BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

Primary Effusion Lymphoma

Secretome Analysis Reveals Novel Candidate Biomarkers with Potential Pathogenetic Significance

Annunziata Gloghini,^{*} Chiara C. Volpi,^{*} Dario Caccia,[†] Ambra V. Gualeni,^{*} Anna M. Cilia,[‡] Antonino Carbone,[‡] and Italia Bongarzoni[†]

From the Department of Diagnostic Pathology and Laboratory Medicine,^{*} and the Proteomics Laboratory,[†] Department of Experimental Oncology and Molecular Medicine, Fondazione IRCCS, Istituto Nazionale dei Tumori, Milano; and the Department of Pathology,[‡] Centro di Riferimento Oncologico Aviano (CRO Aviano National Cancer Institute), Istituto Nazionale Tumori (National Cancer Institute), IRCCS, Aviano, Italy

CME Accreditation Statement: This activity ("ASIP 2014 AJP CME Program in Pathogenesis") has been planned and implemented in accordance with the Essential Areas and policies of the Accreditation Council for Continuing Medical Education (ACCME) through the joint sponsorship of the American Society for Clinical Pathology (ASCP) and the American Society for Investigative Pathology (ASIP). ASCP is accredited by the ACCME to provide continuing medical education for physicians.

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CME Disclosures: The authors of this article and the planning committee members and staff have no relevant financial relationships with commercial interests to disclose.

Accepted for publication
November 26, 2013.

Address correspondence to
Antonino Carbone, M.D.,
Department of Pathology, Cen-
tro di Riferimento Oncologico
Aviano, Istituto Nazionale
Tumori, IRCCS, Via F Gallini
2, 33081 Aviano, Italy. E-mail:
acarbone@cro.it.

Primary effusion lymphoma (PEL) is a rare B-cell neoplasm in which tumor cells are consistently infected by Kaposi's sarcoma-associated herpesvirus and usually grow in body cavities without tumor mass formation. To detect new proteins related to pathogenesis, four established cell lines from PEL (CRO-AP2, CRO-AP3, CRO-AP5, and CRO-AP6) were characterized by proteomics analysis of the secretome. The secretomes were analyzed using two complementary mass spectrometry platforms: liquid chromatography-mass spectrometry and matrix-assisted laser desorption/ionization time-of-flight-based approaches. Among 266 proteins identified from the proteomics analysis, 139 were considered as predicted secreted. Twenty proteins were specifically secreted by PEL cell lines after comparison with secretomes of human cell lines representative of diverse solid tumors and leukemias. More important, 27 additional proteins were shared by all CRO-AP PEL cell lines. The presence of these proteins was confirmed by IHC in CRO-AP cell lines and in six other PEL cell lines, four PEL clinical samples, and three extracavitary Kaposi's sarcoma-associated herpesvirus-positive solid lymphomas included for comparative analysis. Functional classification showed that PEL cell secretomes were enriched in proteins specifically involved in inflammation/immune response, growth/cell cycle, and mRNA processing, in addition to structural/matrix proteins and proteins with enzymatic activity. (*Am J Pathol* 2014, 184: 618–630; <http://dx.doi.org/10.1016/j.ajpath.2013.11.028>)

Primary effusion lymphoma (PEL) is a rare B-cell neoplasm characterized by Kaposi's sarcoma-associated herpesvirus (KSHV) infection of the tumor clone and by liquid growth in fluid-filled body spaces.¹ During its entire clinical course, the lymphoma tends to remain localized to the serous body cavities without mass formation.^{1,2} Pathologically, the basic features of these lymphomas are net predilections for diffuse spreading along the serous membranes without infiltrative or destructive growth patterns.^{3,4} As seen at autopsy or incidentally revealed by computed tomography scan, PEL presents as multiple small

tumor foci involving the serous membranes, which appear irregularly thickened.⁵

PEL is composed of B cells that bridge immunoblastic and anaplastic features and typically display a non-B, non-T

Supported in part by a grant from the Ministry of Health, Rome, within the framework of the Progetto Integrato Oncologia—Advanced Molecular Diagnostics project grant RFPS-2006-2-342010.7 (A.C.) and by a grant from CRO Aviano National Cancer Institute for an intramural project "Infectious Agents and Tumors" (A.C.).

Disclosures: None declared.

Table 1 Antibodies Used for IHC Studies

Antibody (clone)	Source	Manufacturer	Working solution
Ezrin (EPR803)	Rabbit monoclonal	Epitomics Inc. (Burlingame, CA)	1:1000
Moesin (EP1863Y)	Rabbit monoclonal	Epitomics Inc.	1:500
High-mobility group box 1 (EPR3507)	Rabbit monoclonal	Epitomics Inc.	1:1000
Galectin 1	Rabbit polyclonal	Abcam Inc. (Cambridge, MA)	1:1500
Stathmin	Rabbit polyclonal	Cell Signaling Technology Inc. (Danvers, MA)	1:50
Granzyme A (GA6)	Mouse monoclonal	Abcam Inc.	1:100
S100 calcium-binding protein A6 (EPNCIR121)	Rabbit monoclonal	Epitomics Inc.	1:1000
Catalase (EP1929Y)	Rabbit monoclonal	Epitomics Inc.	1:100
Glutathione transferase K class (EPR1939)	Rabbit monoclonal	Epitomics Inc.	1:500
Arginine methyl transferases 1 (EPR3292)	Rabbit monoclonal	Epitomics Inc.	1:500
Poly(rC)-binding protein 2 (5F12)	Mouse monoclonal	Abcam Inc.	1:700

phenotype consistent with late stages of B-cell differentiation.^{1,2,4} PEL pathogenesis is poorly understood, although KSHV is thought to play a major role via expression of several viral latent genes that have the potential to affect B-cell growth.⁶ Other factors involved in PEL pathogenesis include deregulation of cytokine and growth factor autocrine loops, molecular alterations of the tumor DNA, cell cycle abnormalities, stimulation and selection by antigen, and infection by Epstein-Barr virus, which occurs in 70% of PEL cases.^{6,7} KSHV viral infection alone is not sufficient for tumor development, and lesions of cellular genes may be required for PEL lymphomagenesis. In fact, recent studies on pathogenesis of PEL using gene expression profiling, comparative genomic hybridization, and proteomic analyses have shown extensive genomic gains/amplifications and losses and overexpression of proteins, which are critical for cell migration and chemotaxis, and for cell motility.^{8–11}

To detect new proteins related to pathogenesis of PEL, this work performed an in-depth analysis of the secretome (conditioned cell media) of PEL cell lines. A recent strategy for discovery of cancer biomarkers involved in pathogenesis is based on the identification of proteins in cancer tissue—proximal fluids and in the conditioned media of cell lines (secretomes); because these fluids are close to the tumor cells, they may be enriched with secreted or shed proteins. Cell secretomes (cell-conditioned media) comprise proteins that are secreted or shed from the cell surface, as well as intracellular proteins released into the supernatant as the result of vesiculation, cell lysis, apoptosis, and necrosis. Furthermore, the secretome can accurately reflect the functional state of secreting cells at specific time points.^{12–14} It is commonly known that proteins secreted by cancer cells play important roles in cell interaction, adhesion, and invasion. To specifically study the secreted proteins, serum-free culture conditions have been applied successfully in some studies on cancer cell lines, including solid tumors and Hodgkin lymphoma.¹⁵ By secretome analysis of PEL cell lines, we identified specific proteins involved in growth, development, and maintenance of the tumor microenvironment (angiogenesis and inflammation), providing an important resource to study PEL biological characteristics and/

or facilitate the discovery of novel PEL biomarkers with potential pathogenetic significance.

