CD30-positive peripheral T-cell lymphomas share molecular and phenotypic features

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ABSTRACT

Peripheral T-cell lymphoma, not otherwise specified is a heterogeneous group of aggressive neoplasms with indistinct borders. By gene expression profiling we previously reported unsupervised clusters of peripheral T-cell lymphomas, not otherwise specified correlating with CD30 expression. In this work we extended the analysis of peripheral T-cell lymphoma molecular profiles to prototypical CD30$^+$ peripheral T-cell lymphomas (anaplastic large cell lymphomas), and validated mRNA expression profiles at the protein level. Existing transcriptomic datasets from peripheral T-cell lymphomas, not otherwise specified and anaplastic large cell lymphomas were reanalyzed. Twenty-one markers were selected for immunohistochemical validation on 80 peripheral T-cell lymphoma samples (not otherwise specified, CD30$^+$ and CD30$^-$; anaplastic large cell lymphomas, ALK$^+$ and ALK$^-$), and differences between subgroups were assessed. Clinical follow-up was recorded. Compared to CD30$^-$ tumors, CD30$^+$ peripheral T-cell lymphomas, not otherwise specified were significantly enriched in ALK$^-$ anaplastic large cell lymphoma-related genes. By immunohistochemistry, CD30$^+$ peripheral T-cell lymphomas, not otherwise specified differed significantly from CD30$^-$ samples [down-regulated expression of T-cell receptor-associated proximal tyrosine kinases (Lck, Fyn, Itk) and of proteins involved in T-cell differentiation/activation (CD69, ICOS, CD52, NFATc2); upregulation of JunB and MUM1], while overlapping with anaplastic large cell lymphomas. CD30$^-$ peripheral T-cell lymphomas, not otherwise specified tended to have an inferior clinical outcome compared to the CD30$^+$ subgroups. In conclusion, we show molecular and phenotypic features common to CD30$^+$ peripheral T-cell lymphomas, and significant differences between CD30$^-$ and CD30$^+$ peripheral T-cell lymphomas, not otherwise specified, suggesting that CD30 expression might delineate two biologically distinct subgroups.

Introduction

Peripheral T-cell lymphomas (PTCL) are a heterogeneous group of clinically aggressive neoplasms, some of which constitute distinct clinicopathological entities, with more or less stringent diagnostic criteria. Still, the largest group of PTCL is represented by the "not otherwise specified" (NOS) category, characterized by unclear demarcations owing to pronounced morphological and immunophenotypic heterogeneity and absence of defining molecular criteria.[1]

The CD30 antigen has historically been instrumental in defining anaplastic large cell lymphomas (ALCL) as a distinct category, characterized by a frequently cohesive and intrasinusoidal proliferation of large pleomorphic cells with strong and homogeneous expression of CD30.[2] The discovery of recurrent chromosomal translocations involving the anaplastic lymphoma kinase (*ALK*) gene in a subset of these lymphomas led to the delineation of ALK$^+$ and ALK$^-$ ALCL as two disease subtypes.[3,4] Evidence of additional distinguishing clinical and biological features has more recently justified the recognition of ALK$^+$ ALCL as a discrete entity in the 2008 World Health Organization (WHO) classification, and the inclusion of ALK$^-$ ALCL as a provisional category.[1,5-7] As defined in the WHO book, ALK$^-$ ALCL comprises CD30$^+$ T-cell neoplasms that are not reproducibly distinguishable on morphological grounds from ALK$^+$ ALCL, but lack *ALK* gene rearrangement and expression, with most cases expressing T-cell-associated markers and cytotoxic granule-associated proteins.[1] In fact, the definitional criteria remain subject to variations in interpretation, and especially the criteria used for morphological assessment to consider "anaplastic" morphology may be subtle and frequently subjective.[1,8,9] In particular, a subset of PTCL,

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NOS displays large-cell morphology and substantial CD30 expression, rendering the distinction of these lymphomas from ALK$^-$ ALCL problematic.[1,9-11] Thus, although recent clinical and gene expression profiling (GEP) data support their existence as two separate disease entities,[5,12-14] the border between ALK$^-$ ALCL and PTCL, NOS is still imprecise.

Multiple different molecular subgroups have been identified within the spectrum of PTCL, NOS.[12,15-18] We found that spontaneous clustering of PTCL, NOS according to their expression profiles correlated with expression of CD30, and evidenced by supervised analysis that the molecular signature of CD30$^+$ PTCL, NOS, in comparison to that of CD30$^-$ tumors, was characterized by the down-regulation of molecules involved in T-cell differentiation/activation (including CD28, CD52, CD69) and T-cell receptor (TCR) signaling (such as Lck, Fyn, Itk).[19]

The purposes of the present work were: (i) to extend this molecular characterization of PTCL to include ALCL cases, and more specifically to explore the molecular relationship between CD30$^+$ PTCL, NOS and ALK$^-$ ALCL; (ii) to validate our previous GEP findings at the protein level, postulating the existence of significant differences in the protein expression profiles between CD30$^+$ and CD30$^-$ PTCL, NOS; and (iii) to examine clinical outcomes according to pathological classification and immunophenotypic subgroups.

Design and 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]; Lamant *et al.*[7]), the RMA normalized matrices were averaged per gene symbol, concatenated and quantile-normalized. The four PTCL categories were compared for the expression of two gene sets, referred to as "CD30 neg. signature" (*Table S4* of de Leval *et al.*[19]) and "ALK neg. signature" (*Table S3* of Lamant *et al.*[7]). For each gene set, the mean expression across genes was calculated per sample and 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 gene expression profiling data at the protein level

Eighty cases of 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), comprising 36 PTCL, NOS (18 CD30$^+$ and 18 CD30$^-$), 15 ALK$^-$ ALCL and 29 ALK$^+$ ALCL (*Online Supplementary Table S1*). ALK$^-$ ALCL were strictly defined as tumors with a morphology consistent with the common pattern of ALCL, strong CD30 positivity in virtually all tumor cells, negativity for ALK, and a cytotoxic immunophenotype and/or epithelial membrane antigen (EMA) expression. All CD30$^+$ PTCL, NOS were composed of large cells with CD30 staining in >75% of tumor cells and no expression of EMA (*Online Supplementary Figure S1*). CD30$^-$ PTCL, NOS were all essentially negative for CD30. Approval for the study was obtained from the Ethics Committee of the University Hospital of Liège.

