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Expression of p63 protein in anaplastic large cell lymphoma: implications for genetic subtyping

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Abstract

Anaplastic large cell lymphomas (ALCLs) are CD30-positive T-cell non-Hodgkin lymphomas that bear chromosomal rearrangements of the *TP53* homologue, *TP63*, in a subset of cases that demonstrate aggressive clinical behavior. In the present study, we examined the relationship between p63 protein expression by immunohistochemistry and the results of fluorescence *in situ* hybridization (FISH) using *TP63* probes in 116 ALCLs. We also determined the relative expression of full-length TAp63 and truncated ΔNp63 isoforms (e.g. p40) in ALCL cell lines and a subset of clinical cases. Overall, 35.3% of ALCLs were positive for p63 protein. Primary cutaneous and ALK-negative ALCLs were positive more frequently than ALK-positive ALCLs (p=0.0034). As previously reported, cases with *TP63* gene rearrangements expressed p63 uniformly. p63 expression in non-rearranged cases was associated with extra copies of *TP63* on 3q28 (p<0.0001). Extra copies of *TP63* correlated with extra copies of the *DUSP22* locus on 6p25.3 (p<0.0001). Results of immunohistochemistry, Western blotting, and RNA sequencing indicated that p63 expression in non-rearranged cases was entirely attributable to TAp63 isoforms. Taken together, these findings indicate that ALCLs without *TP63* rearrangements may express TAp63 isoforms of p63 and that this expression is associated with extra copies of *TP63*, probably

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due to widespread genomic copy number abnormalities rather than focal gains. Immunohistochemistry for p63 in ALCL is not specific for *TP63* rearrangements, but is useful clinically as a screening test to select cases for further testing by FISH. Immunohistochemistry for ΔNp63 (p40) is not informative in the evaluation of ALCL.

Keywords

Anaplastic large cell lymphoma; p63; p40; *TP63*; copy number abnormality; fluorescence *in situ* hybridization

1. Introduction

Anaplastic large cell lymphomas (ALCLs) represent a heterogeneous group of T-cell non-Hodgkin lymphomas that share morphologic and phenotypic features, including CD30 expression [1]. About half of systemic ALCLs have rearrangements of the *ALK* locus and express ALK fusion proteins, the detection of which has diagnostic, prognostic, and therapeutic significance [2–5]. Detection of ALK fusion proteins by immunohistochemistry (IHC) has obviated the need for genetic studies of the *ALK* locus in most cases. However, about half of systemic ALCLs and nearly all primary cutaneous (pc) ALCLs lack *ALK* rearrangements, and no surrogate test analogous to ALK IHC currently exists to aid in their diagnosis and subclassification.

Recently, we reported that systemic ALK-negative ALCLs were genetically heterogeneous, with rearrangements involving the *DUSP22* locus on 6p25.3 in 30% of cases and rearrangements of the *TP63* locus on 3q28 in 8% [6–9]. While ALK-negative ALCLs with *DUSP22* rearrangements had a favorable overall survival (OS) rate similar to ALK-positive ALCLs, ALK-negative ALCLs with *TP63* rearrangements had clinically aggressive disease and a 5-year OS rate of only 17%. ALCLs lacking rearrangements of *ALK*, *DUSP22*, and *TP63* ("triple-negative" ALCLs) had an intermediate prognosis [9]. Of note, *DUSP22* and *TP63* rearrangements also can occur in pcALCL [6–8, 10, 11].

The *TP63* gene encodes p63, a member of the p53 family of transcription factors with multiple isoforms, some of which share tumor suppressor function with p53 [12, 13]. p63 isoforms comprise combinations of various C-termini with one of two N-termini, which differ based on whether a transactivation (TA) domain or an N-truncated (ΔN) domain is located directly upstream of a common DNA-binding domain (DBD). Thus, these groups of isoforms are referred to as TAp63 and ΔNp63 isoforms, respectively. *TP63* rearrangements in ALCL cause gene fusions, most commonly with *TBL1XR1* on 3q26, that lead to expression of p63 fusion proteins [8]. These fusion proteins contain elements encoded by the partner gene directly upstream of the p63 DBD, and lack both TA and ΔN domains. Antibodies generated from epitopes in or near the p63 DBD, such as the 4A4 clone, react with all known p63 isoforms as well as p63 fusion proteins ("pan-p63" antibodies); in contrast, antibodies directed at the ΔN domain react only with ΔNp63 isoforms (including so-called "p40") [8, 14].

