each state). The distance of the points from the origin (the point representing equivalent expression across all of the states) toward one set of axes or another quantitatively reflects the expression level bias of the protein spots they represent toward one or another state.

Discussion

Previous genome-wide transcriptional analyses of DLBCL, originally pioneered by Staudt, Shipp, and others,[4–7] were able to link disease severity, specific pathological characteristics and prognosis to transcriptional signature. We used normal resting and mitogenically stimulated proliferating B cells in a well-controlled model system to extend this approach by defining axes of gene expression that are related to normal proliferation or malignant proliferation, but not both, which enabled a malignancy-associated transcriptional signature to be deduced.[9] From these studies, the hypothesis followed that a limited and reproducible set of malignancy-associated protein biomarkers could be identified in particular lymphoid malignancies; most importantly, these proteins would be different from the vast majority of proteins associated with normal cellular states, including normal proliferation.

Specifically, here we sought to define a simplified set of potential biomarkers of lymphoid malignancy, exploiting our Tg model to analyze the proteomic signatures of DLBCL, while subtracting out the signatures of both proliferating and resting normal B cells. Using 2D-PAGE, MS and peptide mass fingerprint analyses, we identified highly expressed protein species that were differentially expressed in each of the three B cell proliferative states. More detailed expression profiling and clustering analysis elucidated common expression trends that can be

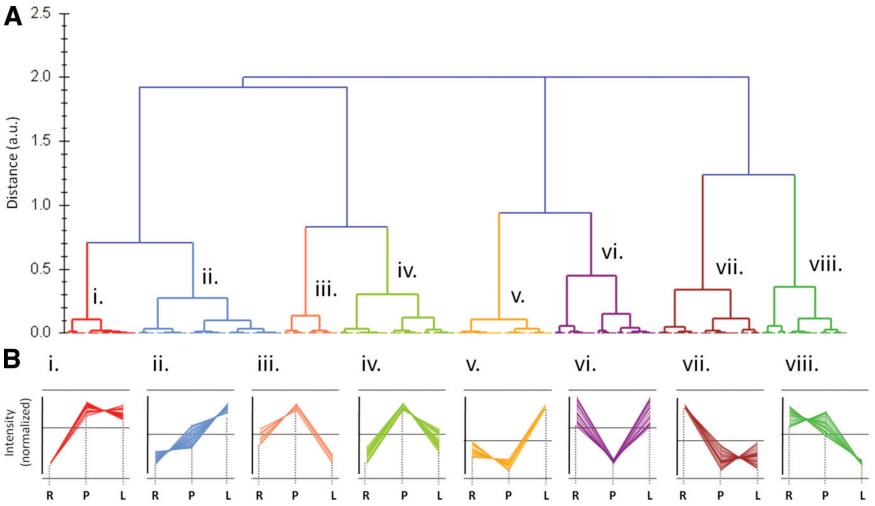

Figure 5. Hierarchical clustering of protein expression profiles across B cell proliferative states. Expression profiles of gel spot features were subjected to unsupervised clustering into expression trend groups. **A:** Dendrogram delineating the relatedness clusters of the expression profiles of the major sets of protein spots that varied between B cell proliferative states. Left axis, relatedness distance metric (in arbitrary units). Clusters at relatedness distance below the arbitrary value of 0.5 are labeled **i–viii**. **B:** Expression trends displayed by each cluster **i-viii** from **A**. Normalized spot intensities are graphed for each member of the cluster. R, normal resting B cells; P, normal mitogenically stimulated proliferating B cells; L, malignant proliferating lymphoma B cells. The average value for each cluster is shown in each graph by a horizontal line. Clusters **i** and **vii** represent a proliferative signature. Clusters **ii**, **v**, and **viii** represent potential malignancy-associated biomarkers.

used for classifying potential malignancy-associated biomarkers and distinguishing them from markers that are shared with normal resting or normal proliferating cells. We defined specific clusters of protein isoform expression that appeared to constitute lymphoma-associated markers, because they were specifically differentially up-regulated in lymphoma, and were different from clusters that constituted merely a proliferative signature, which was nonspecific for lymphoma. We also defined a set of potential markers that are down-regulated in lymphoma with respect to both resting and normal proliferating cells, which might reflect tumor suppressor-like properties of the proteins.

