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***DUSP22*-rearranged anaplastic lymphomas are characterized by specific morphological features and a lack of cytotoxic and JAK/STAT surrogate markers**

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ALK-negative anaplastic large cell lymphoma (ALK-negative ALCL) is a heterogeneous disease with very disparate outcomes. Molecular studies have identified chromosomal rearrangements involving the *DUSP22-IRF4* locus on 6p25.3 (*DUSP22* rearrangements) as a favourable prognostic factor, associated with complete remission after first treatment thereby suggesting that this subgroup of patients may not gain additional benefit from autologous stem cell transplantation in first remission(1-3). Recognition of these cases is critical, and we therefore aimed to study in greater detail the histological and immunophenotypic features of *DUSP22*-rearranged ALK-negative ALCLs.

After approval by the Institutional Review Board of the Hospital Universitario Marqués de Valdecilla and the Fundación Jiménez Díaz (Spain), we collected 91 cases with a diagnosis of systemic or primary cutaneous ALCL made at the participating institutions. Clinical data were retrieved and cases were reviewed by three independent pathologists (AO, SMRP, MAP), using hematoxylin and eosin stains. Immunohistochemistry was performed using a panel of antibodies against ALK, CD3, CD4, CD8, granzyme B, MUM1, perforin, P-STAT3 (D3A7, 1/400 Cell Signaling), TIA1, P-STAT5, TCR-βF1, P63, STAT3 (supplementary data). Of 91 evaluated cases, 18 were primary cutaneous ALCLs (pcALCLs) and 73 cases were systemic ALCLs (19 were ALK-positive ALCLs). ALK-positive cases were not further considered for the study. Only 31 cases were eligible for further study due to tissue scarcity, including 22 ALK- ALCL and 9 pcALCLs. FISH analyses were performed on these cases using an IRF4-DUSP22 (6p25.3) break-apart probe (KBI-10613; Kreatech, Leica, Spain) following standard procedures(4, 5). Cytotoxic markers, pSTAT3, p63 and MUM1 expression were evaluated as described in the supplementary data. Associations of genetic and immunohistochemical subgroups with OS and PFS were assessed using Kaplan-Meier curves. Differences between genetic subgroups in patient characteristics, tumour phenotype and other clinical factors were assessed using the chi-square test and Wilcoxon rank-sum test, as appropriate.

Of the 31 cases tested for *p63* rearrangements, 1 case (1/31, 3.2%) was positive, 26 were negative (26/31, 83.8%), and 3 showed gains of p63 (3/31, 9.7%). One case (1/22, 4.5%) had *DUSP22* gains, and another case had *DUSP22* amplification. Twenty-five cases (25/31, 80.6%) were classified as triple-negative ALCLs, and six cases had *DUSP22* rearrangements, including 4 ALK-negative ALCLs (4/22, 18.2%), and 2 pcALCLs (2/9, 22.2%), representing the study cohort.

Demographic and clinical characteristics of *DUSP22*-rearranged cases are shown in Table 1. The six patients were aged from 39 to 65 years at presentation (mean, 56 years), with a predominance of males (2M:1F). In one of the pcALCL cases (case 5), the lesions were restricted to a single body area – the cheek; the site location was not available for case 6. Systemic *DUSP22*-rearranged cases exhibited a high clinical stage at presentation, with low

ECOG performance status, IPI and PIT indices. One patient had bone marrow involvement at diagnosis and high LDH levels. Two patients received CHOP-based treatment regimens, and another received radiotherapy. All three patients achieved complete remission according to the available clinical information. Only the patient receiving radiotherapy as front-line treatment relapsed 9 months after initial treatment. None of them underwent stem-cell transplantation. After a median follow-up of 55 months, all four patients with systemic *DUSP22*-rearranged ALCL were alive without disease. Patients with pcALCL were treated by excision, and there was no recurrence or progression during follow-up (Table 1). Median follow-up time from diagnosis for systemic ALCL patients who were still alive was 43 months (range, 3 to 126 months).