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 other publicly available sources;[6,7,13,19,21-24] (ii) their involvement in relevant cellular pathways; and (iii) availability of primary antibodies suitable for paraffin-embedded tissues. The 21 molecules

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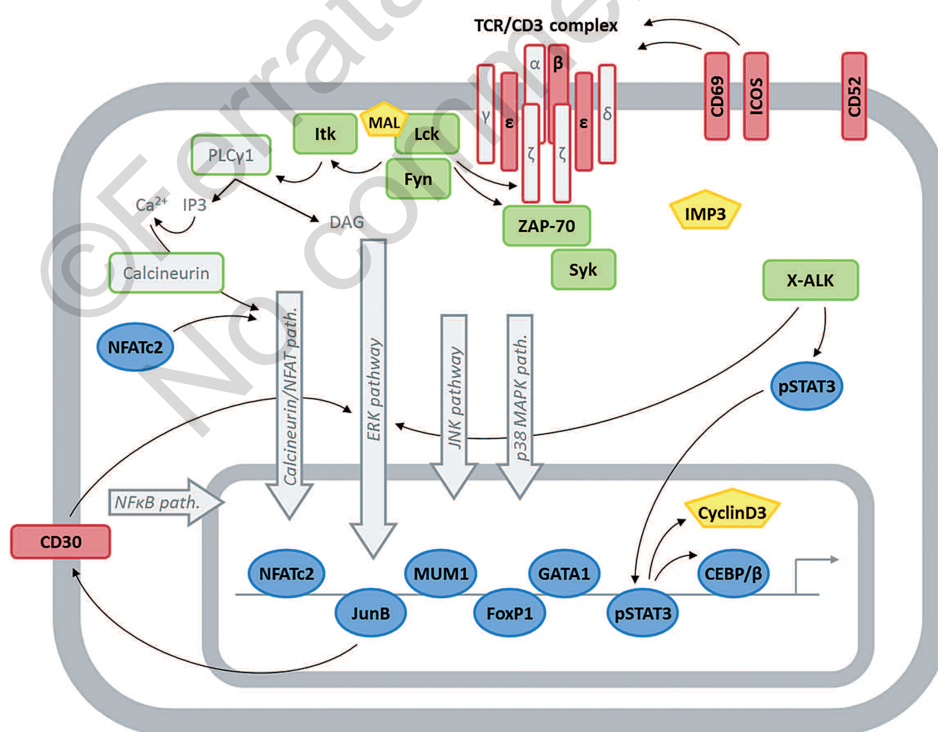

Figure 1. Schematic representation of molecules examined by immunohistochemistry. The proteins explored are depicted in color, including molecules involved in T-cell receptor (TCR) signaling and T-cell differentiation/activation, and others. In brief, antigen ligation to the TCR/CD3 complex initiates a signaling cascade involving the protein tyrosine kinases ZAP-70, Lck, Fyn and Itk. Downstream consequences include Ca^{2+} flux, NFATc2 nuclear translocation, MAP kinase activation (ERK, JNK and p38), transcription and cytokine release. CD69 and ICOS, upregulated following TCR engagement, act as co-stimulatory molecules. Syk is a ZAP-70 homolog physiologically expressed in B cells. CD30 signaling activates the NFκB pathway and also ERK, thereby inducing JunB expression; conversely, JunB activates CD30 transcription. ALK fusion proteins phosphorylate STAT3, in addition to activating the ERK pathway. Downstream targets of pSTAT3 include CEBP/β and cyclin D3. Further information on the molecules explored is provided in *Online Supplementary Table S2.*

explored are depicted in Figure 1 and listed in *Online Supplementary Table S2*.

The immunolabeled sections were evaluated semi-quantitatively, using a scoring scale based on extent and intensity of the stainings.[25] The extent score and the intensity score were multiplied to provide a unique global score, ranging from 0 to 12, for each immunostain. Cases were considered positive for a marker when the corresponding global score was ≥4.

Clinical data

The clinical data recorded for each patient of the validation set included sex, age at diagnosis and date of the diagnostic biopsy. Clinical outcome was determined by overall survival and progression-free survival.[26]

Statistical analyses of clinical and immunohistochemical data

Differences in clinical features and immunostaining scores between the PTCL subgroups were assessed by means of the chi-square, Mann-Whitney and Kruskal-Wallis tests (GraphPad Prism software, San Diego, CA, USA). Distributions of overall and progression-free survival were analyzed by the Kaplan and Meier method and compared using the log-rank test (GraphPad Prism software).[27,28] Hierarchical cluster analysis was conducted on all immunohistochemical data (average linkage clustering, Cluster and TreeView softwares, *http://www.eisenlab.org*).[29,30]

Results

Gene expression analyses reveal molecular similarities between CD30⁺ peripheral T-cell lymphomas, not otherwise specified and ALK⁻ anaplastic large cell lymphomas

In our previous work, comparison between the molecular signatures of six CD30⁺ *versus* ten CD30⁻ PTCL, NOS revealed significant down-regulation of several genes involved in T-cell activation (comprising CD28, CD52 and CD69) and TCR signal transduction (including Lck, Fyn and Itk).[19] Here, we compared the level of expression of that set of genes (down-regulated in CD30⁺ *versus* CD30⁻ PTCL, NOS, i.e. overexpressed in CD30⁻ *versus* CD30⁺ PTCL, NOS, referred to as the "CD30 neg. signature") in the four groups of PTCL. As seen in Figure 2A, the expression of those genes defining the "CD30 neg. signature" was also significantly down-regulated in ALCL (irrespective of ALK status) (Welch *t* test, $P<2x10^{-16}$). The levels of expression in ALCL were slightly lower than in CD30⁺ PTCL, NOS (*P*=0.0013).