DLBCL-Associated Proteome

In particular, we found that several proteins of interest were more highly expressed in resting B cells than in lymphoma cells, including factors that are involved in cell cycle regulation, such as leukemia inhibitory factor (Lif), which is an autocrine/paracrine B cell regulator that me-

Table 2. Proteins Grouped into Eight Co-Expression Clusters Associated with B Cell Proliferative States

Cluster i	Cluster ii	Cluster iii	Cluster iv
Coronin 1A (isoforms)	hnRNP A2/B1 (isoforms)	Hematopoietic cell-specific	Calreticulin
Dihydrolipoyl dehydrogenase	Macrophage capping protein	Lyn substrate 1	Endoplasmin
Alpha enolase	Rho GDI 2	Hsp 90b	Moesin (isoform)
Pyruvate kinase M2	Coronin 1A (isoform)	Moesin (isoform)	Coronin 1A isoforms
	LIM and SH3 domain 1	EF-2 (isoforms)	EF-2 (isoforms)
	Cofilin-1	GRP-78	
	Glutamate dehydrogenase	Coronin 1A (isoform)	
	hnRNP L (isoforms)		
	High mobility group protein B1		
	Tropomyosin α3		
	Peptidyl-prolyl *cis-trans* isomerase A (isoform)		

Cluster v	Cluster vi	Cluster vii	Cluster viii
Aldose reductase	UTP-glucose-1-phosphate	hnRNP U	Chloride intracellular
hnRNP A3 (isoform)	uridylyltransferase 2 (isoform)	Splicing factor 3b	channel protein 1
Serpin b1a protein	High mobility group protein B2	homologue	Prohibitin
hnRNP L (isoform)	Matrin-3	Ig μ C region	Nucleophosmin
Adenylyl cyclase-associated	hnRNP A/B	DEK protein	Unnamed protein product
protein 1	hnRNP H1 (isoform)	Actin-like protein 6A	similar to 30 hypoxia
Ran	Hsp 71	Eukaryotic translation	Up-regulated-1
		initiation factor 3	hnRNP H1 (isoform)
		Vimentin	EF-2 (isoform)
		Peptidyl-prolyl *cis-trans*	Transitional ER ATPase 1
		isomerase A (isoform)	hnRNP K
		Tubulin α6	
		hnRNP H1 (isoform)	
		Splicing factor 3A	

Many of the spot features included in the expression trend groups shown in Figure 5, or their isoforms were assigned by matrix-assisted laser desorption ionization/time of flight MS and peptide mass fingerprint analyses. Listed are the classifications of the assigned proteins or their isoforms into the expression trend groups i–viii.

diates B cell arrest through the Ras and Jak/Stat pathways,[26] prohibitin, a tumor suppressor and cell cycle regulator that plays role in growth regulation of fibroblasts,[27,28] which our group has previously shown is an important negative regulator of E2Fs,[29] and matrin-3 (p130), a cell cycle regulator that mediates cell cycle arrest by p130 and regulates Cdk2.[30] We also identified heat shock cognate 71 kd protein (Hsp70), which is a chaperone protein associated with growth inhibition in lymphoid cells[31] and was more highly expressed in resting B cells than in lymphoma cells. This resting B cell group included proteins that are important for chromatin remodeling, such as actin-like protein 6A (Baf53a), which forms a repressor complex with Rb, a histone deacetylase that inhibits transcription of certain E2F target genes such as *cyclin E*, *cyclin A,* and *CDC2*[32] and suppresses p53-dependent transcription[32]; DEK, a chromatin remodeling protein and proto-oncogene product that is phosphorylated by Cdk2[33,34]; and heterogeneous nuclear ribonucleoprotein U (SAF-A; scaffold attachment factor A), a chromatin remodeling factor together with p300 (and likely p/CAF), which primes sites for transcriptional activation and regulates transcription through scaffold/matrix attachment regions (S/MARs), the chromatin regions that bind the nuclear matrix.[35] We also identified mRNA splicing factors, heterogeneous nuclear ribonucleoprotein H (hnRNP H), and splicing factor 3B subunit 2 (SAP 145). Additionally, in resting B cells we detected elevated levels of vimentin, a B cell intermediate filament protein important for cell structure, that is reduced in B cells as they mature,[36] consistent with the observation that some lymphoma lines lack vimentin altogether. Immunoglobulin μ-chain constant region, membrane-bound and secreted forms, and tubulin α-6 chain were also present in this group.