Consistently with the results of previous studies, patients with ALK-negative ALCL had a poorer outcome than patients with ALK-positive ALCL (3-y OS: 52%, 95% CI: 36-68% vs. 80%, 95% CI: 60-100%; log-rank, p=0.156). Patients with systemic *DUSP22*-rearranged ALCL showed better OS rates than the triple-negative ALCL genetic subtype (3-y OS: 100% vs. 28%, 96% CI: 4-72%; log-rank p=0.05, for triple-negative patients), and similar to ALK-positive ALCL patients (3-y OS: 80%, 96% CI: 60-100%; log-rank, p=0.422) (Figure 1).

As previously described(6), *DUSP22*-rearranged ALCLs showed unusual histological features that were consistent among all cases. In the systemic cases, lymph node architecture was effaced, with neoplastic infiltration by intermediate cells that were smaller than those observed in triple-negative and ALK-positive ALCLs, with a sheet-like growth pattern, and a monomorphic appearance. Histopathological findings were consistent among all cases. Neoplastic cells exhibited prominent nucleoli and pseudo-inclusions in the so-called "doughnut" cells, although they were not specific to this group. Hallmark cells, mitotic figures and apoptotic bodies were abundant. Tumor cells were predominant, with no lymphohistiocytic or inflammatory background infiltrate. No sinusoidal involvement was observed, in contrast to the pattern commonly observed in ALK-positive ALCLs (Figure 2). Triple-negative ALCL cases had a more variable morphology, with the presence of hallmark cells and large pleomorphic and multinucleated cells.

The two pcALCL cases with *DUSP22* rearrangements had a biphasic pattern, as previously reported by our group(7). A prominent dermal nodule with a dense lymphoid infiltrate and overlying ulceration was noted at low magnification. The neoplastic infiltrate was composed of medium-to-large atypical cells, with abundant finely granular cytoplasm, intermingled with abundant hallmark cells. A characteristic pagetoid reticulosis-like intraepidermal lymphocytosis pattern was also present, along with intraepidermal small atypical lymphocytes featuring hyperchromatic and irregular nuclei. Mitotic figures and apoptotic bodies were abundant

within the dermal infiltrate. Eosinophils and neutrophils were absent (Figure 3).

Among *DUSP22*-rearranged cases, neoplastic cells were positive in all cases for at least one T-cell antigen (Table 1), CD3 and/or the T-cell receptor (TCR) β chain (TCRβF1), negative for ALK, and strongly and diffusely positive for CD30. TCRβF1 stain was not available in case 2, but CD3 was positive. Case 5 was CD3-negative but TCRβF1-positive. These markers accentuated the sheet-like growth pattern in the systemic cases, and the epidermotropic pagetoid reticulosis-like infiltrate in the primary cutaneous cases. All cases had a non-cytotoxic phenotype. TIA-1 was negative in all cases, being found in 5-25% of the tumoral cells. Granzyme B and perforin were also negative in all cases (<5% of tumoral cells). MUM1 was positive in four cases (median expression in 95% of tumoral cells, range: 75-100%), and only case 6 was completely negative. P63 expression was more variable, being positive in 2/5 cases tested (85-100% of tumoral cells), and negative (<15% of tumoral cells) in 3/5 cases. The three surrogate markers of the JAK/STAT pathway (phosphorylated STAT1, STAT3 and STAT5) were consistently negative in all six cases (expression in <20% of tumoral cells).

In this study, we report six cases of *DUSP22*-rearranged ALCL (systemic and cutaneous) with common histological features, with the presence of intermediate cells with a doughnut-like morphology, and abundant hallmark cells, apoptotic and mitotic figures, as previously reported(6). In addition, both primary cutaneous cases exhibited a biphasic pattern (7, 8), which has also been described in lymphomatoid papulosis cases carrying the same translocation(8).