Materials and Methods

Cell Lines

Four PEL cell lines, named CRO-AP2,¹⁶ CRO-AP3,¹⁷ CRO-AP5,¹⁷ and CRO-AP6,¹⁸ were the basis of this study. All cell lines have been authenticated^{19,20} and comparatively reviewed⁷ (Supplemental Figure S1). All cell lines were cultured in RPMI 1640 medium (GIBCO, Carlsbad, CA) supplemented with 20% heat-inactivated fetal bovine serum (FBS; GIBCO), 100 IU/mL penicillin/streptomycin (PenStrep; GIBCO), and

Figure 1 Methods used in the present study. MLC, mass spectrometry liquid chromatography.

Table 2 Cell Culture Conditions, Number of Cells Counted, Mortality, and Amount of Secretome

Cell line	FBS (%)	No. of cells (approximation)	Mortality (%)	Secretome volume (μL)	Absorbance (nm)	Protein concentration (mg/mL)
CRO-AP2	0	33 × 10 ⁶	9	300	0.4956	12,755.77
CRO-AP3	0	31 × 10 ⁶	10	300	0.1766	3553.85
CRO-AP5	0	84 × 10 ⁶	9.5	300	0.2303	5082.69
CRO-AP6	0	53 × 10 ⁶	5	300	0.4714	12,034.62

2 mmol/L L-glutamin (Lonza, Verviers, Belgium) under a 5% CO₂ atmosphere at 37°C. Formalin-fixed, paraffin-embedded cell blocks from PEL cell lines were used for immunohistochemistry (IHC) and *in situ* hybridization (ISH) analyses.

Before secretome preparation, the viral status of PEL cell lines was confirmed by IHC and ISH.

Cell blocks obtained from other PEL-derived cell lines, BC-1,²¹ and its sister cell line, HBL-6,²² BC-2,²¹ BC-3,²³ BCBL-1,²⁴ and BCP-1,²⁵ obtained from Prof. Gianluca Gaidano (Division of Hematology, Department of Translational Medicine, Amedeo Avogadro University of Eastern Piedmont, Novara, Italy), were also used for IHC analyses.

Primary Neoplastic Samples

Four PEL clinical samples and three primary extracavitary KSHV-positive solid lymphomas⁴ were investigated for the

expression of proteins identified in the secretome of PEL cell lines. The cases belonged to a single institution series observed at the Centro di Riferimento Oncologico, National Cancer Institute of Aviano, Italy. All cases were diagnosed and classified according to morphological, immunophenotypical, and virological features previously reported^{1,4} and acknowledged by the 2008 World Health Classification of Tumors of Hematopoietic and Lymphoid Tissues.²

Secretome Collection and SDS-PAGE

Cell lines were cultured in FBS-free RPMI 1640 medium, without phenol red and with 100 UI PenStrep and 2 mmol/L L-glutamine, to have at least 3 × 10⁷ cells. After 16 to 18 hours, cells were collected and their viability was verified. If the cell death rate was <10%, the secretome samples in conditioned media were collected. The conditioned media

Table 3 Proteins Shared by PEL Secretomes

Protein (symbol)	Function/biological process*
α-Actinin-4 (ACTN4)	F-actin cross-linking protein that is thought to anchor actin to a variety of intracellular structures. This is a bundling protein that is probably involved in vesicular trafficking via its association with the CART complex. The CART complex is necessary for efficient transferrin receptor recycling but not for EGFR degradation.
Fructose-bisphosphate aldolase A (ALDOA)	Plays a key role in glycolysis and gluconeogenesis. In addition, may also function as scaffolding protein.
β-2-Microglobulin (B2MG)	Component of the class I MHC. Involved in the presentation of peptide antigens to the immune system.
Calreticulin (CALR)	Molecular calcium-binding chaperone promoting folding, oligomeric assembly, and quality control in the ER via the calreticulin/calnexin cycle. This lectin interacts transiently with almost all of the monoglucosylated glycoproteins that are synthesized in the ER. Interacts with the DNA-binding domain of NR3C1 and mediates its nuclear export.
Clathrin heavy chain 1 (CLH1)	Clathrin is the major protein of the polyhedral coat of coated pits and vesicles. Two different adapter protein complexes link the clathrin lattice to either the plasma membrane or the trans-Golgi network.
Cofilin-1 (COF1)	Positive regulation of actin filament depolymerization and protein phosphorylation. Regulation of actin cytoskeleton dynamics. Important for normal progress through mitosis and normal cytokinesis. Establishment of cell polarity and response to amino acid stimulus.
α-Enolase (ENOA)	Glycolysis. Growth control, hypoxia tolerance, and allergic responses. Receptor and activator of plasminogen on the cell surface of several cell types, such as leukocytes and neurons. Stimulates immunoglobulin production. Transcription and transcription regulation.
EZRI	Probably involved in connections of major cytoskeletal structures to the plasma membrane. In epithelial cells, required for the formation of microvilli and membrane ruffles on the apical pole. Cell shape.
Glyceraldehyde-3-phosphate dehydrogenase (G3P)	Gluconeogenesis, glycolysis, protein stabilization, transcription, RNA transport, DNA replication, and apoptosis.
Glucose-6-phosphate isomerase (G6PI)	Angiogenesis, gluconeogenesis, glycolysis, hemostasis, humoral immune response. Function as a tumor-secreted cytokine and an angiogenic factor (AMF) that stimulates endothelial cell motility.
HMGB1	DNA fragmentation involved in apoptotic nuclear change, DNA topological change, V(D)J recombination, base-excision repair, DNA ligation, dendritic cell chemotaxis, inflammatory response to antigenic stimulus, innate immune response, negative regulation of RNA polymerase II transcriptional preinitiation complex assembly, positive regulation of DNA binding, positive regulation of apoptosis, positive regulation of caspase activity, and positive regulation of transcription from RNA polymerase II promoter.