TP63 rearrangements can be detected by genetic studies, such as fluorescence *in situ* hybridization (FISH) [8, 9, 15–17]. Although p63 fusion proteins can be detected by IHC in ALCL [8, 9, 15], the role of p63 IHC in the evaluation of ALCLs remains unclear. Gualco et al reported that 44% of ALCLs showed p63 nuclear positivity [18]. Although this study preceded the discovery of *TP63* rearrangements, this expression frequency is unlikely to be solely attributable to *TP63* rearrangements based on our reported rearrangement frequency of 8%. We have confirmed that a subset of ALCLs lacking *TP63* rearrangements but positive for p63 by IHC [9]. We undertook the present study in an extended series of cases to characterize the spectrum of p63 protein expression in the context of *TP63* FISH results; to investigate the relative expression of TAp63 and ΔNp63 isoforms; and to clarify the clinical role of p63 IHC in the evaluation of ALCL.

2. Materials and methods

2.1. Patients

Patients included 116 ALCLs comprising 22 systemic ALK-positive ALCLs, 62 systemic ALK-negative ALCLs, and 32 pcALCLs. There were 75 males and 41 females, with an overall mean age of 56 years (range, 6–93 years). Additional demographic data and the distribution of *DUSP22* and *TP63* rearrangements are shown in Table 1. Studies were approved by the respective Institutional Review Boards at the participating institutions.

2.2. IHC and FISH

IHC for pan-p63 was performed in all cases using the 4A4 clone (Biocare, Concord, CA), which recognizes all known native p63 isoforms as well as p63 fusion proteins, as previously described [8]. IHC for ΔNp63 (“p40”) was performed in a subset of cases with available tissue sections using a rabbit polyclonal antibody (Biocare) as previously described [16]. p63 expression was scored by decile for percentage of tumor cell nuclei with positive staining. IHC also was performed on paraffin tissue sections of cell blocks prepared from ALCL cell lines (see below). Interphase FISH on paraffin tissue sections using a breakapart probes to the *TP63* and *DUSP22* loci and/or a dual-fusion probe for *TBL1XR1*/*TP63* either was performed as previously described, or had been performed for clinical purposes or in prior studies [6, 8–10, 15] One hundred interphase nuclei were evaluated for each case. Upper limits of normal for extra copies were based on 95% confidence intervals derived from FISH results in 25 normal samples and were: *DUSP22* breakapart probe, 8%; *TP63* breakapart probe, 9%; and *TP63* dual-fusion probe, 5%. Copy numbers for TP63 and DUSP22 loci were recorded when these loci were not involved in rearrangements. When a range of copy numbers was present in different cells, the highest value in the range was used in comparative analyses.

2.3. Western Blotting

The ALCL cell lines Karpas 299 (ATCC, Gaithersburg, MD), SU-DHL-1 (DSMZ, Braunschweig, Germany), MAC1 (established by M.E.K. [19]), and FE-PD (generously provided by Dr. K. Pulford, Oxford, U.K., with kind permission from Dr. A., Del Mistro, Padova, Italy) were maintained in DMEM medium containing 10% fetal bovine serum. HEK-293 cells (ATCC) were transfected with pcDNA3 (Invitrogen) either as an empty

vector or containing FLAG-tagged ΔNp63α (a gift from David Sidransky; Addgene plasmid #26979) [20] using Lipofectamine 2000 (Invitrogen) per the manufacturer's instructions. Western blotting was performed on cell lysates using antibodies to p63 (clone 4A4; Biocare), ΔNp63 (goat polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (clone AC-15; Novus Biologicals, Littleton, CO).

2.3. RNA Sequencing

RNA sequencing was performed on total RNA extracted from ALCL cell lines, and normalized read-count data and bridging reads that spanned exons were used to evaluate *TP63* splicing patterns and assess the relative presence of isoforms containing the TA domain and ΔN leader sequence, respectively, as previously described [21, 22].

2.4. Statistics

Relationships between groups were evaluated using the Wilcoxon test, Pearson χ^2 test, or Cochran-Mantel-Haenszel non-parametric trend test (modified to use Ridit scores), as appropriate, and p values less than 0.05 were considered statistically significant.