Furthermore, our results support results recently published by other groups (9), identifying lack of activation of the JAK/STAT pathway in *DUSP22*-rearranged cases, despite initially proposed to be a universal finding in ALK-positive and ALK-negative ALCLs (10).

We describe histological and immunophenotypic features that may help recognize *DUSP22*-rearranged cases. The presence of sheets of intermediate-to-large cells, with relatively monomorphous large-cell cytology, including hallmark and doughnut cytology, with no expression of cytotoxic markers, is useful for further FISH testing in systemic cases. In the pcALCL cases, the presence of the previously described biphasic pattern is a useful indicator of *DUSP22*-rearrangement. The same translocation involving locus 6p25 was also described in lymphomatoid papulosis (LyP)(8, 11), suggesting that this molecular alteration could determine a better outcome, both in cutaneous and systemic ALK-negative ALCL.

Constant expression of T-cell markers and a lack of cytotoxic markers and markers of activation of the STAT pathways seem to be linked to *DUSP22* translocation in this series.

It would be of interest to explore whether this combination of markers in other ALK-negative ALCLs identifies cases with specific morphology, immunophenotype or clinical features.

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Table 1. Clinical, Histological, Immunophenotypic, and Genetic Features of 6 Patients with *DUSP22*-rearranged ALCL

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Clinical presentation Age at Diagnosis Sex Site	 65 M Lymph node	 71 F Lymph node	 50 M Lymph node	 39 M Lymph node	 73 F Skin (right cheek)	 39 M Skin
Ann-Arbor Stage	III	IV	------	III	------	------
ECOG Status	0-1	0-1	------	------	------	------
IPI	Low-intermediate	------	------	Low	------	------
PIT	Group 1 (PIT=0)	------	------	------	------	------
Extranodal involvement	Pleural effusion	Skin	------	Tonsil	------	------
Bone Marrow involvement	Absent	Present	Absent	Absent	Absent	Absent
Histologic features Cell morphology	Hallmark cells, doughnut cells	Hallmark cells, doughnut cells	Hallmark cells, doughnut cells	Hallmark cells, doughnut cells	Hallmark cells, doughnut cells	Hallmark cells, doughnut cells
Pattern	Sheet-like growth pattern	Sheet-like growth pattern	Sheet-like growth pattern	Sheet-like growth pattern	Biphasic pattern (dermal nodule and pagetoid reticulosis-like epidermal infiltrate)	Biphasic pattern (dermal nodule and pagetoid reticulosis-like epidermal infiltrate)
Background	Inflamatory infiltrate absent. Macrophages with tingible bodies. Apoptotic bodies and mitotic figures.	Inflamatory infiltrate absent. Apoptotic bodies and mitotic figures.	Inflamatory infiltrate absent. Apoptotic bodies and mitotic figures.	Inflamatory infiltrate absent. Apoptotic bodies and mitotic figures.	Inflamatory infiltrate absent. Apoptotic bodies and mitotic figures.	Inflamatory infiltrate absent. Apoptotic bodies and mitotic figures.
Pathological Diagnosis	Systemic ALK-negative ALCL	Systemic ALK-negative ALCL	Systemic ALK-negative ALCL	Systemic ALK-negative ALCL	pcALCL	pcALCL
Immunophenotype ALK	 Negative	 Negative	 Negative	 Negative	 Negative	 Negative
CD3	Positive	Positive	Positive	Positive	Negative	Positive
CD30	Positive	Positive	Positive	Positive	Positive	Positive
TCRβF1	Positive	------	Positive	Positive	Positive	Positive
TIA-1	Negative	Negative	Negative	Negative	Negative	Negative