(table continues)

Table 3 (continued)

Protein (symbol)	Function/biological process*
Heat shock cognate 71 kDa protein (HSP7C)	Cellular membrane organization, interspecies interaction between organisms, mRNA metabolic process, negative regulation of transcription, DNA-dependent, neurotransmitter secretion, post-Golgi vesicle-mediated transport, protein folding, response to unfolded protein, transcription, DNA dependent. Inhibits the transcriptional coactivator activity of CITED1 on Smad-mediated transcription. Chaperone. Isoform 2 may function as an endogenous inhibitory regulator of HSC70 by competing with cochaperones.
Eukaryotic initiation factor 4A-I (IF4A1)	Nuclear-transcribed mRNA poly(A) tail shortening. ATP-dependent RNA helicase that is a subunit of the eIF4F complex involved in cap recognition and is required for mRNA binding to small ribosomal subunit, and subsequent scanning for the initiator codon.
L-Lactate dehydrogenase A chain (LDHA)	Glycolysis.
L-Lactate dehydrogenase B chain (LDHB)	Glycolysis.
LEG1	May regulate apoptosis, cell proliferation, and cell differentiation, binds β -galactoside and a wide array of complex carbohydrates. Inhibits CD45 protein phosphatase activity and, therefore, the dephosphorylation of Lyn kinase.
MOES	Leukocyte cell-cell adhesion, leukocyte migration, membrane to membrane docking. Probably involved in connections of major cytoskeletal structures to the plasma membrane.
Nucleophosmin (NPM)	Host-virus interaction, CenH3-containing nucleosome assembly at centromere, DNA repair, antiapoptosis, cell aging, centrosome cycle, interspecies interaction between organisms, intracellular protein transport, negative regulation of cell proliferation, negative regulation of centrosome duplication, nucleocytoplasmic transport, positive regulation of NF- κ B transcription factor activity, protein oligomerization, regulation of endodeoxyribonuclease activity, regulation of endoribonuclease activity, ribosome biogenesis, signal transduction, protein chaperoning, histone assembly, and regulation of tumor suppressors p53/TP53 and ARF.
Proliferating cell nuclear antigen (PCNA)	Involved in the control of eukaryotic DNA replication, induces a robust stimulatory effect on the 3'-5' exonuclease and 3'-phosphodiesterase, stimulates APEX2. Cell proliferation, mismatch repair, nucleotide-excision repair, DNA gap filling, phosphatidylinositol-mediated signaling, positive regulation of deoxyribonuclease activity, regulation of transcription involved in G ₁ /S phase of mitotic cell cycle, telomere maintenance via recombination, telomere maintenance via semiconservative replication, transcription-coupled nucleotide-excision repair, and translesion synthesis.
Plastin-2 (PLSL)	Actin-binding protein. Plays a role in the activation of T cells. Modulates the cell surface expression of IL2RA/CD25 and CD69, and regulates intracellular protein transport.
Peptidyl-prolyl <i>cis-trans</i> isomerase A, cyclophilin A (PPIA)	RNA-dependent DNA replication, entry into host cell, leukocyte migration, platelet activation, platelet degranulation, protein folding, provirus integration, regulation of viral genome replication, uncoating of virus. It catalyzes the <i>cis-trans</i> isomerization of proline imidic peptide bonds in oligopeptides.
Peroxiredoxin-1 (PRDX1)	Redox regulation of the cell. Role in eliminating peroxides generated during metabolism. Participates in the signaling cascades of growth factors and tumor necrosis factor- α . Cell proliferation, cell redox homeostasis, hydrogen peroxide catabolic process, and skeletal system development.
GTP-binding nuclear protein Ran (RAN)	Involved in nucleocytoplasmic transport. Required for the import of protein into the nucleus and for RNA export. Involved in chromatin condensation and control of cell cycle. Plays a role in mitotic spindle formation and enhances AR-mediated transactivation. DNA metabolic process, androgen receptor signaling pathway, cell division, viral genome transport in host cell, and viral infectious cycle.
Adenosylhomocysteinase (SAHH)	Role in the control of methylations and one-carbon metabolism.
STMN1	Involved in the regulation of the MT filament system. Prevents assembly and promotes disassembly of microtubules. Cell differentiation, intracellular signal transduction, microtubule depolymerization, mitotic spindle organization, and response to virus.
Tubulin β -2C chain (TBB2C)	<i>De novo</i> post-translational protein folding, G ₂ /M transition of mitotic cell cycle, cellular component movement, microtubule-based movement, natural killer cell mediated cytotoxicity, and protein polymerization.
Ubiquitin-like modifier-activating enzyme 1 (UBA1)	Activates ubiquitin, cell death, and protein modification process.

*Derived from UniProt/Swiss-Prot.

AR, androgen receptor; CART, cytoskeleton-associated recycling or transport; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; MHC, major histocompatibility complex; MT, microtubule; redox, reduction-oxidation.

were then centrifuged at $3116 \times g$ for 15 minutes at 4°C to remove cells debris, and finally were concentrated and desalted with 5-kDa cutoff spin concentrators (Agilent Technologies Inc., Wilmington, DE). Protein concentrations of the secretome samples were determined by bicinchoninic acid assay (Bio-Rad Laboratories Srl, Segrate, Italy).

All electrophoresis (SDS-PAGE) experiments were performed with precast 4% to 12% Bis-Tris polyacrylamide NuPAGE NOVEX gels (Invitrogen, Segrate, Italy) and stained with colloidal Coomassie Blue, according to standard procedures.²⁶

Protein identification was performed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and liquid chromatography coupled to high-resolution Orbitrap MS (LC-Orbitrap MS), as previously described.¹³ The same amount of protein (ie, 20 μg) from all cell lines was profiled for secretome analysis.

MS Data

MALDI-TOF MS Protein Identification

For MALDI-TOF MS identification of proteins, secreted proteins were resolved by either SDS-PAGE or OFFGEL fractionator (Agilent Technologies, Milan, Italy) and visualized by silver or colloidal Coomassie Blue staining, according to standard procedures. Protein bands were excised and processed, as previously described.²⁶ Briefly, proteins were in-gel digested with trypsin (13 ng/ μL) for 18 hours at 37°C . The peptide mixture obtained from each band was analyzed by a MALDI-TOF Voyager-DE STR (Applied Biosystems, Framingham, MA) equipped with a nitrogen laser (337 nm). The resulting spectra were analyzed with MASCOT peptide Mass Fingerprint (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF, last accessed August 5, 2011). Input was searched according to the following database: SwissProt; taxonomy, Mammalia; peptide tolerance, 20 ppm; spectrometer internal error maximum, 25. Only proteins identified in at least three separate experiments were considered.