3. Results

3.1. p63 Protein Expression Varies by ALCL Subtype

Representative ALCLs with and without p63 protein expression are shown in Figures 1–3. Among all 116 ALCLs the mean percentage (± standard deviation) of tumor cells with positive nuclear staining for p63 was 27.3% ± 38.1%. When stratified by WHO subtype, p63 protein expression was significantly lower in ALK-positive ALCL (5.0% ± 17.4%) than either ALK-negative ALCL (33.4% ± 40.6%) or pcALCL (30.9% ± 38.6%; p=0.0065, Wilcoxon test; Figure 4a). Even after excluding cases with *TP63* rearrangements, p63 expression in the 3 ALCL subtypes was 5.0% ± 17.4%, 24.3% ± 35.0%, and 27.0% ± 36.6%, respectively (p=0.026). Therefore, the lower expression of p63 in ALK-positive ALCLs could not be attributed only to the lack of *TP63* rearrangements in this subtype (Table 1 and references [8, 9]). Similar results were obtained when p63 protein expression was assessed as a dichotomous variable using a cutoff for p63 positivity of ≥30% staining as previously used [15]. Using this criterion, 35.3% of ALCLs were positive for p63. Stratified by ALCL subtype, percentages of positive cases were 4.6%, 43.6%, and 40.6%, for ALK-positive, ALK-negative, and pcALCL, respectively (p=0.0034, Pearson χ^2 test; Supplemental Figure 1). Excluding cases with *TP63* rearrangements, these values were 4.6%, 35.2%, and 36.7%, respectively (p=0.017). *DUSP22* rearrangements were present in 30.6% of ALK-negative ALCLs and 40.6% of pcALCLs (Table 1). No significant association was observed between *DUSP22* rearrangement status and either percentage of p63-positive cells or percentage of p63-positive cases (data not shown).

3.2. p63 Protein Expression in ALCL is Associated with Extra Copies of the *TP63* Locus

We previously have demonstrated that p63 protein expression is associated with extra copies of the *TP63* locus in lung adenocarcinomas [16]. Thus, we investigated whether this association was observed in ALCLs lacking *TP63* rearrangements. Indeed, cases with increasing copies of the *TP63* locus demonstrated increasing percentages of p63-positive

tumor cells (p=0.011, Cochran-Mantel-Haenszel trend test; Figure 4b). Overall, ALCLs with extra (≥3) copies of *TP63* had 47.9% ± 40% p63-positive cells, compared to 11.4% ± 24.6% for cases without extra copies (p<0.0001, Wilcoxon test; Figure 4c). Stratified by WHO subtype, significant differences were observed both for ALK-negative ALCL (48.8% ± 40% vs. 13.9% ± 27.2%, respectively; p-0.0013) and pcALCL (54.0% ± 42.0% vs. 13.5% ± 25.2, respectively; p=0.014); a trend in the same direction was observed for ALK-positive ALCL (10.0% ± 14.1% vs. 4.5% ± 17.9%, respectively), but this was not statistically significant. To explore whether extra copies of *TP63* were more likely due to focal gains or more widespread genomic complexity and/or aneuploidy, we then examined the correlation with the number of copies of the *DUSP22* locus. We found that 15/27 ALCLs (55.6%) with extra copies of *TP63* had extra copies of *DUSP22*. In contrast, 2/10 cases (20.0%) with *TP63* rearrangements and only 5/77 cases (6.5%) without rearrangements or extra copies had extra copies of *DUSP22* (p<0.0001, Pearson χ^2 test; Figure 4d and Supplemental Table 1). These findings suggest that extra copies of *TP63* are more likely due to aneuploidy or other structural complexity than to isolated, focal gains in the majority of cases.

3.3. p63 Protein Expression in ALCLs without *TP63* Rearrangements Represents TAp63 Isoforms

Slides were available to perform immunohistochemistry for ΔNp63 in 59 ALCLs (Supplemental Table 2). All cases were negative for ΔNp63, regardless of p63 protein expression status or *TP63* rearrangement status (Figures 1–3). We also examined cell block sections derived from 4 ALCL cells, including 2 ALK-positive lines (Karpas 299 and SU-DHL-1) and 2 ALK-negative lines (MAC1 and FE-PD). Karpas 299 and MAC1 were positive for p63 (stronger in Karpas 299) and SU-DHL-1 and FE-PD were negative (Figure 5a). Sections from all 4 cell lines were negative for ΔNp63. To further confirm these results, we performed Western blotting on cell line lysates. Bands detectable with the 4A4 pan-p63 antibody were seen for Karpas 299 (stronger) and MAC1 (weaker), but not for SU-DHL-1 or FE-PD (Figure 5b). No band corresponding to ΔNp63 was seen in any of the cell lines. Finally, we examined exonic and junctional reads mapping to the *TP63* locus in RNAseq data from all 4 cell lines. The results corroborated those from IHC and Western blot (Figure 5c). Notably, no exonic reads were detected that mapped to the ΔNp63-specific exon 3′, nor were junctional reads spanning exons 3′ and 4 identified. Taken together with the lack of ΔNp63 staining in any clinical ALCL sample, these findings indicate that ΔNp63 is not expressed in ALCL, and strongly suggest that positivity for p63 by IHC in ALCLs lacking *TP63* rearrangements is due to expression of TAp63 isoforms.