Since the defective expression of TCR-related molecules has been suggested to be a distinguishing feature of ALCL,[21,22] we wanted to specifically search for molecular similarities between CD30⁺ PTCL, NOS and ALK⁻ ALCL. Thus, we looked at the expression of a set of genes defining the "ALK neg. signature" (up-regulated in ALK⁻ ALCL compared to ALK⁺ ALCL). As seen in Figure 2B, the expression levels of this gene set were slightly lower but not significantly different in CD30⁺ PTCL, NOS compared to ALK⁻ ALCL (*P*=0.088), whereas they were significantly reduced in CD30⁻ PTCL, NOS (*P*=0.0002). Accordingly, GSEA using the "ALK neg. signature" showed significant enrichment for expression in the group of CD30⁺ PTCL, NOS as compared to CD30⁻ PTCL, NOS (141 genes; *P*=0.0415).

CD30⁺ peripheral T-cell lymphomas share common phenotypic features

A summary of the immunostaining results for the tested markers is provided in Table 1, and the details of all immunohistochemical scores can be consulted in *Online Supplementary Figure S2* and *Online Supplementary Table S3*. Figure 3 and *Online Supplementary Figure S3* illustrate representative immunostainings of a selection of markers.

Within the TCR/CD3 complex, CD3 showed the highest levels of expression in CD30⁻ PTCL, NOS (median of global scores: 10; 94% of samples positive), and was mostly preserved in CD30⁺ PTCL, NOS (median 8; 82% positive), but with significantly lower expression levels. Conversely, ALK⁺ ALCL were predominantly negative for CD3 (median 0; 17% positive), while ALK⁻ ALCL showed

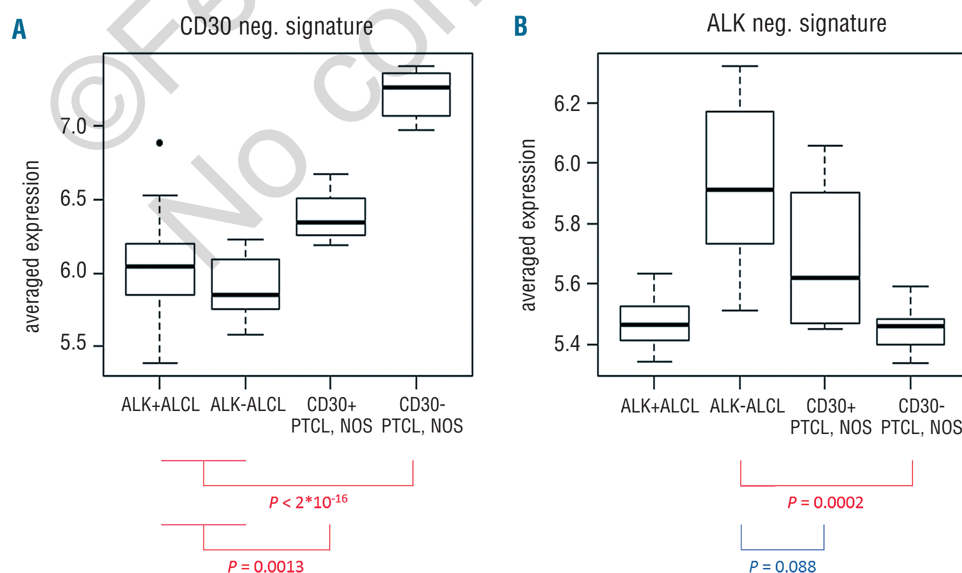

Figure 2. (A) Boxplots of the averaged expression of the genes of the "CD30 neg. signature" in four groups of samples: ALK⁺ ALCL; ALK⁻ ALCL; CD30⁺ PTCL, NOS; CD30⁻ PTCL, NOS. These values are significantly lower in ALCL (irrespective of the ALK status) than in CD30⁻ PTCL, NOS (Welch t test, $P<2x10^{-16}$), and slightly lower than in CD30⁺ PTCL, NOS (*P*=0.0013). (B) Boxplots of the averaged expression of the genes of the "ALK neg. signature" in the same four groups of samples. These values are not significantly different between ALK⁻ ALCL and CD30⁺ PTCL, NOS (*P*=0.088), while a significant difference is observed between ALK⁻ ALCL and CD30⁻ PTCL, NOS (*P*=0.0002). The boxplots represent the median and interquartile range.

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more heterogeneous results (median 1; 42% positive), significantly lower than those for CD30+ PTCL, NOS. TCRβF1 was undetectable in the majority of the samples analyzed, among both CD30- and CD30+ PTCL.

The levels of expression of most of the other molecules involved in proximal TCR signaling and T-cell differentiation/activation were significantly different between CD30- PTCL, NOS and the whole group of CD30+ PTCL (PTCL, NOS and ALCL cases). The differences were most striking for the proximal tyrosine kinases Lck, Fyn and Itk: their expression was mostly conserved in the CD30- PTCL, NOS samples (medians 7.5, 8.0 and 12.0; 73%, 88% and 100% positive, respectively), while it was markedly reduced or completely negative in all CD30+ PTCL (all medians 0; 4-9% positive).