Nanoscale LC-MS/MS Analysis

For nanoscale LC-MS/MS analysis, 20 μg of reduced and carbamylated proteins from secretome samples was digested with Trypsin Gold (1:20, w/w; Promega Corporation, Fitchburg, WI). Peptide separations were performed on a 12-cm reverse-phase spraying fused silica capillary column (75 μm i.d. $\times$ 15 cm), which was packed with 3- μm ReproSil 100 Å C18 (Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany). The LC system was connected to an LTQ Orbitrap MS (ThermoScientific, Bremen, Germany) equipped with a nanoelectrospray ion source (Proxeon Biosystems, Odense, Denmark). Full-scan mass spectra were acquired in the LTQ Orbitrap MS with the resolution set to 60,000. The lock-mass option was used for accurate mass measurements. The acquisition mass/charge ratio range for each sample was from 350 to 1700 Da. The six

most intense doubly and triply charged ions were automatically selected and fragmented in the ion trap after accumulation to a target value of 10,000. Target ions already selected for the MS/MS were dynamically excluded for 45 seconds. All MS/MS samples were analyzed using the Mascot search engine, version 2.2.06 (Matrix Science, London, UK) and X! Tandem, version 2007.01.01.1. X! Tandem and Mascot were set up to search the SwissProt database. For LTQ-Orbitrap data, mass tolerance was set to 5 ppm and 0.6 Da for precursor and fragment ions, respectively. Scaffold, version Scaffold_2_06_00 (Proteome Software Inc., Portland, OR), was used to validate MS/MS-based peptide and protein identifications. Protein thresholds were set to a 99.0% minimum and a two-peptide minimum, whereas peptide thresholds were set to a 95% minimum.

Bioinformatics Analysis

The lists of identified proteins were analyzed and classified using bioinformatics software: Signal Peptide Predictor (SignalP version 4.1; <http://www.cbs.dtu.dk/services/SignalP>, last accessed October 20, 2013) was used to analyze the secretion features of the identified proteins.^{27,28} SignalP uses amino acid sequences to predict the existence and location of signal peptide cleavage sites. A protein is considered to be classically secreted if it receives a signal peptide probability of >0.9 . To identify non-classical, or leaderless, protein secretion, SecretomeP (<http://www.cbs.dtu.dk/services/SecretomeP>, last accessed October 24, 2013) was used.²⁹ SecretomeP uses a neural network that combines six protein characteristics to determine whether a protein is non-classically secreted. These characteristics include the following: number of amino acids, number of positively charged residues, presence of transmembrane helices, presence of low-complexity regions, presence of propeptides, and subcellular localization. A protein is considered non-classically secreted if it receives an NNScore of >0.5 . Finally, it was also possible that proteins located on the plasma membrane were shed and released to the extracellular space. Therefore, TMHMM (<http://www.cbs.dtu.dk/services/TMHMM>, last accessed October 24, 2013) was used to predict transmembrane helices. Some secretory proteins, such as proteins secreted through exosome vesicles, may not pass the SignalP and SecretomeP score cutoffs.^{30–32}

The list of PEL-specific proteins was accomplished by filtering the list of identified proteins against a background database of proteins found in the conditioned medium of tumor cell lines of a different histotype generated in-house.¹³ The database includes 1370 proteins, 717 (52%) of which are either classically or non-classically secreted, and 125 (9%) of which are transmembrane proteins; the remaining proteins are intracellular or nonconventional secreted proteins.

Finally, the PEL secretomes were compared with the secretome from the B-cell lymphoma cell line, DOOH-2 [KSHV⁻/Epstein-Barr virus (EBV)⁻]. Data from DOOH-2 were obtained from an in-house database of secreted

Table 4 Proteins Specifically Detected in Secretomes of PEL Cell Lines

PEL cell line	Protein (symbol)	Function/biological process*
CRO-AP2	Protein arginine N-methyltransferase 1 (ANM1)	Constitutes the main enzyme that mediates monomethylation and asymmetric dimethylation of histone H4 (Arg-4). It represses STAT1 transcriptional activity, in the late phase of interferon γ signaling. May be involved in the regulation of TAF15 transcriptional activity, acts as an activator of estrogen receptor-mediated transactivation and as a negative regulator of megakaryocytic differentiation. Cell surface receptor-linked signaling pathway.
	Catalase (CATA)	Occurs in almost all aerobically respiring organisms and serves to protect cells from the toxic effects of hydrogen peroxide. Promotes growth of cells, including T cells, B cells, myeloid leukemia cells, melanoma cells, mastocytoma cells, and normal and transformed fibroblast cells.
	Glutathione S-transferase κ 1 (GSTK1)	Significant glutathione conjugating activity is found only with the model substrate, 1-chloro-2,4-dinitrobenzene. Outer membrane-bounded periplasmic space, peroxisom.
	Poly(rC)-binding protein 2 (PCBP2)	Involved in innate immune response, negative regulation of defense response to virus, negative regulation of type I interferon production, nuclear mRNA splicing, via spliceosome, proteasomal ubiquitin-dependent protein catabolic process, response to virus.
CRO-AP3	Putative protein FAM10A4 (ST134)	Unknown.
	Cellular nucleic acid-binding protein (CNBP)	Single-stranded DNA-binding protein. Involved in transcription, transcription regulation.
	60S ribosomal protein L24 (RL24)	Endocrine pancreas development, translational elongation, translational termination, and viral transcription.
	60S ribosomal protein L27 a(RL27A)	Same as above.
	60S ribosomal protein L8 (RL8)	Same as above.
	40S ribosomal protein S13 (RS13)	Endocrine pancreas development, negative regulation of RNA splicing, translational elongation, translational termination, and viral transcription.
	40S ribosomal protein S2 (RS2)	Endocrine pancreas development, translational elongation, translational termination, and viral transcription.
	40S ribosomal protein S3a (RS3A)	Involved in cell differentiation, endocrine pancreas development, induction of apoptosis, translational elongation, translational initiation, translational termination, and viral transcription.
CRO-AP5	Chromobox protein homolog 3 (CBX3)	Chromatin remodeling, negative regulation of transcription, DNA dependent, transcription, DNA dependent.
	Ig κ chain C region (IGKC)	Complement activation, classical pathway, innate immune response.
CRO-AP6	Chromobox protein homolog 3 (CBX3)	Chromatin remodeling, negative regulation of transcription, DNA dependent, transcription, DNA dependent.
	Complement C4-A (C04A)	Complement pathway, immunity, inflammatory response, and innate immunity.
	Complement C4-B (C04B)	Same as above.
	Cochlin (COCH)	Hearing.