4. Discussion

This study is the first to address in a comprehensive fashion the distribution and origin of p63 protein expression in ALCL in the context of the recent discovery of *TP63* rearrangements in a subset of these cases. The prognostic role of *TP63* and *DUSP22* rearrangements in ALCL has become recognized [9, 23], and has led to clinical FISH testing for these alterations in some centers. ALCLs with *TP63* rearrangements produce p63 fusion proteins that can be detected by routine p63 IHC, have been exclusively ALK-negative, and have poor outcomes. Here, we show unequivocally that a subset of ALCLs without *TP63*

rearrangements express p63 protein and that this expression occurs predominantly in ALK-negative ALCL and pcALCL, is associated with extra copies of the *TP63* gene, and represents TAp63 isoforms. Overall, these findings support the use of p63 IHC as a screening test in the evaluation of ALCL, but also emphasize the need for *TP63* FISH in IHC-positive cases for prognostic purposes.

Gualco et al reported in 2008 that p63 was detected by IHC in a subset of ALCLs, but not cases of classical Hodgkin lymphoma [18]. However, that study preceded our group's discovery of *TP63* rearrangements in ALCL and therefore the proportions of p63-positive cases attributable to rearranged and non-rearranged cases in the series reported by Gualco et al cannot be ascertained. Of note and in contrast to our study, Gualco et al did not identify a significant association between p63 positivity and ALK negativity in systemic ALCLs. This may be due in part to the lower threshold for p63 positivity in the earlier study (nuclear staining in >5% of the neoplastic cells, in contrast to ≥30% in the current study); when Gualco et al used a cutoff of >50% positive cells the proportions of positive cases were 4.1% and 14.8% for systemic ALK-positive and -negative cases, respectively. In addition to systemic ALCL and pcALCL, breast implant-associated ALCL represents a new provisional entity in the WHO classification [23]. The present study did not include any examples of this entity and further study of the p63 status of these neoplasms is warranted.

In a previous study, we reported a p63 protein expression in a *TP63*-rearranged lung adenocarcinoma and in non-rearranged cases with extra copies of the intact *TP63* locus [16]. Therefore, we examined p63 expression and *TP63* copy number in ALCL and found these to be correlated significantly. p63 expression also has been reported in B-cell lymphomas with or without *TP63* rearrangements, but an association with *TP63* copy number has not been reported [8, 17, 24–29]. Though our FISH approach did not allow precise mapping of the regions of chromosomal gain in ALCLs with extra copies of *TP63* (on 3q28), this finding was associated significantly with extra copies of the *DUSP22* locus on 6p25.3, suggesting aneuploidy rather than focal gains as the cause of these extra copies. Interestingly, Gualco et al reported that 3 of 5 cases of T-cell ALCL with aberrant expression of the B-cell transcription factor PAX5 also were p63 positive, a finding likely explained by structural complexity in p63-positive cases given our previous finding that PAX5 expression in ALCL is associated with extra copies of the *PAX5* gene on 9p13.2 [18, 30].

Immunohistochemical staining for ΔNp63 (p40) is used widely in surgical pathology practice but its expression in ALCL has not been reported. We found that p63 expression in ALCLs lacking *TP63* rearrangements is attributable solely to the expression of TAp63 isoforms. Importantly, this result was not assumed based only on the lack of immunohistochemical staining for ΔNp63, but also was validated by analysis of *TP63* transcripts in RNA sequencing in ALCL cell lines. In fact, no exonic or junctional reads corresponding to a ΔNp63 isoform were identified. TAp63 isoforms have tumor suppressor properties in some experimental models, and their expression has been associated with favorable outcomes in diffuse large B-cell lymphoma [27, 29]. However, while *TP63* rearrangements are associated with poor prognosis in ALCL, we previously reported a lack of significant differences in outcomes between p63 protein-positive and -negative cases [9]. Similarly, ALCLs included in the present study, excluding those with *TP63* rearrangements,

did not demonstrate a significant association between p63 IHC result and overall survival (data not shown). The functional roles of p63 isoforms in ALCL cells, as well as the possibility that any tumor suppressor effect conferred by TAp63 expression is negated by prognostic effects relating to aneuploidy and/or underlying genomic instability, merit further investigation.

