Online Supplementary Data for

Molecular and phenotypic features are shared by CD30-positive peripheral T-cell lymphomas

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Online Supplementary Methods

Gene expression analyses
In order to compare the expression profiles of 16 PTCL, NOS (6 CD30+ and 10 CD30-) and 35 ALCL (25 ALK+ and 10 ALK-) from our two previously published datasets (de Leval et al.19 / E-TABM-783; Lamant et al.7 / E-TABM-117), the two corresponding RMA normalized expression matrices were averaged per HUGO Gene Symbol and restricted to the set of common HUGO Gene Symbols (n=13211). Both matrices were then concatenated and quantile normalized.

The four PTCL categories (CD30+ PTCL, NOS; CD30- PTCL, NOS; ALK+ ALCL; ALK- ALCL) were compared for the expression of two gene sets, referred to as “CD30 neg. signature” (75 genes overexpressed in CD30- compared to CD30+ PTCL, NOS, as defined by Table S4 of de Leval et al.19) and “ALK neg. signature” (173 genes overexpressed in ALK- compared to ALK+ ALCL, as defined by Table S3 of Lamant et al.7). For each gene set, the mean expression across genes was calculated per sample. These values were compared using Welch t tests. The “ALK neg. signature” was also used for gene set enrichment analysis (GSEA), as previously described.19,20

Validation of GEP data at the protein level

Tumor samples
Routinely processed paraffin-embedded tissue samples of 80 PTCL were selected from the files of the Pathology Departments of the University Hospital of Liège (Belgium), the Henri Mondor Hospital, Créteil (France) and the University Hospital Purpan, Toulouse (France). This series comprised 36 PTCL, NOS (18 CD30+ and 18 CD30-), 15 ALK- ALCL and 29 ALK+ ALCL. Aside from 3 ALK+ ALCL presenting as soft tissue mass lesions, all other cases were primarily nodal diseases.

Lymphomas were classified based on morphologic and immunophenotypic criteria, according to current WHO criteria.1 The staining panel included at least CD20, CD3 (usually with other T-cell markers) and CD30. CD30+ cases were composed of monomorphic or pleomorphic populations of large cells, with CD30 staining in >75% of tumor cells and no expression of EMA. A cytotoxic immunophenotype was demonstrated in only three samples. A few representative cases are illustrated in Online Supplementary Figure S1.

Most ALK+ ALCL were of the common type, 9 were of the small cell/lymphohistiocytic variant and one was sarcomatoid. ALK- ALCL were strictly defined as tumors with a morphology consistent with the common pattern of ALCL including hallmark cells and cohesive growth, strong CD30 positivity in virtually all tumor cells, negativity for ALK, and a cytotoxic immunophenotype (granzyme B, perforin and/or TIA-1). All cases were reviewed by two to four hematopathologists (BB, LL, PG and/or LdL). Relevant additional information is available in Online Supplementary Table S1.

Fifty-nine of the 80 samples were included in tissue microarray (TMA) blocks. Approval for the study was obtained from the Ethics Committee of the University Hospital of Liège.
**Immunohistochemistry**

For immunohistochemical validation of GEP findings, the selection of markers was based on i) the most differentially expressed genes across distinct PTCL subgroups, according to our GEP datasets and to other publicly available sources; ii) their involvement in relevant cellular pathways; and iii) availability of primary antibodies suitable for paraffin-embedded tissues.

The 21 molecules explored are depicted in Figure 1 and listed in Online Supplementary Table S2, along with their physiological and/or pathophysiological role and their expected expression patterns in the PTCL subgroups analyzed.

Immunostainings were performed according to standard manual or automated methods. For the automated labeling procedures, BenchMark XT or Discovery XT autostainers (Ventana Medical Systems, Tucson, AZ, USA) were employed. Details regarding primary antibody clones and dilutions are provided in Online Supplementary Table S2. Immunostainings were performed primarily on TMA slides. Cases with insufficient material or equivocal results on TMA were analyzed on whole tissue sections.

**Scoring system**

The immunolabeled sections were evaluated semi-quantitatively, by comparison with hematoxylin-eosin, CD3 and/or CD30-stained sections, using a scoring scale based on both the extent and intensity of the stainings. For staining extent, less than 10% of stained tumor cells were considered negative (score 0), and scores 1, 2, 3 and 4 corresponded to positivity in 10-25%, 26-50%, 51-75% and 76-100% of tumor cells, respectively. Negative cores devoid of an internal positive control were not interpreted. For staining intensity, scores 1, 2 and 3 denoted weak, moderate and strong intensities, respectively; undetectable staining was scored 0. The extent score and the intensity score were multiplied to provide a unique global score for each immunostaining, ranging from 0 to 12. Cases were considered positive for a marker when the corresponding global score was 4 or higher.