ZAP-70 displayed the same general tendency, although several CD30+ samples, particularly among PTCL, NOS cases, were still positive for this marker (median for CD30- PTCL, NOS: 12; median for the whole group of CD30+ PTCL: 6; 100% of CD30- PTCL, NOS positive; 62% of all CD30+ PTCL positive). The dissimilarities between CD30- and CD30+ PTCL were also highly significant for other pro-

Table 1. Summary of immunostaining scores by PTCL subgroup.

Immunomarker		Diagnostic category CD30- PTCL, NOS	CD30+ PTCL, NOS	ALK- ALCL	ALK+ ALCL
CD3	Median*	10	8	1	0
	Pos/Tot (%)**	15/16 (94%)	14/17 (82%)	5/12 (42%)	4/23 (17%)
TCRβF1	Median	0	0	0	0
	Pos/Tot (%)	5/11 (45%)	2/12 (17%)	1/8 (13%)	2/19 (11%)
ZAP-70	Median	12	8	4.5	4
	Pos/Tot (%)	16/16 (100%)	11/15 (73%)	7/11 (64%)	11/21 (52%)
Syk (inactive)	Median	0	0	0	4
	Pos/Tot (%)	1/13 (8%)	1/14 (7%)	0/10 (0%)	10/17 (59%)
Syk (total)	Median	7.8	10.5	12	12
	Pos/Tot (%)	13/14 (93%)	14/16 (88%)	10/11 (91%)	20/21 (95%)
Lck	Median	7.5	0	0	0
	Pos/Tot (%)	11/15 (73%)	2/15 (13%)	0/10 (0%)	0/21 (0%)
Fyn	Median	8	0	0	0
	Pos/Tot (%)	14/16 (88%)	2/16 (13%)	0/12 (0%)	1/22 (5%)
Itk	Median	12	0	0	0
	Pos/Tot (%)	15/15 (100%)	1/15 (7%)	0/9 (0%)	3/22 (14%)
CD69	Median	3	0	0	0
	Pos/Tot (%)	7/15 (47%)	0/16 (0%)	1/10 (10%)	2/20 (10%)
CD52	Median	9.5	0	0	0
	Pos/Tot (%)	11/14 (79%)	2/15 (13%)	1/11 (9%)	4/21 (19%)
ICOS	Median	2	0	0	0
	Pos/Tot (%)	6/13 (46%)	1/17 (6%)	0/11 (0%)	1/18 (6%)
NFATc2	Median	10	4.3	2.5	0
	Pos/Tot (%)	14/15 (93%)	9/16 (56%)	4/10 (40%)	3/21 (14%)
IMP3	Median	3.8	11	11	8
	Pos/Tot (%)	4/8 (50%)	14/14 (100%)	3/4 (75%)	12/14 (86%)
GATA1	Median	6	6	6	6
	Pos/Tot (%)	8/11 (73%)	11/13 (85%)	6/6 (100%)	17/17 (100%)
FoxP1	Median	0	0	0	0
	Pos/Tot (%)	3/13 (23%)	1/14 (7%)	1/10 (10%)	6/19 (32%)
Cyclin D3	Median	5	3	4.5	10
	Pos/Tot (%)	9/13 (69%)	6/16 (38%)	8/11 (73%)	21/23 (91%)
JunB	Median	0	10	10	10
	Pos/Tot (%)	4/15 (27%)	17/17 (100%)	9/12 (75%)	21/23 (91%)
pSTAT3	Median	0	0	5.5	12
	Pos/Tot (%)	0/10 (0%)	1/10 (10%)	7/8 (88%)	18/19 (95%)
C/EBPβ	Median	0	2	0	12
	Pos/Tot (%)	4/15 (27%)	8/17 (47%)	3/9 (33%)	20/21 (95%)
MUM1/IRF4	Median	0	12	9.5	6
	Pos/Tot (%)	3/13 (23%)	15/16 (94%)	8/8 (100%)	15/21 (71%)
MAL	Median	2	1	1	0
	Pos/Tot (%)	8/16 (50%)	5/14 (36%)	4/10 (40%)	0/19 (0%)

*Median: median of individual staining scores for each immunomarker. Scores were based on staining extent and intensity, and ranged from 0 to 12 (see Design and Methods section for details). **Pos/Tot: number of positive cases (i.e. score ≥ 4) / number of total evaluable cases in the corresponding diagnostic category.*

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teins associated with T-cell differentiation/activation, comprising the cell-surface antigens CD69, ICOS and CD52, and the transcription factor NFATc2 (medians for CD30⁻ PTCL, NOS: 3.0, 9.5, 2.0 and 10.0, respectively; all medians for the whole group of CD30⁺ PTCL: 0; 46-93% of CD30⁻ PTCL, NOS positive; 4-34% of all CD30⁺ PTCL positive).

Using a polyclonal antibody that recognizes all Syk forms (Santa Cruz, C-20),[23] expression of this tyrosine kinase was observed in the majority of cases within all PTCL categories (medians: between 7.8 and 12; 88-95% positive). Conversely, using an antibody targeting Syk phosphoTyr323, i.e. the inactive form of the kinase (Epitomics, clone EP573Y),[22,31] phosphorylated Syk was mostly undetectable in all categories (medians 0; 0–8% positive) except for ALK⁺ ALCL (median 4; 59% positive).

Among the transcription factors investigated, JunB was significantly up-regulated in all CD30⁺ PTCL categories (all medians 10; 90% of all CD30⁺ PTCL positive) compared to CD30⁻ PTCL, NOS (median 0; 27% positive). MUM1/IRF4 was also over-expressed in CD30⁺ PTCL (median for the whole group of CD30⁺ PTCL: 10; 84% positive) relative to CD30⁻ PTCL, NOS (median 0; 23% positive). In contrast to the foregoing GEP prediction,[7] we did not observe a significant difference in MUM1 expression between ALK⁻ (median 9.5; 100% positive) and ALK⁺ ALCL (median: 6; 71% positive).