	(10%)	(15%)	(25%)	(5%)	(10%)	(5%)
Granzyme B	Negative (1%)	Negative (0%)	Negative (15%)	Negative (5%)	Negative (1%)	Negative (1%)
Perforin	Negative (1%)	Negative (5%)	Negative (5%)	Negative (0%)	Negative (0%)	Negative (0%)
MUM1	Positive (95%)	Positive (85%)	Positive (100%)	Positive (75%)	Positive (95%)	Negative (0%)
p63	Negative (15%)	Positive (100%)	Positive (85%)	Negative (0%)	------	Negative (0%)
P-STAT1	Negative (<1%)	------	Negative (<1%)	Negative (<1%)	------	Negative (<1%)
P-STAT3	Negative (0%)	------	Negative (15%)	Negative (7%)	Negative (2%)	Negative (10%)
P-STAT5	Negative (0%)	------	Negative (2%)	Negative (2%)	------	Negative (2%)
STAT3	------	Negative (15%)	------	------	------	------
Cytotoxic phenotype	Absent	Absent	Absent	Absent	Absent	Absent
Follow-up Treatment	CHOP	RT	------	CHOEP	Excision	Excision
Treatment response	CR	CR	CR	CR	------	------
Recurrence/Progression	No	Yes (skin)	No	No	No	No
SCT	No	No	No	No	No	No
Status at last follow-up	NED	NED	NED	NED	NED	NED
Months since onset	68	66	43	7	1423	12
Months disease-free	68	9	43	7	1423	12

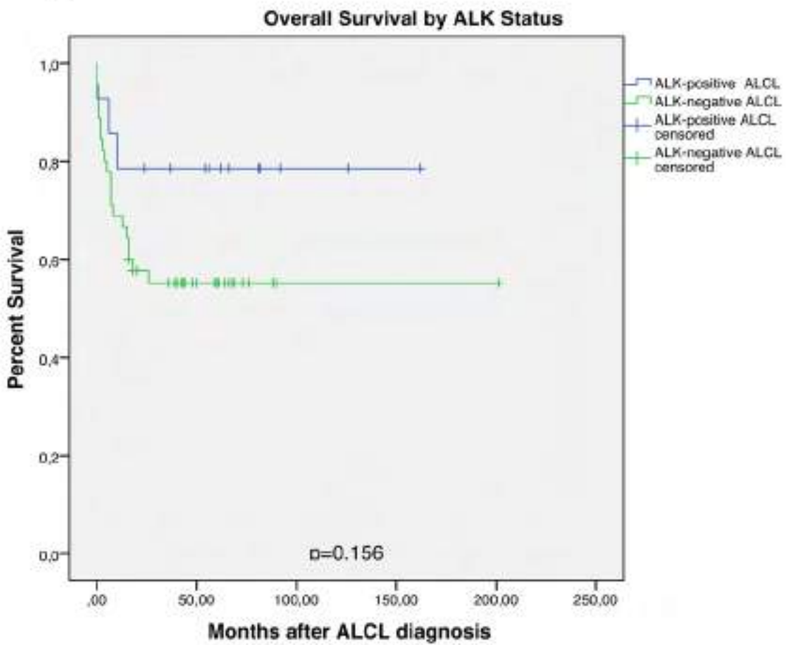
pcALCL: Primary cutaneous ALCL; M: Male; F: Female; CHOP: Cyclophosphamide + hydroxydaunorubicin + vincristine prednisone; RT: Radiotherapy; CHOEP: Cyclophosphamide + hydroxydaunorubicin + vincristine + etoposide + prednisone; RT: Radiotherapy; SCT: Stem-cell transplantation; NED: No evidence of disease

Figure 1. Outcome in patients with ALCL based on genetic subtype. (A) OS rates in patients with ALCL, stratified by ALK status. (B) OS rates in patients with systemic ALK-negative ALCL, stratified by rearrangements. (C) OS rates in patients with ALK-positive ALCL and *DUSP22*-rearranged ALCL.