*Derived from UniProt/Swiss-Prot.

proteins. We applied a clustering method to protein expression matrix in which rows corresponded to proteins and columns to cell lines. Unsupervised clustering only organizes the data based on their expression similarity. In this study, hierarchical clustering was performed on both genes and experiments by using the average linkage method. Eisen's CLUSTER and TREEVIEW software version 1.2 (<http://rana.lbl.gov/EisenSoftware.htm>, last accessed October 24, 2013) was used to perform this analysis and display the results.

Pathway Enrichment Analysis

Functional categories of proteins found in the conditioned media were analyzed using MetaCore version 6.8 build 30387 (Thomson Reuters, St. Joseph, MI), including gene ontology (GO) and canonical pathway maps. The MetaCore program uses GeneGo's MetaBase knowledgebase containing approximately 300,000 protein-protein and protein-small molecule interactions manually extracted from the literature

Table 5 Enrichment Analysis Report of Proteins Identified in Secretomes of PEL Cell Lines

No.	Processes	Total	<i>P</i> value	FDR
Enrichment by GO processes				
1	Cellular macromolecular complex subunit organization	708	1.07×10^{-64}	3.00×10^{-61}
2	Macromolecular complex subunit organization	1352	1.00×10^{-53}	1.41×10^{-50}
3	Catabolic process	2270	6.79×10^{-47}	6.34×10^{-44}
4	Cellular component organization at cellular level	3810	1.01×10^{-42}	3.53×10^{-40}
5	Cellular component organization or biogenesis at cellular level	4596	1.76×10^{-41}	4.12×10^{-39}
		3947	5.09×10^{-41}	9.50×10^{-39}
6	Cellular catabolic process	1910	5.41×10^{-40}	7.58×10^{-38}
7	Cellular component organization or biogenesis	4755	9.84×10^{-40}	1.25×10^{-37}
8	Establishment of localization in cell	1759	2.71×10^{-39}	3.03×10^{-37}
9	mRNA metabolic process	688	4.30×10^{-38}	4.02×10^{-36}
Enrichment by pathway maps				
1	LRRK2 in neurons in Parkinson's disease	33	1.16×10^{-13}	3.73×10^{-11}
2	Glycolysis and gluconeogenesis (short map)	66	2.13×10^{-11}	3.59×10^{-9}
3	Glycolysis and gluconeogenesis p.3/human version	24	7.32×10^{-8}	3.68×10^{-6}
4	Glycolysis and gluconeogenesis p.3	24	7.32×10^{-8}	3.68×10^{-6}
5	Cytoskeleton remodeling_regulation of actin cytoskeleton by ρ GTPases	23	2.79×10^{-7}	2.48×10^{-5}
6	Development_Slit-Robo signaling	30	3.09×10^{-7}	1.24×10^{-5}
7	Immune response_antigen presentation by MHC class I	28	4.85×10^{-7}	3.00×10^{-5}
8	Transcription_role of Akt in hypoxia-induced HIF1 activation	27	7.84×10^{-7}	4.41×10^{-5}
9	Apoptosis and survival_Granzyme A signaling	30	1.61×10^{-6}	7.44×10^{-5}
10	Cytoskeleton remodeling_neurofilaments	25	3.19×10^{-6}	1.07×10^{-4}

(table continues)

The table shows top-ranked pathways from the MetaCore pathway analysis for each cell line. GO processes and pathways were ranked according to *P* values, and FDR adjusted *P* < 0.05 was considered significant.

HIF, hypoxia-inducible factor; MHC, major histocompatibility complex.

by a group of experts.³³ The *P* value is essentially the probability of a particular mapping arising by chance given the number of proteins in the set relative to all proteins on maps/processes, proteins on a particular map/process, and proteins in the analyzed experiment. A pathway or network with *P* ≤ 0.01 was considered as significant.

Version 9.05 of the Search Tool for the Analysis of Interacting Genes/Proteins (STRING; <http://string-db.org>, last accessed October 16, 2013) resource was used.³⁴ The STRING algorithm links genes or proteins into networks based on published functional or informatics-predicted interactions. Herein, reported interactions are predicted with a medium confidence threshold of 0.400. Overrepresented categories were identified relative to the *Homo sapiens* background gene set using a hypergeometric test with a Benjamini and Hochberg false-discovery rate (FDR) correction to define significance.

Moreover, a map of Top Canonical Pathways that may be overrepresented in the PEL secretome was obtained using Ingenuity software (Ingenuity Pathway Analysis, version 9; IngenuitySystems, Redwood City, CA).

IHC and ISH

Both IHC and ISH were performed on an automated immunostainer (BenchMark Ultra; Ventana Medical Systems, Inc., Tucson, AZ), according to the manufacturer's instructions.

The presence of KSHV was ascertained and confirmed in all PEL cell lines by IHC using a mouse monoclonal antibody against the latency-associated nuclear antigen encoded by viral open reading fragment 73 (Mouse Monoclonal; Novacastra Laboratories Ltd, Newcastle Upon Tyne, UK). The infection by EBV was investigated by ISH using a probe against EBER (EBV-encoded small RNA; INFORM EBER—Epstein-Barr Virus Early RNA Probe; Ventana Medical Systems, Inc.). As expected, EBV infection was demonstrated only in CRO-AP2 and CRO-AP5 cells.^{7,16,17}

The expression of panel of protein identified in the secretome of PEL was tested by IHC in both PEL cell lines and primary PEL samples and in extracavitary KSHV-positive solid lymphomas from patients. The selected antibodies used for IHC studies, together with their clone designation, source, and working dilution, are listed in Table 1. The work flow and methods used in the present study are illustrated in Figure 1.

Results

Profiling of the Secretome from PEL Cell Lines

The four cell lines of PEL were maintained in culture to allow the achievement of an adequate number of cells ($>3 \times 10^7$) in the experiment. Subsequently, all cell lines were maintained in a culture medium free of FBS. Our

Table 5 (continued)