Other proteins were present in relatively equal amounts in resting and normal proliferating B cells, a group that defines normal B cell biology, including the transitional endoplasmic reticulum ATPase, p97 (valosin-containing protein), which is a molecular chaperone[37] with cell cycle control properties that is responsible for degradation of cyclin E, is involved in the transcriptional activation of NFκB, and is expressed at high levels in rapidly dividing cells.[37] Valosin is up-regulated after B cell stimulation and is required for normal cell growth control.[37] This normal B cell group also included nucleophosmin, which binds the tumor suppressors p53 and p19^{arf} and is essential for ribogenesis, cell proliferation and survival after DNA damage, but is often mutated in human lymphoma and leukemia, and is associated with induction of proliferation in B cells[38,39]; and elongation factor 2, which promotes the GTP-dependent translocation of the nascent protein chain from the A-site to the P-site of the ribosome.[40]

In normal proliferating B cells, as expected, we identified factors that are important for cell cycle control such as calreticulin, which is a calcium-dependent regulator of p53 transcription[41]; endoplasmin (polymorphic tumor rejection antigen 1, tumor rejection antigen gp96), which is necessary for the Raf-1-MEK-MAPK signaling pathway; heat shock protein Hsp 90-β (tumor-specific transplantation 84-kd antigen), also necessary for Raf-1-MEK-MAPK signaling[42]; hematopoietic lineage cell-specific protein (HS1), which is required for B cell activation and B cell antigen receptor-mediated signaling[43]; and adenylyl cyclase-associated protein 1 (CAP-1), which is a multifunctional protein that activates Ras by posttranslational modification, directly regulates filament dynamics, and has been implicated in a number of complex developmental and morphological processes.[44] We identified proteins with roles in translation:78 kd glucose-regulated protein Hspa5 (Bip), which is required for B cell maturation into antibody secreting cells,[45] and splicing factor 3A subunit 3 (SAP 61). We identified proteins involved in energy metabolism, such as pyruvate kinase isozyme M2, which controls glycolysis necessary for cell proliferation.[46] We found that normal proliferating B cells also overexpress a large group of transcription-related proteins, including heterogeneous nuclear ribonucleoprotein K, which assembles either transcriptional repressors or activators on DNA, accounting for the observations that K protein can either increase or decrease rates of transcription and that K protein is required for a p53 mediated cell cycle checkpoint.[47,48]

The malignancy-associated proteome identified several up-regulated proteins known not to be up-regulated during normal proliferation. The proteins up-regulated in lymphoma cells include proteins that are important for energy metabolism, such as aldose reductase, which has been shown to be up-regulated in many cancers, specifically liver and colon cancer, and indirectly mediates the expression of COX-2 and production of PGE[49,50]; α-enolase, a glycolytic enzyme that has been implicated in the differentiation of lymphoma[51]; dihydrolipoyl dehydrogenase, a mitochondrial enzyme that is a component of the glycine cleavage system and the α-ketoacid dehydrogenase complexes associated with the pyruvate dehydrogenase complex; glutamate dehydrogenase 1, a mitochondrial enzyme that supplements energy metabolism in mitogenically stimulated B cells and lymphoid cells; and UTP-glucose-1-phosphate uridylyltransferase 2. The lymphoma-associated group also included up-regulated isoforms of proteins of importance for the cell cycle such as coronin-1A (p57), which is a tumor suppressor gene and is known to be involved in lymphoid malignancies[52]; the GTP-binding nuclear protein Ran, which directly regulates hepatocarcinoma-up-regulated protein, which interacts with several mitotic spindle assembly factors[53]; peptidyl-prolyl *cis-trans* isomerase A (cyclophilin), which is overexpressed in many cancers[54] and has been found to stimulate cell proliferation through CD147 and activation of ERK1/2 and p38 MAPKs[54]; and rho GDP-dissociation inhibitor 2, which has been implicated as a tumor suppressor gene.[55] The malignancy-associated signature also included proteins that are important for cell structure, such as cofilin-1 (non-muscle isoform), a lim-K1 substrate that has been shown by gene array to be unregulated in metastases[56,57]; LIM and SH3 domain protein 1 (Lasp-1), which plays a role in cell motility by changing cell structure and is implicated in breast cancer[58]; macrophage capping protein (myc basic motif homolog 1) (CapG), which shows increased expression in certain cancers and appears to be a tumor promoter[59,60]; and membrane-orga-