We conclude that p63 protein expression in ALCLs without *TP63* rearrangements is a phenomenon associated with extra copies of *TP63* and other structural chromosomal abnormalities, is due to expression of TAp63 isoforms, and is of uncertain clinical and biologic significance. Immunohistochemistry for ΔNp63 (p40) is not informative in the evaluation of ALCL. IHC for p63 using pan-p63 antibodies is a useful screening test to select cases of systemic ALK-negative ALCL for *TP63* FISH, which is more costly and less readily available. In our experience, a threshold for positivity of ≥30% has 100% sensitivity for ALCLs with *TP63* rearrangements (Supplemental Figure 2). A possible algorithm for the use of p63 IHC and *TP63* FISH in the evaluation of ALCL is shown in Supplemental Figure 3. The lack of specificity of IHC for *TP63* rearrangements should be emphasized, as should the fact that no prognostic significance of p63 IHC has yet been demonstrated in ALCL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- IHC for p63 is highly sensitive but not specific for *TP63* rearrangements in ALCL.
- p63-positive ALCLs lacking *TP63* rearrangements often have extra copies of *TP63*.
- IHC for p63 can be used to select cases for FISH testing for *TP63* rearrangements.
- IHC for ΔNp63 (p40) is not informative in the evaluation of ALCL.

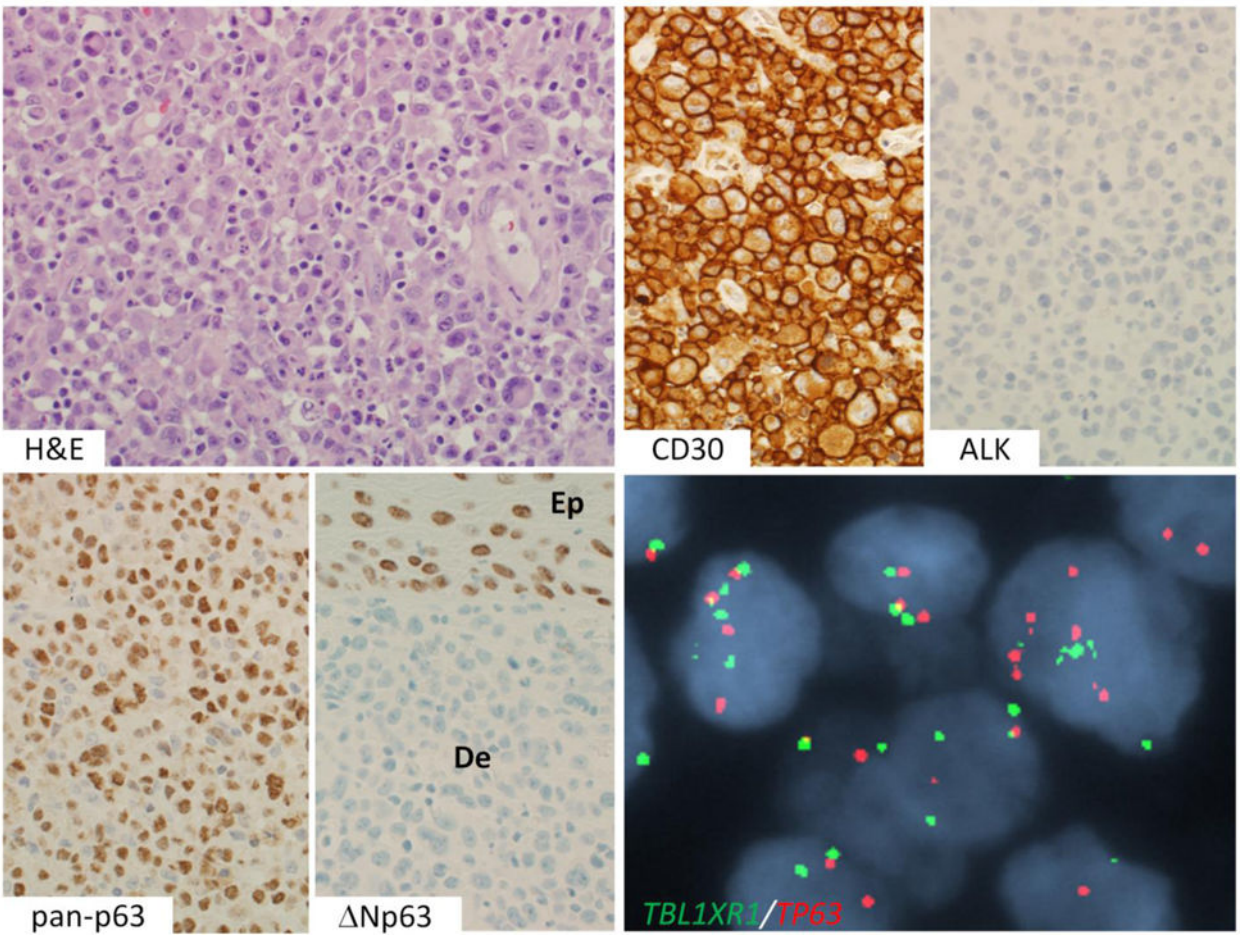

Figure 1.