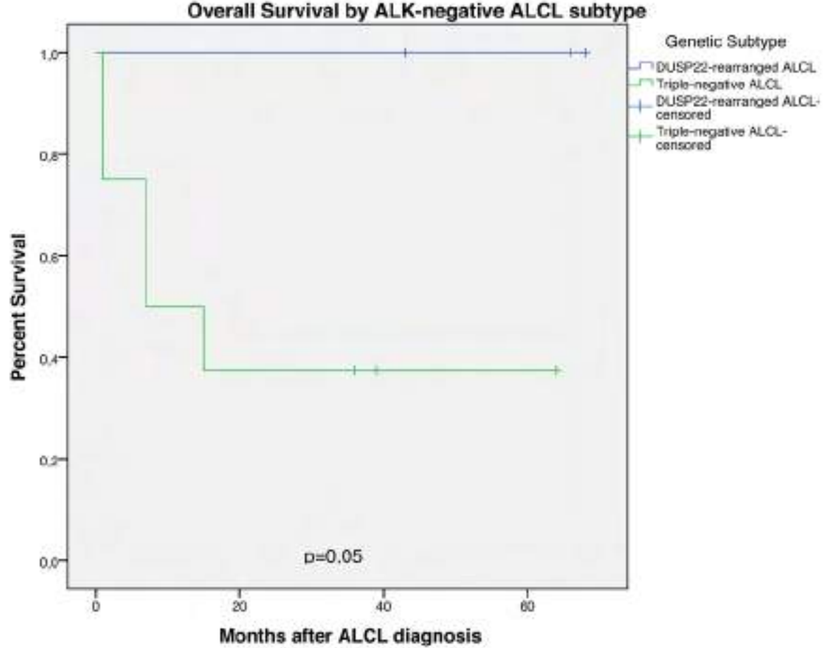
Figure 2. **Histological and immunophenotypic features of systemic ALK-negative anaplastic large cell lymphoma with *DUSP22* rearrangement (case 1)**. (A) Low-power microscopic image of a lymph node with effaced architecture. (B) Sheets of medium-to-large neoplastic cells (C) with abundant hallmark cells, apoptotic cells and doughnut cells (inset), with an eosinophilic nuclear inclusion (D, E and F). Neoplastic cells were diffusely positive for CD3 (G), TCRβF1 (H), and CD30 (I), and negative for ALK (J), cytotoxic markers TIA-1 (K), granzyme B (L) and perforin (M), and for p-STAT3 (N). (O) FISH using a break-apart probe at the *DUSP22* locus shows a rearrangement, with one normal fusion signal and an abnormal split signal.

Figure 3. **Histological and immunophenotypic features of a primary cutaneous anaplastic large cell lymphoma with *DUSP22* rearrangement (case 5)**. (A) Low-power microscopic image of the skin biopsy showing diffuse dermal infiltration, characterized histologically by a dense dermal infiltrate with epidermal involvement by small lymphocytes (B, C). (D) Dermal infiltrate of medium-sized and atypical lymphocytes, with a monomorphic appearance, including hallmark and occasional doughnut cells (E, inset; F, inset). Neoplastic cells were CD3-negative (G), TCRβF1-positive (H), and CD30-positive (I). ALK (J), TIA-1 (K), granzyme B (L), and perforin (M), and P-STAT3 (N) were negative. (O) FISH using a break-apart probe at the *DUSP22* locus shows a rearrangement, with one normal fusion signal and an abnormal split signal.