nizing extension spike protein (moesin), which is involved in connecting major cytoskeletal structures to the plasma membrane and in cell motility. Last, the malignancy-associated group included numerous heterogeneous nuclear ribonucleoproteins, which have a variety of biological functions, have many implications in cancer, and have been suggested as potential cancer biomarkers, including heterogeneous nuclear ribonucleoprotein A/B, which interacts with the OPN promoter, decreasing OPN promoter activity and mRNA levels,[61] which correlates with metastatic behavior, motility, and invasion in breast cancer.[62] This ribonucleoprotein is also involved in telomere biogenesis and is important for B cell proliferation; its up-regulation has been found to be consistent with cancer and cell proliferation.[61] Also identified were the heterogeneous nuclear ribonucleoproteins A3, L, and A2/B1, which have been shown to be unregulated in many cancers and play a role in mRNA splicing and cell cycle control,[61,63] along with high mobility group (HMG) proteins B1 and B2,[64] which are oncogenes and cause highly aggressive lymphoid malignancy in mice,[65] are required for c-*myc* function, are overexpressed in human leukemia, and bind chromatin as architectural regulators of transcription.[64]

A Generalizable Approach to Cancer-Associated Proteomes

Proteomic and transcriptional profiling tools have the potential to reveal the signatures of cancer. Combining proteomic and transcriptional signatures of the same murine B cell malignancies yields largely orthogonal sets of biomarkers, consistent with the experience of others.[18,66,67] We found that proteomic signatures correlated with differentially expressed genes[9] for heat shock protein 8, immunoglobulin heavy chain, vimentin, splicing factor 3b and heterogeneous nuclear ribonucleoprotein U in normal, resting cells. Proteomic identifications agreed with transcriptome for pyruvate kinase isozyme M2 in normal proliferating cells and for Ran GTP-binding nuclear protein, high mobility group protein B1, LIM, and SH3 domain protein 1 and serpin b1a in proliferating malignant cells. Discordance between proteomic profiling and gene expression profiling datasets derived from the same animal model will likely resolve as both technologies advance. Within each methodological approach, robust correlations can be obtained between clinical variables and transcriptional signatures, such as the DLBCL analyses already discussed.[5] However, we concur that transcriptional data alone must be used with great caution in drug discovery efforts, because transcriptional readouts are often of greater distance from biological function than are proteomic, phosphoproteomic, or metabolomic readouts.[16–18,66] These methods of profiling are complementary and non-redundant.[16] Although gene expression profiling provided information on the differential expression of a vastly larger number of features (22,690 transcriptional probe sets[9]), proteomic analysis arguably provides a more direct view of the cell's architecture and protein machinery that constitute the normal and disease-altered cell states. Thus, the cancer-specific proteome offers a unique and highly useful set of biomarkers for disease detection and differential diagnosis.

Multiple biomarkers that constitute a signature of a disease state ultimately can have much stronger diagnostic and predictive power among a heterogeneous population than any single biomarker alone.[4–6] Thus, a constellation of protein markers, each associated with a specific subtype of lymphoma, would provide a biomarker set for the accurate detection and determination of the malignancy status of a patient, and when correlated with International Prognostic Index markers, would establish criteria for prognostic classification. Although we have shown that several proteins that we have identified through MS-based proteomic methodologies appear to constitute a malignancy-associated signature, the usefulness of these proteins as potential biomarkers of lymphoma remains to be qualified, verified and validated in this and other models, as well as in human DLBCL cell lines and patient samples.[68] Similarly, although these state-specific markers hold great promise for our understanding of lymphomagenesis, maintenance, and progression, functional hypothesis testing will be required to reveal their role in the pathogenesis of DLBCL.

Nevertheless, we demonstrate the potential utility of comparing the proteome of malignant B cells with normal resting and proliferating counterparts. Subtraction of non-malignancy signals effectively simplifies proteomic biomarker discovery and analysis. This approach represents a paradigm ideally applied in personalized medicine and diagnosis of B cell malignancies; a patient's unique lymphoma-associated protein signature may thus be resolved from the background profile of normal B cell proliferative proteins. Significant progress toward this goal in genomic terms has been reported recently for the genetic signature of M1 subtype acute myeloid leukemia compared with normal skin from the same patient.[69] Given the importance of phosphorylation cascades elicited by abnormal growth factor receptor activity and cytokine signal transduction in lymphoid and myeloid cancers, the malignancy-associated phosphoproteome is clearly of crucial importance to deduce for individual patients as well. Biomarker discovery of this type should aid personalized diagnosis and allow individualized tracking of chemotherapeutic efficacy and improved, earlier detection of relapse. Notably, it may be extended to biomarker discovery strategies in many other types of lymphoid and myeloid malignancy, including cancers of the macrophage, monocyte, neutrophil, and granulocyte lineages, as well as endothelial cells.

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