p63 protein-positive primary cutaneous ALCL without *TP63* rearrangement. An H&E stain shows sheets of large tumor cells, including “hallmark” cells. By IHC, the cells are positive for CD30 and negative for ALK. They show uniform nuclear staining using a pan-p63 antibody (4A4 clone). The tumor cells in the dermis (De) are negative for ΔNp63 (“p40”); epithelial cells in the basal layer of the epidermis (Ep) are positive. Interphase FISH using a dual-fusion probe for *TBL1XR1* (green) and *TP63* (red) shows extra copies of both genes without evidence of *TBL1XR1-TP63* fusion.

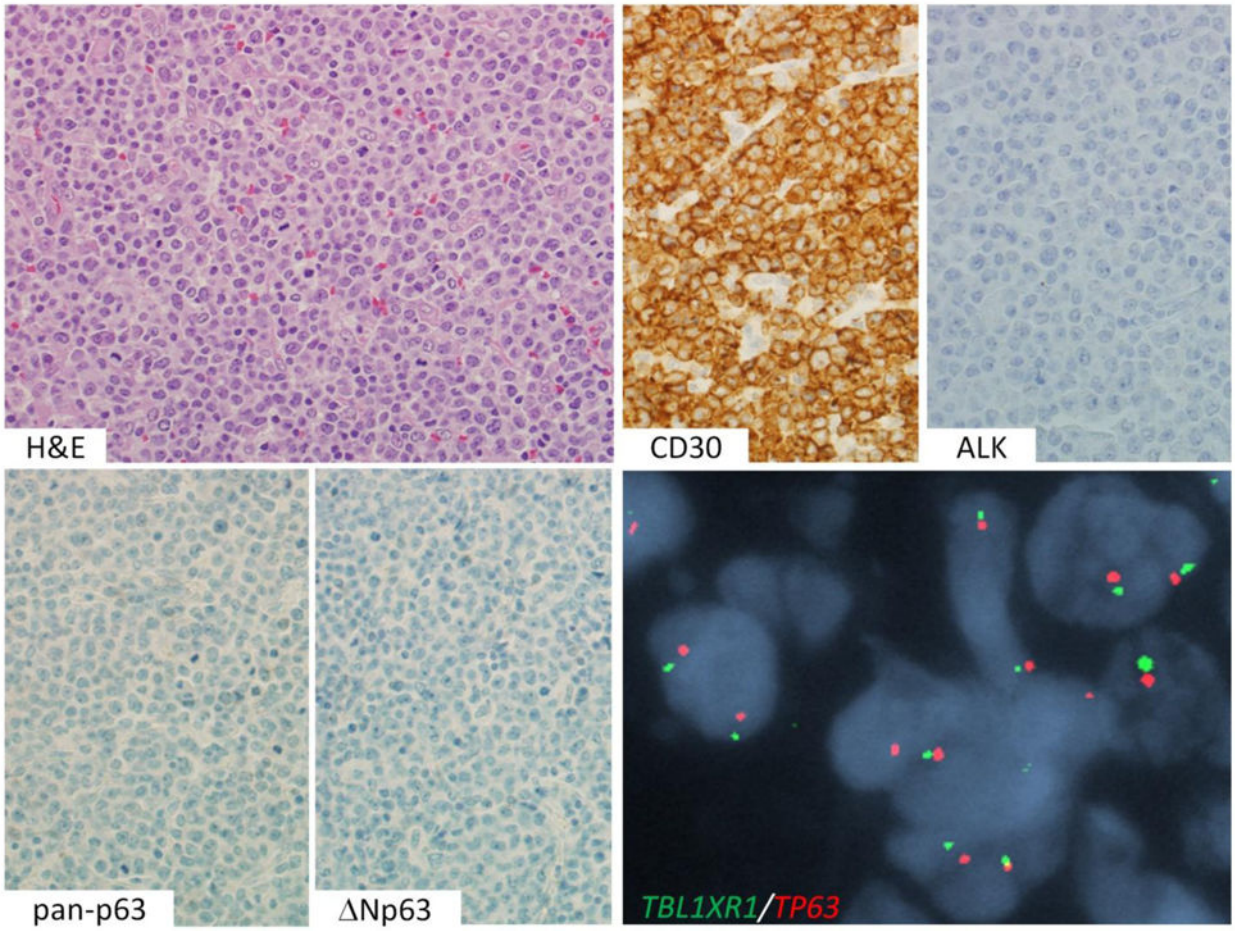

Figure 2.