A


Overall Survival by ALK Status
Percent Survival
1,0
0,8
0,6
0,4
0,2
0,0
ALK-positive ALCL
ALK-negative ALCL
ALK-positive ALCL censored
ALK-negative ALCL censored
p=0.156
,00
50,00
100,00
150,00
200,00
250,00
Months after ALCL diagnosis

B


Overall Survival by ALK-negative ALCL subtype
Percent Survival
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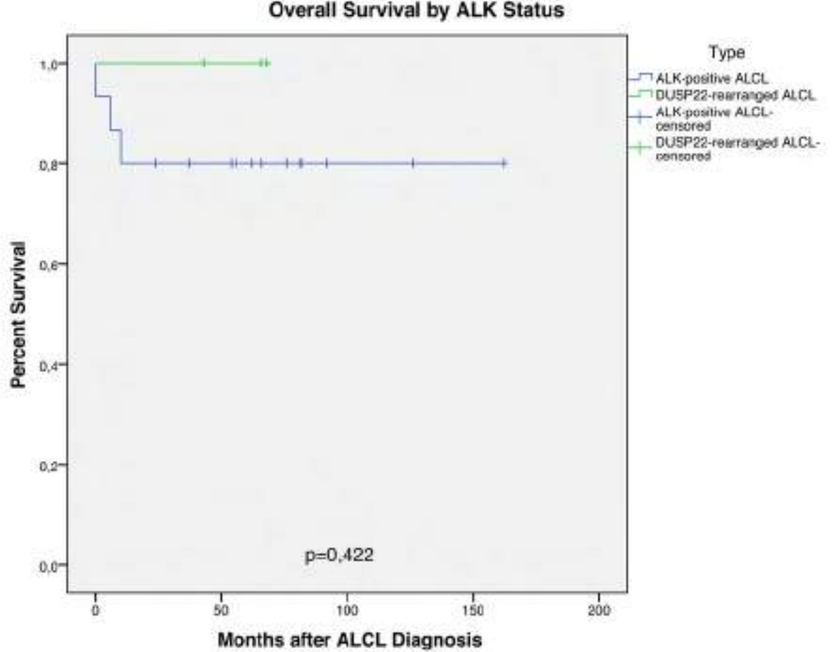
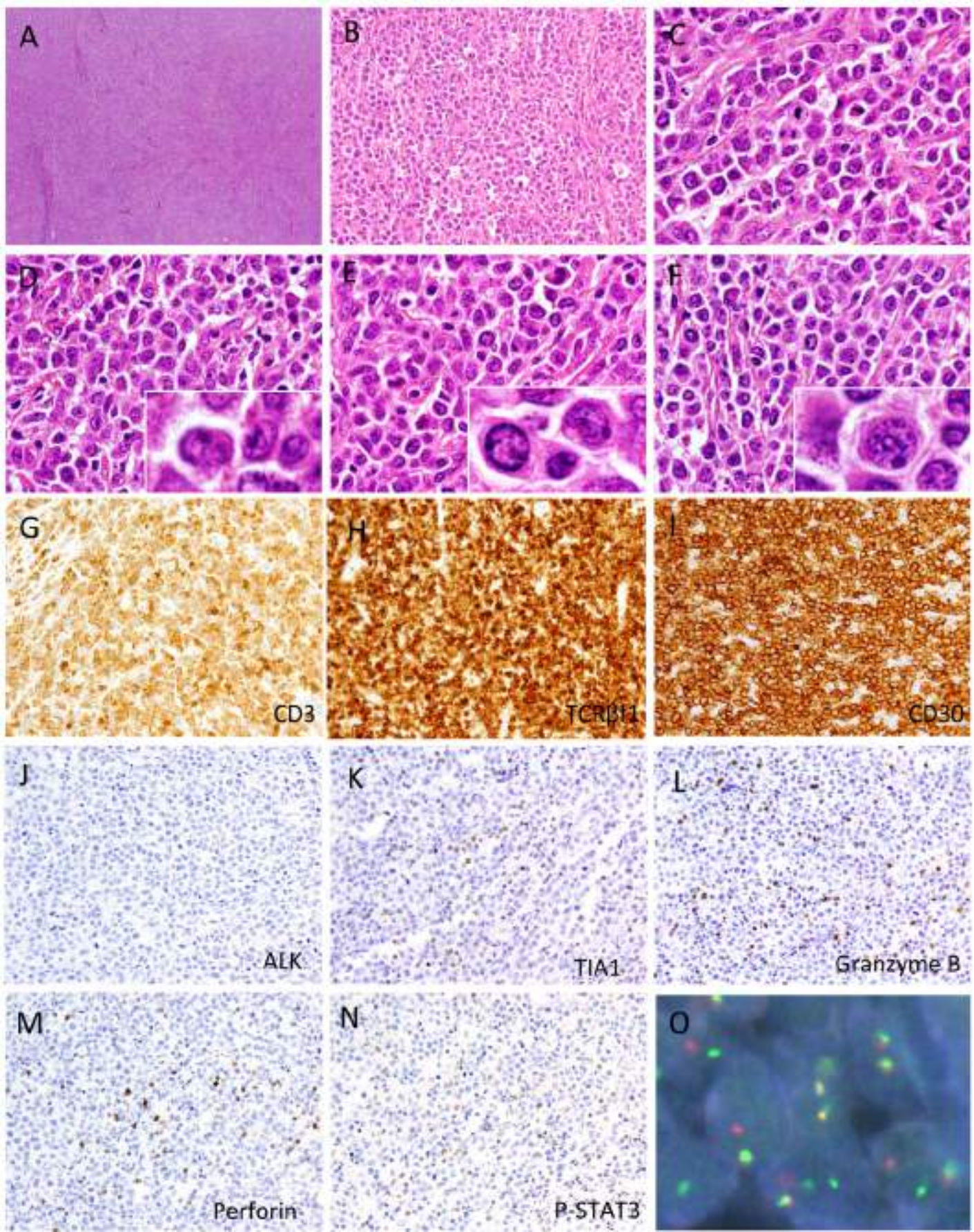
0,8
0,6
0,4
0,2
0,0
Genetic Subtype
DUSP22-rearranged ALCL
Triple-negative ALCL
DUSP22-rearranged ALCL-censored
Triple-negative ALCL-censored
p=0,05
0
20
40
60
Months after ALCL diagnosis