C/EBPβ and pSTAT3 were both highly up-regulated in ALK⁺ ALCL (both medians 12; both 95% positive) compared to the other PTCL categories (medians for C/EBPβ: 0-2; medians for pSTAT3: 0-5.5). It was noteworthy that

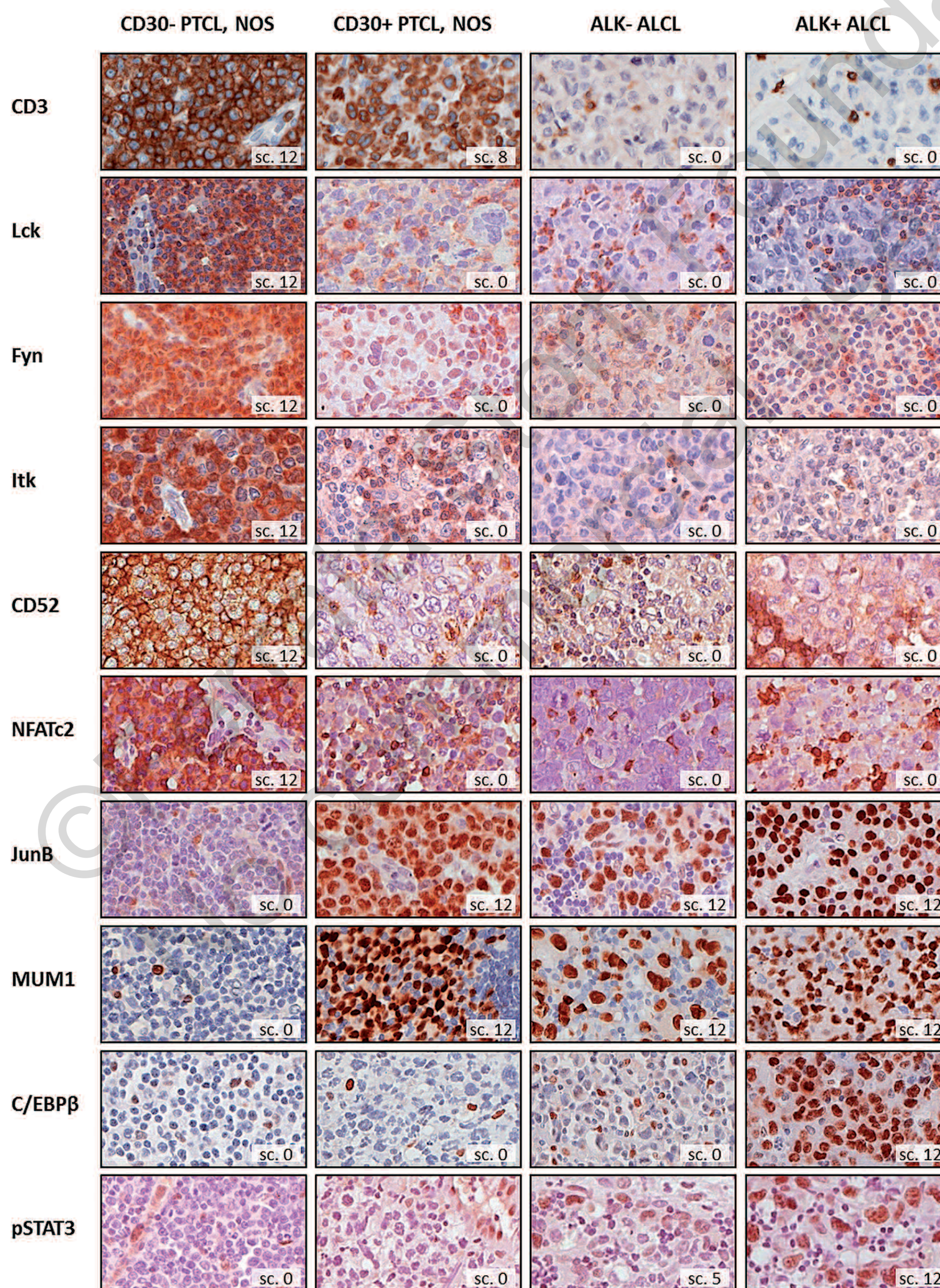

Figure 3. Selection of immunostains, depicting the most representative expression patterns for each of the four PTCL subgroups explored (magnification 200x). Corresponding immunohistochemical scores (sc.) are provided.

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pSTAT3 expression was also significantly higher in ALK− ALCL (median 5.5; 88% positive) than in CD30+ PTCL, NOS (median 0; 10% positive). Conversely, FoxP1 and GATA1 did not show any difference in expression across the various PTCL groups analyzed (all medians for FoxP1: 0; all medians for GATA1: 6).

The cell cycle regulator cyclin D3 was overexpressed in ALK+ ALCL (median 10; 91% positive) compared to the other PTCL (medians 3-5; 38-73% positive), while the difference in expression was not significant between CD30− and CD30+ PTCL, NOS (medians 5 and 3, respectively; 69% and 38% positive, respectively).

Finally, we observed a higher expression of IMP3 in CD30+ PTCL, NOS (median 11; 100% positive) compared to CD30− cases (median: 3.8; 50% positive); and a slight but significant down-regulation of MAL in ALK+ ALCL (median 0; 0% positive) relative to the other PTCL categories (medians 1-2; 36-50% positive).