Samples not evaluable for >10 stainings were completely excluded from all the analyses involving immunohistochemical data. According to these prerequisites, 68 of 80 samples (85%) were adequate for interpretation by immunohistochemistry, comprising 16 CD30- PTCL, NOS, 17 CD30+ PTCL, NOS, 12 ALK- ALCL and 23 ALK+ ALCL.

**Clinical data**

The clinical data recorded for each patient of the validation set included sex, age at diagnosis and date of the diagnostic biopsy. When available, details on therapy response, progression or relapse, date of last information and survival status were also annotated.

Clinical outcome was determined by overall survival (OS) and progression-free survival (PFS). OS was defined as time from diagnostic biopsy to death from any cause. PFS was defined as time from diagnostic biopsy to disease progression, relapse, or death from any cause. Follow-up of patients not experiencing any of these events was censored at the date of last information.

**Statistical analyses of clinical and immunohistochemical data**

Differences in clinical features and immunostaining scores between the PTCL subgroups were assessed by means of Chi-square (for qualitative variables), Mann-Whitney (for comparison of two groups) and Kruskal-Wallis (for comparison of more than two groups) tests (GraphPad Prism software, San Diego, CA, USA).

OS and PFS distributions were analyzed by the Kaplan and Meier method and compared using the log-rank test (GraphPad Prism software).

Hierarchical cluster analysis was conducted to integrate all immunohistochemical data and explore the correlations within and across the recognized PTCL entities (average linkage clustering, Cluster and TreeView softwares, http://www.eisenlab.org).

**References**

See manuscript.
**Online Supplementary Figures**

**Supplementary Figure S1.** Diverse morphologic and immunophenotypic features of CD30+ PTCL, NOS. (A) Neoplastic cells displaying a pleomorphic morphology, admixed with a reactive cellular component (A1, HE, magnification 400x). CD30 staining of moderate to strong intensity in at least 75% of the tumor cells (A2, CD30, 200x). (B) Case showing a centroblastic-like morphology with oval nuclei and one or several nucleoli (B1, HE, 400x), and homogeneously strong CD30 positivity (B2, CD30, 200x). (C) Case composed of large cells with scattered cells suggestive of hallmark cells (arrows) (C1, HE, 400x) and strong diffuse positivity for CD30 (C2, CD30, 200x). This case lacked however expression of EMA, ALK and all cytotoxic markers.
**Supplementary Figure S2.** Immunostaining results. For each marker, the diagrams display the immunohistochemical (IHC) staining score of individual samples subdivided by diagnostic category (global IHC score combining staining extent and intensity, ranging from 0 to 12). The median of the scores for each diagnostic category is indicated by a horizontal line. The P value of a Kruskal-Wallis test comparing all 4 diagnostic groups is provided.
Supplementary Figure S3. Representative immunostainings for TCRβF1 (A1 and A2) and ZAP-70 (B1 and B2) in PTCL, NOS and ALCL samples. (A) Negative TCRβF1 staining of a PTCL, NOS (A1, magnification 200x) and an ALCL (A2, 200x). (B) Positive ZAP-70 staining of a PTCL, NOS (B1, 200x) and an ALCL (B2, 200x).
Supplementary Figure S4. Venn diagram illustrating the differences and similarities of marker expression between the PTCL subgroups. The four ellipses represent the four diagnostic PTCL subgroups, superimposed in order to create all possible intersections across them. Each immunomarker tested was placed in a compartment of each ellipse according to the similarities or differences of expression between the diagnostic categories. As an example, CD3 was differentially expressed in CD30+ PTCL, NOS compared to the other PTCL subgroups; it was also differentially expressed in CD30- PTCL, NOS compared to the other PTCL subgroups; but no significant difference was observed in CD3 expression between ALK+ and ALK- ALCL; as a consequence, CD3 has been placed in the “CD30+ PTCL, NOS distinct” compartment, in the “CD30- PTCL, NOS distinct” compartment, and at the intersection between ALK+ ALCL and ALK- ALCL.

Supplementary Figure S5. Kaplan-Meier survival curves (A, overall survival; B, progression-free survival) stratified by pathological category.
Online Supplementary Tables

Supplementary Tables S1 and S3: see separate Excel files.
### Supplementary Table S2. Molecules explored by immunohistochemistry and expected expression patterns in PTCL subgroups according to literature data.