C


Overall Survival by ALK Status
Percent Survival
1,0
0,8
0,6
0,4
0,2
0,0
Type
ALK-positive ALCL
DUSP22-rearranged ALCL
ALK-positive ALCL-censored
DUSP22-rearranged ALCL-censored
p=0,422
0
50
100
150
200
Months after ALCL Diagnosis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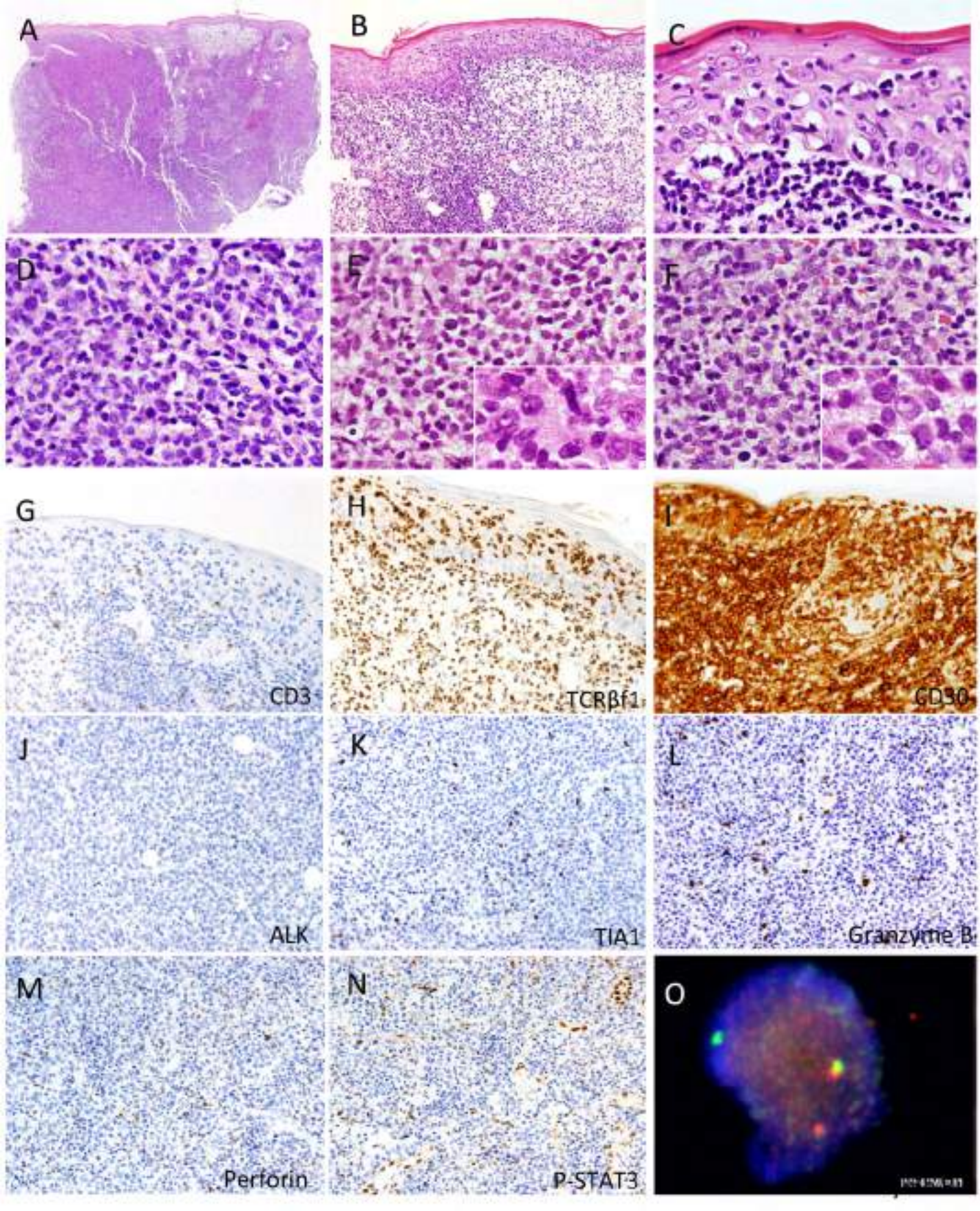
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Perforin
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P-STAT3
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Supplementary data

Immunohistochemistry was performed on 3-µm paraffin sections using the Envision method (Dako, Glostrup, Denmark) on an automated immunostainer (Dako), using a panel of antibodies against ALK (ALK-1, RTU; Dako), CD3 (rabbit polyclonal, RTU; Dako), CD4 (4B12, RTU; Dako), CD8 (C8/144B, RTU; Dako), CD30 (Ber-H2, RTU; Dako), granzyme B (GRB7, 1/25, Dako), MUM1 (RTU, Dako), perforin (5B10, 1/10, Thermo Fisher Scientific), P-STAT3 (D3A7, 1/400 Cell Signaling), TIA1 (TA-1, 1/50, Abcam), P-STAT5 (D2A37, 1/200, Cell Signaling), TCR-βF1 (8A3, 1/40 dilution; Thermo Scientific), P63 (RTU, Dako), STAT3 (F-2, 1/100, Santa Cruz Biotechnology)

Cytotoxic markers were evaluated by measuring the percentage of positive tumoral cells, taking 25% of positive neoplastic cells to be the threshold. A cytotoxic phenotype was ascribed when two of the three cytotoxic markers were positive, or when only one of them was positive in >75% of tumoral cells. MUM 1 was considered positive when expressed in >25% of tumoral cells(1); the cut-off value for p63 positivity was 30%, as proposed by another group(2).

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