When comparing the PTCL subgroups in pairs (Mann-Whitney tests), CD30− PTCL, NOS and CD30+ PTCL, NOS showed significantly different immunohistochemical scores for 11/21 (52%) markers. As expected, the CD30− PTCL, NOS category was even more divergent when compared to the whole group of CD30+ PTCL, with significant differences for 16/21 (76%) markers. Conversely, the large majority of immunostains gave similar scores for CD30+ PTCL, NOS and ALK− ALCL, with only 2/21 markers (10%) being significantly different (CD3 and pSTAT3). ALCL entities (ALK− *versus* ALK+) differed from each other by 6/21 markers (29%). The differences and similarities of marker expression between the PTCL subgroups are illustrated as a Venn diagram in *Online Supplementary Figure S4*.

The differences observed between CD30− PTCL, NOS and the various groups of CD30+ PTCL were to a large extent accounted for by molecules involved in proximal T-cell signaling (CD3, TCRβF1, ZAP-70, Lck, Fyn, Itk) and T-cell differentiation/activation (CD52, CD69, ICOS, NFATc2), the expression of which proved to be significantly reduced in all CD30+ samples. As predicted by our GEP findings,[19] most of these dissimilarities also pertained to the CD30− *versus* CD30+ comparison within the PTCL, NOS group, while the CD30+ PTCL, NOS cases were not distinguishable from the ALCL samples on the basis of these pathways, except for a mostly retained expression of CD3.

CD30+ and CD30− peripheral T-cell lymphomas are segregated by hierarchical cluster analysis

Hierarchical clustering was performed starting from all immunohistochemical scores obtained with the 21 markers specifically explored in this study, including CD3 (*Online Supplementary Table S2*), after exclusion of diagnostic classifiers (ALK, CD30, EMA and cytotoxic markers). This analysis corroborated the marked divergence between CD30− and CD30+ PTCL, by segregating them into the main two branches of the dendrogram (Figure 4). Pursuant to the immunostaining results, the heat map visually highlighted the relative over-expression of TCR-associated and T-cell differentiation/activation-related proteins in CD30− PTCL, NOS compared to CD30+ cases. Conversely, the latter shared high levels of the transcription factors JunB and MUM1/IRF4.

Within the spectrum of CD30+ PTCL, ALK+ ALCL tended to cluster together, distinguished by up-regulation of pSTAT3, C/EBPβ and cyclin D3, all described to be activated or induced by chimeric ALK.[32] Conversely, the two CD30+ ALK− PTCL categories (comprising NOS and ALCL cases) were mostly intermingled with one another, reflecting considerable similarities in their protein expression profiles. The levels of CD3, ZAP-70 and NFATc2 were generally higher in these samples than in ALK+ ALCL.

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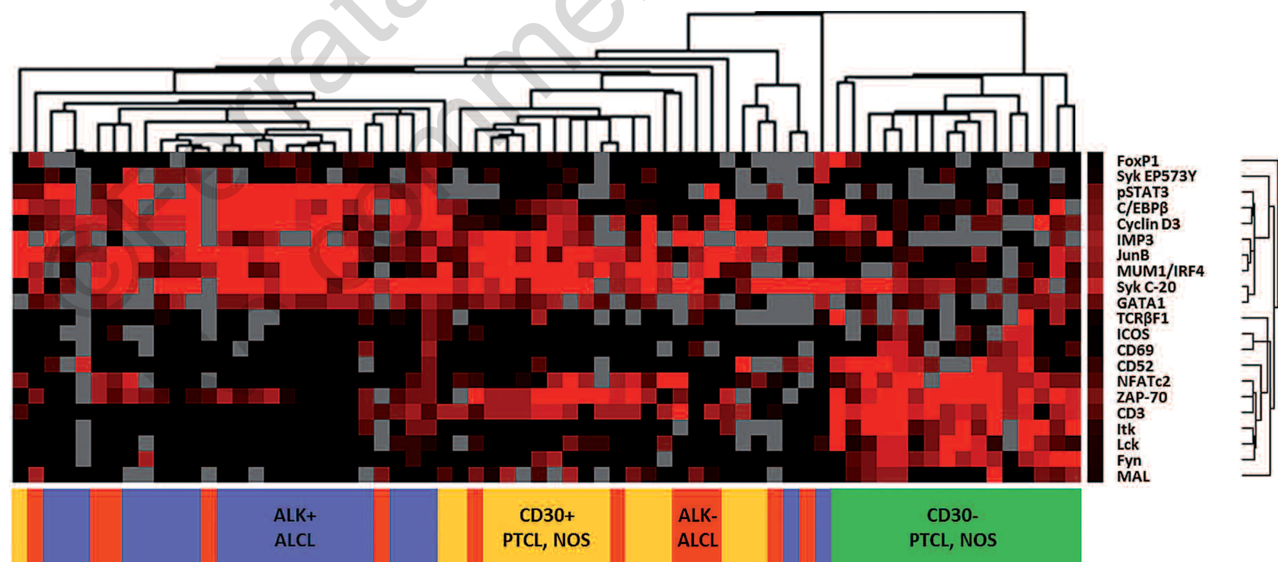

Figure 4. Hierarchical clustering of PTCL samples according to immunostaining results. The dendrogram was generated by the software on the grounds of the immunohistochemical staining scores, without any *a priori* knowledge of the pathological diagnoses (average linkage clustering, Cluster and TreeView softwares, *http://www.eisenlab.org*).[29] The immunomarkers explored are listed at the right of the heat map. The pathological diagnoses of the samples are shown at the bottom [68 PTCL comprising 23 ALK+ ALCL (blue), 12 ALK- ALCL (orange), 17 CD30+ PTCL, NOS (yellow) and 16 CD30- PTCL, NOS (green)]. The color tones of the squares, ranging from black to bright red, represent increasing immunostaining scores between 0 and 12. Gray squares indicate non-evaluable stains.

Table 2. Clinical features and survival data by diagnostic category.