CRO-AP2		CRO-AP3		CRO-AP5		CRO-AP6	
P value	FDR	P value	FDR	P value	FDR	P value	FDR
Enrichment by GO processes							
3.67×10^{-38}	8.21×10^{-35}	1.07×10^{-64}	3.00×10^{-61}	1.88×10^{-33}	2.30×10^{-30}	3.37×10^{-16}	1.17×10^{-14}
9.81×10^{-38}	9.18×10^{-35}	1.00×10^{-53}	1.41×10^{-50}	2.48×10^{-26}	5.06×10^{-24}	2.45×10^{-15}	6.90×10^{-14}
5.86×10^{-38}	8.21×10^{-35}	6.79×10^{-47}	6.34×10^{-44}	5.66×10^{-29}	1.54×10^{-26}	2.36×10^{-29}	5.31×10^{-26}
6.41×10^{-27}	7.82×10^{-25}	1.01×10^{-42}	3.53×10^{-40}	1.16×10^{-21}	1.14×10^{-19}	1.26×10^{-18}	8.59×10^{-17}
7.05×10^{-29}	1.16×10^{-26}	1.76×10^{-41}	4.12×10^{-39}	1.15×10^{-19}	7.02×10^{-18}	5.34×10^{-17}	2.48×10^{-15}
1.95×10^{-26}	2.27×10^{-24}	5.09×10^{-41}	9.50×10^{-39}	2.34×10^{-21}	2.05×10^{-19}	1.61×10^{-18}	1.04×10^{-16}
2.55×10^{-28}	3.74×10^{-26}	5.41×10^{-40}	7.58×10^{-38}	1.30×10^{-21}	1.22×10^{-19}	2.14×10^{-21}	2.67×10^{-19}
2.67×10^{-28}	3.74×10^{-26}	9.84×10^{-40}	1.25×10^{-37}	2.53×10^{-19}	1.51×10^{-17}	7.52×10^{-17}	3.32×10^{-15}
1.32×10^{-18}	7.38×10^{-17}	2.71×10^{-39}	3.03×10^{-37}	4.84×10^{-15}	1.98×10^{-13}	8.98×10^{-15}	2.20×10^{-13}
1.12×10^{-14}	3.17×10^{-13}	4.30×10^{-38}	4.02×10^{-36}	8.49×10^{-10}	1.34×10^{-8}	1.83×10^{-6}	1.22×10^{-5}
Enrichment by pathway maps							
4.34×10^{-12}	1.46×10^{-9}	1.16×10^{-13}	3.73×10^{-11}	1.27×10^{-12}	3.94×10^{-10}	2.73×10^{-13}	5.48×10^{-11}
2.13×10^{-11}	3.59×10^{-9}	4.54×10^{-10}	7.33×10^{-8}	1.09×10^{-10}	1.70×10^{-8}	9.88×10^{-9}	9.93×10^{-7}
3.68×10^{-7}	2.48×10^{-5}	3.89×10^{-7}	3.14×10^{-5}	1.79×10^{-7}	1.39×10^{-5}	7.32×10^{-8}	3.68×10^{-6}
3.68×10^{-7}	2.48×10^{-5}	3.89×10^{-7}	3.14×10^{-5}	1.79×10^{-7}	1.39×10^{-5}	7.32×10^{-8}	3.68×10^{-6}
2.79×10^{-7}	2.48×10^{-5}	1.75×10^{-4}	2.83×10^{-3}	1.06×10^{-4}	2.19×10^{-3}	5.92×10^{-5}	1.01×10^{-3}
1.53×10^{-6}	6.43×10^{-5}	1.61×10^{-6}	7.44×10^{-5}	7.50×10^{-7}	3.88×10^{-5}	3.09×10^{-7}	1.24×10^{-5}
9.88×10^{-7}	4.76×10^{-5}	1.05×10^{-6}	6.75×10^{-5}	4.85×10^{-7}	3.00×10^{-5}	5.76×10^{-6}	1.45×10^{-4}
7.84×10^{-7}	4.41×10^{-5}	1.87×10^{-5}	5.04×10^{-4}	9.94×10^{-6}	3.85×10^{-4}	4.77×10^{-6}	1.37×10^{-4}
4.89×10^{-4}	5.89×10^{-3}	1.61×10^{-6}	7.44×10^{-5}	3.09×10^{-4}	4.80×10^{-3}	3.29×10^{-2}	1.07×10^{-1}
1.20×10^{-5}	3.11×10^{-4}	1.26×10^{-5}	4.06×10^{-4}	1.49×10^{-4}	2.57×10^{-3}	3.19×10^{-6}	1.07×10^{-4}

experiments have been performed under serum-free conditions to increase the ability to detect secreted proteins and to gain more information on how cancer cells can survive. Cell cultures were maintained in the previously mentioned conditions for approximately 16 to 18 hours, time necessary to allow the cells to accumulate extracellular proteins.

The day of the experiment, the lines of PELs were harvested and counted, to verify that the threshold value of mortality of 10% had not been exceeded. We then proceeded to the separation, purification, and concentration of the secretome. [Table 2](#) shows the culture conditions, the number of cells counted and mortality, and the amount of secretome obtained.

SDS-PAGE analysis of proteins from each cell line was performed. Stained bands were numbered and excised for tryptic digestion and MALDI-TOF MS analysis. In parallel, a second aliquot of secretome underwent LC-Orbitrap MS analysis. A total of 266 proteins were identified: 152 in CRO-AP2, 188 in CRO-AP3, 128 in CRO-AP5, and 99 in CRO-AP6. The complete list is reported in [Supplemental Table S1](#), along with their predicted localization and the cell line origin.

Cellular compartment assignment was performed in a multistep manner using SignalP 3.0, SecretomeP 2.0, and TMHMM 2.0 (as described in [Materials and Methods](#)). Among the 266 identified proteins, 139 (52%) were either classically or non-classically secreted. Within this group, five transmembrane proteins were included. Among the total

proteins identified, the secreted proteins accounted for 50% (76/152) in CRO-AP2, 48% (90/188) in CRO-AP3, 50% (64/128) in CRO-AP5, and 67% (66/99) in CRO-AP6 ([Supplemental Table S2](#)). In addition to secreted proteins, 127 proteins that were considered to be intracellular or unconventionally secreted were also detected. Probably a fraction of these proteins derives from dead cells; our cell lines had a mortality ranging from 5% (CRO-AP6) to 10% (CRO-AP3), and this finding could contribute to the presence of intracellular proteins in the final secretome repertoire. Interestingly, CRO-AP6 had the lower mortality rate (5%) and the higher percentage (67%) of secreted proteins. Another fraction may include secretory proteins, such as proteins secreted through exosome vesicles, which may not pass the SignalP and SecretomeP score cutoffs.³⁰ A fraction of proteins identified in CRO-AP secretomes has been found in exosomes of B cells catalogued in the ExoCarta database ([Supplemental Table S3](#)).

Twenty-seven proteins were found in PEL secretomes from all PEL cell lines ([Table 3](#)). The comparison of PEL secretomes with secretomes from other tumor cell lines, using an in-house database of secreted proteins (as described in [Materials and Methods](#)), sorted out 20 proteins present only in the secretome of PEL ([Table 4](#)).

Furthermore, we compared the secretome of a cell line lacking of EBV and KSHV infection (DOOH-2) with that of CRO-AP cell lines KSHV⁺/EBV⁺ (CRO-AP2 and CRO-AP5) and CRO-AP cell lines KSHV⁺/EBV⁻ (CRO-AP3 and CRO-AP6).