p63 protein-negative ALCL without *TP63* rearrangement. An H&E stain shows sheets of tumor cells. By IHC, the cells are positive for CD30 and negative for ALK, pan-p63, and ΔNp63. Dual-fusion FISH shows two copies each of *TBL1XR1* and *TP63* (normal signal pattern).

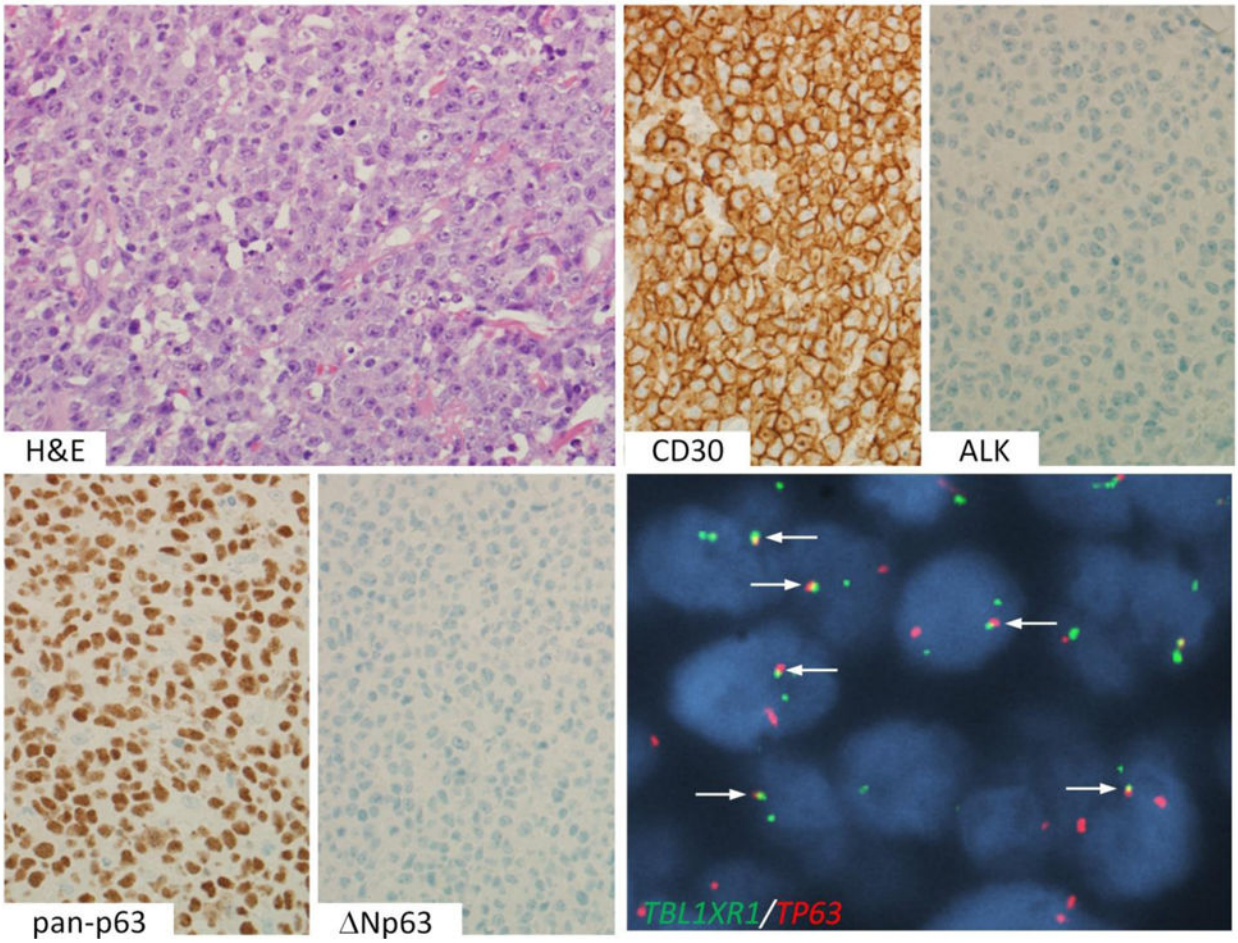

Figure 3.