Clinical feature	All patients	Diagnostic category CD30⁻ PTCL, NOS	CD30⁺ PTCL, NOS	ALK⁻ ALCL	ALK⁺ ALCL	P value (4 groups)
N. of patients*	n=80	n=18	n=18	n=15	n=29	
Median age at diagnosis, years (range)	53 (2-85)	60 (21-77)	58 (42-85)	62 (6-85)	22 (2-70)	<0.0001 (Kruskal-Wallis test)
Male:female ratio	1.47	1.25	2.60	1.50	1.15	0.629 (Chi-square test)
Median follow-up, months (range)	30 (1-153)	20.5 (1-153)	29 (1-87)	26 (1-62)	46 (1-142)	0.413 (Kruskal-Wallis test)
Median overall survival, months	Not reached** (n=62)	24 (n=16)	59 (n=17)	58 (n=8)	Not reached** (n=21)	0.252 (Log-rank Mantel-Cox test)
Median progression-free survival, months	26 (n=59)	10.5 (n=16)	59 (n=15)	58 (n=7)	Not reached** (n=21)	0.186 (Log-rank Mantel-Cox test)

**N. of patients: the first row indicates the total number of patients (n) included in the study. However, while information about age and sex was available for all the patients, survival data were not. Therefore, a different n is reported in the rows regarding survival. **Not reached: median survival could not be determined if >50% of the patients were alive (for overall survival) or stable (for progression-free survival) at the date of last information.*

CD30⁻ peripheral T-cell lymphomas, not otherwise specified tend to have an inferior clinical outcome

Available clinical characteristics and survival data for the various PTCL subgroups are summarized in Table 2. The median age of the ALK⁺ ALCL patients (22 years) was significantly lower than that of the other categories (58-62 years; $P<0.0001$, Kruskal-Wallis test). A male predominance was observed in all subgroups (overall male:female ratio 1.47). The median follow-up time for all patients was 30 months (range, 1-153 months).

Overall and progression-free survival curves, stratified by diagnostic category, are illustrated in *Online Supplementary Figure S5*. When comparing the median overall and progression-free survivals, ALK⁺ ALCL tended to have a better outcome (medians not reached for ALK⁺ ALCL). Conversely, patients with CD30⁻ PTCL, NOS were characterized by shorter median overall survival and progression-free survival (24 and 10.5 months, respectively; compared to approximately 60 months for CD30⁺ PTCL, NOS and ALK⁻ ALCL). However, none of the differences was statistically significant, either when comparing all four subgroups at once (P=0.252 for overall survival and P=0.186 for progression-free survival; log-rank Mantel-Cox tests), or when comparing the various subgroups in pairs (lowest P=0.053 for overall survival, when comparing ALK⁺ ALCL and CD30⁻ PTCL, NOS; lowest P=0.081 for progression-free survival, when comparing ALK⁺ ALCL and CD30⁻ PTCL, NOS; log-rank Mantel-Cox tests).

Discussion

The present work was based on our previous observations suggesting conspicuous dissimilarities between the molecular profiles of PTCL, NOS according to CD30 expression.[19] Within the spectrum of PTCL, a diagnostic gray zone exists between PTCL, NOS and ALK⁻ ALCL, accounted for by a subset of PTCL composed of large CD30⁺ cells.[1] To further explore the possible relatedness of CD30⁺ nodal PTCL at the molecular level, we extended here the analysis to a series of ALCL, and found substantial overlaps between the signatures of CD30⁺ PTCL, NOS and ALK⁻ ALCL, while the profile of CD30⁻ samples was confirmed to be clearly divergent.

With the main purpose of corroborating transcriptional data at the protein level, we studied a larger series of CD30⁻ and CD30⁺ PTCL (NOS type and ALCL) by immunohistochemistry, focusing primarily on those molecules whose expression had appeared to be discriminating between CD30⁺ and CD30⁻ PTCL, NOS subgroups.

The CD30⁺ PTCL, NOS group featured a substantial loss of several molecules involved in TCR signaling and T-cell differentiation/activation (the proximal tyrosine kinases Lck, Fyn and Itk, the surface antigens CD69, CD52 and ICOS, and the transcription factor NFATc2), which were, in contrast, mostly conserved in CD30⁻ samples. Conversely, the transcription factors JunB and MUM1/IRF4 showed an opposite expression pattern, being highly expressed in most CD30⁺ PTCL, NOS and largely absent in the majority of CD30⁻ cases. Interestingly, by studying these same proteins in ALK⁺ and ALK⁻ ALCL samples, we observed expression scores that were strikingly similar to those of CD30⁺ PTCL, NOS.

In line with these observations, hierarchical clustering of the whole set of immunohistochemical scores segregated the samples into two groups according to the expression of CD30. Although the organization of the data was carried out blindly by the software without any *a priori* information ("unsupervised analysis"), the immunomarkers had been selected on the basis of their differential expression ("supervised approach"), consequently implying some bias by the marker selection itself. Nevertheless, the immunohistochemical clustering mirrors the GEP clustering somewhat and underscores distinctive immunophenotypic features between CD30⁻ PTCL, NOS and the whole group of CD30⁺ PTCL, independently of the commonly used diagnostic classifiers (namely ALK, CD30, EMA and cytotoxic proteins) which had been excluded from the analysis.