To further characterize the expression patterns in PEL secretomes, we used the MetaCore pathway/network enrichment analysis tool to identify GO processes and pathways that were most significantly represented. By comparing GO biological processes, we found several processes significantly enriched (Table 5). Specifically, the process cellular macromolecular complex subunit organization was the most significant, but many others were significantly represented in all of the cell lines. The top 10 GO processes and pathways were identified as significantly activated, with $P < 0.001$ (Table 5).

2.12×10^{-9}) (Table 5, Figure 2 and Supplemental Figure S3). LRRK2 has been associated with the process of autophagy regulation. Autophagy is an intracellular catabolic mechanism by which a cell recycles or degrades damaged proteins and cytoplasmic organelles. This pathway is emerging as the most significant, also considering the total number ($N = 266$) of identified proteins. Another top-scored pathway map is the apoptosis and survival_Granzyme A signaling ($P = 5.54 \times 10^{-11}$; FDR = 1.02×10^{-8}) (Table 5, Figure 2, and Supplemental Figure S3). Granzymes are a family of serine proteases with cytotoxic and noncytotoxic roles found in the granules of lymphocytes.

When we analyzed the GO biological overrepresented processes in the total number of the identified proteins using STRING software, we found that the highest-scored function was the virus infectious cycle ($P = 2.47 \times 10^{-21}$). Moreover, within the protein list referred to cellular macromolecular complex subunit organization term, other overrepresented biological processes ontologically associated to virus infection (viral transcription, viral genome

expression, and others) were identified and visualized in STRING (Supplemental Figure S4). In other words, these findings could reflect the capability of the virus in recruiting cellular proteins to sustain its own viral infection cycle.

By using Ingenuity software, we also categorized the top canonical pathways that may be overrepresented in the PEL secretome. The most significant pathway was eukaryotic initiation factor 2 ($P = 3.2 \times 10^{-26}$; ratio = 33/201 = 0.164) (Supplemental Table S4).

Protein Expression in PEL Cell Lines, Primary PEL Samples, and Extracavitary KSHV-Positive Solid Lymphomas

The expression of 11 proteins predicted to be secreted (five shared and six specific for CRO-AP PEL cell lines) was also tested by IHC analysis in paraffin sections obtained from cell and tissue blocks.

As expected, the shared proteins tested [ezrin (EZRI), moesin (MOES), high-mobility group box 1 (HMGB1), galectin 1 (LEG1), and stathmin 1 (STMN1)] were detected by IHC in all CRO-AP PEL cells (Figure 3). Interestingly, specific proteins [protein arginine methyltransferases 1 (ANM1), granzyme A (GRAA), glutathione S-transferase κ 1 (GSTK1), catalase (CATA), poly(rC)-binding protein 2 (PCBP2), and S100 calcium-binding protein A6 (S10A6)], which have been identified in only one PEL cell line secretome, resulted positive in all CRO-AP PEL cell lines (Figure 4).

Overall, the IHC analysis confirmed the results obtained from the proteomic analysis performed with both MALDI-TOF MS and LC-Orbitrap MS. Moreover, we demonstrated that the cells express the secreted proteins, even in standard culture conditions. Finally, IHC analysis allowed us to identify the presence of proteins that probably MS has not detected because of a score assigned lower than the established threshold value ($P < 0.05$).

IHC performed in cell blocks from other PEL-derived cell lines, as well as PEL primary samples and extracavitary KSHV-positive solid lymphomas (as described in Materials and Methods), confirmed the presence of both shared and specific proteins in PEL cells (data not shown).

Discussion

By applying proteomics techniques to analyze PEL secretomes, we aimed at identifying putative new players in the interaction between PEL cells and microenvironmental cells and proteins that might be relevant for PEL pathogenesis. This approach has great advantages for the detection and separation of low-abundance proteins secreted by the cells into the culture medium. A disadvantage of culturing cells in medium without serum is the reduced viability and proliferation potential. Careful monitoring of cell viability and secreting potential is, thus, essential for a reliable secretome analysis. In this study, we checked cell viability and we decided to culture the cells for 16 to 18 hours in medium

without serum to achieve a high viability with reasonable production of secreted proteins (Table 2). Based on confirmation of selected proteins in PEL cells of primary samples, we could establish that the use of cell lines is an appropriate method to study the secretome of PEL.

Among the 266 proteins identified from the proteomic analysis (Supplemental Table S1), 139 were considered as predicted secreted (Supplemental Table S2), whereas the other 127 were considered to be intracellular or unconventionally secreted. Furthermore, a fraction of the identified proteins could be related to exosomes (Supplemental Table S3). Interestingly, a recent article³⁵ reported that exosome protein content may provide diagnostic markers specific for EBV- and KSHV-associated malignancies. Many changes common to the exosome proteomes of both EBV and KSHV indicate the modulation of cell death and survival, with additional predicted effects on ribosome function and protein synthesis. Our analysis is neither quantitative nor exosome specific; nevertheless, by using Ingenuity Pathway Analysis software (IPA, Ingenuity Systems, Redwood City, CA), we found an enrichment for proteins predicted to affect the eukaryotic initiation factor 2 signaling pathway ($P = 3.2 \times 10^{-26}$; ratio = 33/201 = 0.164). Thus, it is likely that secretomes may reflect exosome content and functions.

Most proteins recognized in the secretome of PEL are new with respect to previous studies based on conventional proteomic analysis and gene expression profiling. According to biological and molecular classification, proteins

Figure 3 The IHC expression of proteins shared by PEL secretomes. CRO-AP5 cell lines were strongly positive for Ezrin (EZRI), moesin (MOES), high mobility group protein B1 (HMGB1), galectin 1 (LEG1), and stathmin (STMN1). All tumor cells were stained. The staining pattern was membranous for EZRI and MOES, membranous and cytoplasmic for STMN1, cytoplasmic and nuclear for LEG1, and nuclear for HMGB1 (IHC, hematoxylin counterstain).

involved in catalytic activity and cell-cell and cell-microenvironment interactions were identified in the secretomes of PEL-derived cell lines. On the other hand, we also found four proteins, termed coronin 1A, cytosol aminopeptidase, malate dehydrogenase cytoplasmic, and malate dehydrogenase mitochondrial, that were already reported in PEL by conventional proteomic analysis.¹⁰ Moreover, we recognized proteins encoded by up-regulated genes that were identified in PEL by gene expression profiling analysis. This latter group included ADP-ribosylation factor 1, cyclophilin B, cytochrome *c*, glucose-6-phosphate isomerase, GRAA, histidyl-tRNA synthetase, and leukotriene A-4 hydrolase.^{8,9} Interestingly, LKHA4 expression in BCBL1 PEL cells leads to increased leukotriene B-4 production. The biological effect of leukotriene B-4 on PEL cells may lead to activation of the NF- κ B pathway.³⁶