<table>
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<tr>
<th>Main function / family of molecules</th>
<th>Protein investigated by immunohistochemistry</th>
<th>Expected differential expression in PTCL subgroups</th>
<th>Source of information regarding expression in PTCLs</th>
<th>Clone of primary antibody (source), dilution</th>
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<td><strong>CD3</strong> (antibody recognizes cytoplasmic portion of CD3ε chain)</td>
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<td>Bonzheim et al.²</td>
<td>Rabbit polyclonal (Dako), 1/300</td>
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<td><strong>TCRβF1</strong> (β chain constant region of the αβ T-cell antigen receptor)</td>
<td><strong>ZAP-70</strong> (ζ-chain associated protein kinase 70 kDa)³⁴⁶</td>
<td>Down in ALCL <strong>versus</strong> PTCL, NOS</td>
<td>Bonzheim et al.²</td>
<td>8A3 (Thermo Scientific), 1/20</td>
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<td><strong>Protein tyrosine kinases (PTKs) involved in proximal TCR signaling</strong></td>
<td><strong>Lck</strong> (lymphocyte-specific protein tyrosine kinase; p56⁵⁶∥)⁵⁶³⁸</td>
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<td><strong>Fyn</strong> (FYN oncogene related to SRC, FGR, YES; p59⁵⁹∥)⁵⁶³⁸</td>
<td><strong>Itk</strong> (IL-2-inducible T-cell kinase)¹⁰</td>
<td>Down in CD30+ <strong>versus</strong> CD30- PTCL, NOS</td>
<td>de Leval et al.⁷</td>
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<td><strong>PTK involved in B-cell receptor (BCR) signaling (ZAP-70 homologue)</strong></td>
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<td>Geissinger et al.⁸</td>
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<td><strong>Syk</strong> (spleen tyrosine kinase), total form</td>
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<td><strong>CD52</strong> (CD52 antigen): unknown function, expressed on most normal T cells¹⁶</td>
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<td><strong>Transcription factors</strong></td>
<td><strong>NFATC2</strong> (nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 2; also known as NFAT1or NFATp): translocates from the cytoplasm to the nucleus following TCR stimulation¹⁸</td>
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<td><strong>GATA1</strong> (GATA binding protein 1 (globin transcription factor 1)): plays an important role in erythroid lineage development¹⁰</td>
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<td><strong>FoxP1</strong> (forkhead box P1): widely expressed; associated with the maintenance of quiescence in naïve T cells²¹</td>
<td>Up in CD30- <strong>versus</strong> CD30+ PTCL, NOS</td>
<td>de Leval et al.⁷</td>
<td>JC12 (LRF Immunodiagnostics Unit), 1/80</td>
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**Molecular and phenotypic features of CD30+ PTCL**

<table>
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<tr>
<th>Gene/Protein</th>
<th>Function</th>
<th>Expression Pattern</th>
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<td>JunB (jun B proto-oncogene)</td>
<td>Binds to the CD30 gene promoter thereby activating its transcription; conversely, CD30 signaling induces JunB expression via the activation of ERK1/2p22</td>
<td>Up in CD30+ versus CD30- lymphomas</td>
<td>Rassidakis et al.23</td>
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<td>pSTAT3 (phosphorylated form of signal transducer and activator of transcription 3 (acute-phase response factor)): major substrate of ALK fusion proteins and required for survival of ALK-transformed cells24</td>
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<td>C/EBPβ (CCAAT enhancer-binding protein β): involved in the regulation of inflammatory responses; induced by chimeric ALK via STAT3, leading to increased cell proliferation25-28</td>
<td>Up in ALK+ versus ALK-ALCL</td>
<td>Lamant et al.27</td>
<td>H-7 (Santa Cruz), 1/100</td>
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<td>MUM1/IRF4 (multiple myeloma oncogene-1/ interferon regulatory factor-4): critical regulator of normal lymphoid development including T-helper-cell differentiation29</td>
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<td>MUM1p (Dako), 1/100</td>
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<td>Cyclin D3</td>
<td>Plays a pivotal role in the G1/S transition;30 transcriptionally regulated by pSTAT331</td>
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<td>DCS-22 (Novocastra), 1/10</td>
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<td>IMP3 (insulin-like growth factor II mRNA-binding protein 3; also known as IGF2BP3): associated with an aggressive behavior in many diverse malignancies22,33</td>
<td>Up in CD30+ versus CD30- PTCL, NOS</td>
<td>de Leval et al.1</td>
<td>6H9.1 (Dako), 1/300</td>
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<td>MAL (malt, T-cell differentiation protein): required for the efficient transport of Lck to the plasma membrane34</td>
<td>Up in ALK- versus ALK+ALCL</td>
<td>Lamant et al.27</td>
<td>6D9 (gift from MA Alonso, Centro de Biología Molecular, Madrid, Spain), 1/2000</td>
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</table>

**Cell cycle regulator**

**Oncofetal RNA-binding protein**

**Membrane proteolipid**

**References**


AbD Serotec, Düsseldorf, Germany; Atlas Antibodies AB, Stockholm, Sweden; Cell Signaling Technology, Boston, MA, USA; Dako, Glostrup, Denmark; Epitomics, Burlingame, CA, USA; Leica Microsystems, Newcastle Upon Tyne, UK; LRF Immunodiagnostics Unit, Oxford University, Oxford, UK; Novocastra, Newcastle Upon Tyne, UK; Santa Cruz Biotechnology, Santa Cruz, CA, USA; Sigma-Aldrich, St. Louis, MO, USA; Thermo Fisher Scientific, Rockford, IL, USA; Upstate Biotechnology, Temecula, CA, USA;
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