The disturbed expression of molecules associated with the TCR/CD3 complex and downstream signaling previously evidenced in ALCL has been interpreted as a unifying feature of these neoplasms, irrespective of their *ALK* gene status, suggesting that this characteristic might enable ALCL to be distinguished from PTCL, NOS.[21,22] However, the relationship between the down-regulation of TCR-associated molecules and the expression of CD30 has not been well characterized. Indeed, most PTCL, NOS cases included in those studies did not express CD30, and it could

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not, therefore, be determined whether CD30$^+$ PTCL, NOS would display features more similar to the remaining PTCL, NOS, or to the ALCL categories. Our findings establish that the defective expression of TCR-related signaling molecules is also a characteristic feature of CD30$^+$ PTCL, NOS, thus pointing towards the impairment of TCR signaling as a pathway common to all CD30$^+$ PTCL, including NOS cases, and raising consideration of whether the up-regulation of CD30 and disturbed expression of TCR-associated molecules might be mechanistically related.[33]

ALK$^+$ ALCL differed from other CD30$^+$ PTCL by virtue of the up-regulation of several molecules known to be activated or induced by the kinase activity of ALK chimera. These comprised most notably the transcription factors pSTAT3 and C/EBPβ, and the cell cycle regulator cyclin D3.[6,7,13,32,34]

Despite the limitations inherent to the selection of the markers studied, CD30$^+$ PTCL, NOS and ALK$^-$ ALCL had markedly overlapping profiles, with differential expression of only two markers (CD3 and pSTAT3). Accordingly, in cluster analysis most CD30$^+$ PTCL, NOS and ALK$^-$ ALCL samples clustered together. It could be opposed that the stringency of the criteria utilized for categorization as ALK$^-$ ALCL, which ensured that our ALK$^-$ ALCL group was strictly defined, might have led to some cases being classified as PTCL, NOS that others might consider consistent with ALK$^-$ ALCL. Indeed, six of the samples that we classified as PTCL, NOS had some hallmark-like cells, while not showing evidence of EMA and/or cytotoxic marker expression, hence not categorized as ALK$^-$ ALCL according to our criteria. Interestingly however, when we excluded these "borderline" samples from the analyses, only the score of pSTAT3 was significantly different between CD30$^+$ PTCL, NOS and ALK$^-$ ALCL (*data not shown*), enabling us to exclude the hypothesis of an eventual bias introduced by the inclusion of these six cases. Moreover, when applying the molecular classifiers developed by Piva *et al.*[13] and Agnelli *et al.*[14] to discriminate ALCL from PTCL, NOS, we found that the levels of mRNA expression of three of the four genes which could be analyzed in our dataset (*TMOD1*, *PERP*, *TNFRSF8*) were significantly lower in CD30$^+$ PTCL, NOS than in ALK$^-$ ALCL, while one of the genes (*BATF3*) was expressed at similar levels in CD30$^+$ PTCL, NOS and ALCL. Interestingly, the levels of expression of *TMOD1*, *PERP* and *TNFRSF8* were also significantly different between CD30$^-$ and CD30$^+$ PTCL, NOS, being lower in the CD30$^-$ subgroup (*data not shown*).

To what extent CD30$^+$ PTCL, NOS and ALK$^-$ ALCL have overlapping and distinctive features remains to be characterized further. ALK$^-$ ALCL is currently considered a provisional entity, defined by often subtle morphological and immunophenotypic criteria.[1] The distinction from CD30$^+$ PTCL, NOS, particularly from those cases composed of large pleomorphic cells, may be fragile and subjective. The molecular findings presented here further substantiate the biological continuum across CD30$^+$ PTCL.

From a clinical perspective, while it is well established that ALK$^+$ ALCL patients have a more favorable prognosis than patients with other systemic PTCL (although this difference may at least partially be dictated by the younger age at presentation),[5,35] survival data are conflicting with regard to ALK$^-$ ALCL and PTCL, NOS. ten Berge *et al*. reported a comparable poor prognosis for these two entities (5-year overall survival: <45%), proposing that the segregation of the two entities might be of limited clinical relevance.[36] Conversely, in a larger study by the International Peripheral T-cell Lymphoma Project, ALK$^-$ ALCL patients had a significantly better outcome than PTCL, NOS patients (5-year overall survival: 49% *versus* 32%), with an even more marked difference when the analysis of PTCL, NOS was restricted to CD30$^+$ cases (5-year overall survival: 19%).[5] The survival data available in the present study showed no significant differences between CD30$^+$ PTCL, NOS and ALK$^-$ ALCL, but particularly for the latter category the cohort size was too small and the follow-up duration too limited to allow any significant conclusions to be drawn on this issue.

A more notable finding in our series, albeit not statistically significant, was the tendency of CD30$^+$ PTCL, NOS patients to have a better outcome than those with CD30$^-$ PTCL, NOS, suggesting that their segregation might be not only biologically but also clinically relevant, yet contrasting with the survival data reported by Savage *et al*.[5] Conversely, in a recent study from the North American T-cell lymphoma Consortium, describing the clinicopathological features of 159 PTCL cases (74 CD30$^-$ PTCL, NOS, 21 CD30$^+$ PTCL, NOS, 37 ALK$^-$ ALCL and 27 ALK$^+$ ALCL) the authors reported, like us, that the overall survival of patients with CD30$^+$ PTCL, NOS was similar to that of patients with ALK$^-$ ALCL, and superior to that of patients with CD30$^-$ PTCL, NOS.[37] The discordance in the outcome of the CD30$^+$ PTCL, NOS cases between different studies remains unexplained. The fact that not all studies have used the same cutoffs for CD30 positivity, the retrospective nature of multicenter cohorts, the heterogeneity of the treatments delivered, and the relatively small numbers of patients are possible compounding factors that may account for the heterogeneous clinical outcomes. Altogether, however, the discordant observations emphasize the need to collect data from larger series of cases in a controlled setting.

In conclusion, following-up on previous GEP data our findings suggest that the expression of CD30 might constitute a valuable criterion to define two distinct biological subgroups (CD30$^+$ and CD30$^-$) within the heterogeneous category of PTCL, NOS. The putative clinical relevance of these subgroups needs to be confirmed in larger series of patients, but might be reinforced by the potential benefits of incorporating anti-CD30 immunoconjugates into the treatment strategies of CD30$^+$ PTCL.[38]

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