Among the 139 secreted proteins, 27 were shared by all CRO-AP PEL cell lines (Table 3). Another 20 proteins were specifically secreted by PEL cell lines when compared with secretomes of cell lines derived from solid tumors and leukemias (Table 4). Within the group of secreted proteins that were shared by PEL secretomes, five (EZRI, MOES, HMGB1, LEG1, and STMN1) were involved in functions that are potentially related to PEL pathogenesis and cell adhesion. Both EZRI and MOES belong to the ezrin/radixin/moesin family of actin-binding proteins, which act as both linkers between the actin cytoskeleton and plasma membrane proteins and signal transducers in responses involving cytoskeletal remodeling. EZRI plays a key role in cell surface structure adhesion, migration, and organization, and it has been implicated in various human cancers. It is functionally involved in pathways that regulate cell survival, proliferation, migration, and cell morphogenesis.³⁷ EZRI has been identified in various cell types, including lymphoid cells. The MOES, another member of the ezrin/radixin/moesin family, is involved in maintaining cell shape of leukocytes as well as in chemotaxis and cell-cell communication.³⁸ HMGB1, initially identified as a nonhistone protein bound to DNA, also has an action similar to that of cytokines. This protein is actively secreted or passively released from necrotic cells. It has been hypothesized that this protein is capable of supporting the growth of lymphoma cells.³⁹ LEG1 belongs to a class of adhesion molecules. It has a crucial role in the modulation of biological processes in lymphoid cells, such as apoptosis, cell growth, RNA splicing, differentiation, and adhesion.⁴⁰ STMN1 is a protein involved in the process of assembly/disassembly of the mitotic spindle and is, thus, involved in the regulation of the cell cycle.⁴¹

Within the group of secreted proteins that were specifically found in PEL secretomes, six (ANM1, GRAA, GSTK1, CATA, PCBP2, and S10A6) were involved in functions that are potentially related to PEL pathogenesis and cell growth. ANM1 (alias PRMT1) is a methyltransferase that regulates the processing and maturation of mRNA, modulating, by methylation, the activity of the RNA binding protein.⁴² CATA is an enzyme capable of converting the hydrogen peroxide into water and oxygen, thus protecting cells from

cytotoxic effects. GSTK1 is a member of the family of glutathione transferases, enzymes that play a vital role in cellular detoxification processes.⁴³ PCBP2 belongs to the family of heterogeneous nuclear ribonucleoprotein-E2, proteins that bind the pretransporting RNA in the cytoplasm for splicing.⁴⁴ Furthermore, it seems involved in the innate immune response. S10A6 protein (alias caliclin) belongs to the family of S100 proteins, and S10A6 links two molecules of Ca^{2+} . The function of the protein is not clear, but seems to be involved in processes of cell proliferation, the dynamics of the cytoskeleton, and oncogenesis.⁴⁵ GRAA is a protein with lytic activity, which is released during the immune response by the natural killer cells.⁴⁶ By IHC analysis, all these proteins were expressed in the PEL cell lines (Figures 3 and 4), in the four primary PEL samples, and in the three extracavitary KSHV-positive solid lymphomas.

MetaCore analytical software version 6.16 revealed multiple pathways activated in PEL cell lines, the top scored being the LRRK2 pathway. LRRK2 is a member of the ROCO family of proteins, containing an ROC (Ras of complex proteins)/GTPase, a C-terminal of ROC, and a kinase domain, flanked by several protein-protein interaction motifs, including a WD40 domain and leucine-rich repeats.⁴⁷ The ROC domain interacts with β -tubulin, a key component of microtubules and MOES. LRRK2 is highly expressed in immune cells, including monocytes, B cells, and T cells, and this expression has been shown to increase after both

Figure 4 IHC expression of proteins specifically detected in secretomes of PEL cell lines (CRO-AP2). Cell lines were strongly positive for protein arginine methyltransferases 1 (ANM1), granzyme A (GRAA), glutathione S-transferase kappa 1 (GSTK1), catalase (CATA), poly(rC)-binding protein 2 (PCBP2), and S100 calcium-binding protein A6 (S10A6). The staining pattern was cytoplasmic for GRAA, CATA, and GSTK1, nuclear for ANM1, and nuclear and cytoplasmic for S10A6 (IHC, hematoxylin counterstain).

lipopolysaccharide and lentiviral particle treatment in macrophages.⁴⁸ Recent data support the idea that LRRK2 regulates autophagy.⁴⁹ Autophagy is also an important innate immunity mechanism that some viruses can evade by subverting or hijacking the autophagy process. KSHV exhibits two phases in its replication cycle: latent and lytic. During latency, KSHV is capable of evading the immune surveillance to persist in host cells without viral production; however, infectious viral particles can be produced and released after the induction of lytic reactivation from latency. KSHV adopts various cellular machineries and recruits different cellular proteins to maintain latency or to initiate lytic replication. Recent data indicate that autophagy is involved in KSHV lytic replication⁵⁰; it will be of interest to determine whether the KSHV persistent infection in our cell lines is intersecting molecular mechanisms for LRRK2 deregulation of autophagy. The second top-scored pathway was apoptosis and survival_granzyme A signaling. Most reports have focused on granzyme cell death—inducing properties; however, granzymes likely have extracellular functions in promoting inflammation and degrading extracellular matrix.⁵¹ The known extracellular activities of GRAA suggest a proinflammatory effect. GRAA can also proteolytically activate macrophages to secrete cytokines.⁵² The extracellular functions of granzyme A might be involved in the particular tropism of PEL and its cell growth. Further studies are needed to confirm and validate the importance of these pathways/processes and their roles in PEL tumorigenesis and progression.

In conclusion, this study has shown that PEL secretomes were enriched in proteins specifically involved in inflammation/immune response (eg, HMGB1, GRAA, and PCBP2), growth/cell cycle (eg, LEG1, STMN1, and S10A6), and mRNA processing (eg, ANM1 and PCBP2), in addition to structural/matrix proteins (eg, EZRI and MOES) and proteins with enzymatic activity (eg, CATA and GSTK1). More important, proteins involved in catalytic activity and cell-cell and cell-microenvironment interactions were identified in secretomes of all PEL-derived cell lines. This study, defining the secretome of PEL, has identified many proteins potentially involved in the pathogenesis of PEL, and in its peculiar cell growth. Coherently with the pathophysiological characteristics of PEL, many identified proteins are involved in growth, development, and maintenance of the tumor microenvironment (angiogenesis and inflammation), and responsible for the particular tropism (cell adhesion and migration) of PEL.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2013.11.028>.

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