ALK-negative ALCL with *TP63* rearrangement. An H&E stain shows sheets of tumor cells. By IHC, the cells are positive for CD30 and pan-p63, and negative for ALK and ΔNp63. Dual-fusion FISH shows abnormal fusion signals (arrows), indicative of *TBL1XR1-TP63* fusion.

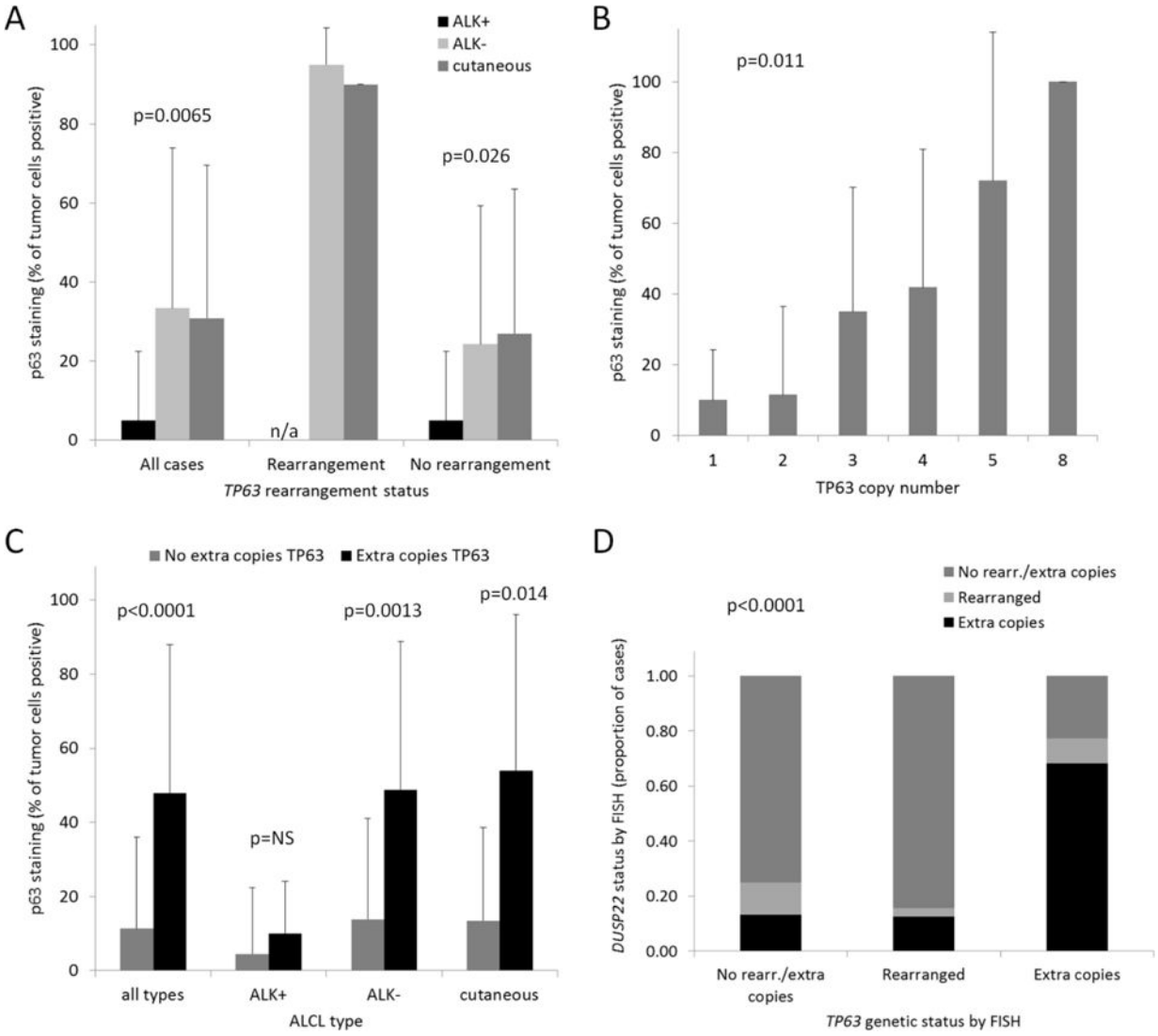

Figure 4.

p63 protein expression in ALCL. (A) Percentages of tumor cell nuclei staining for pan-p63 in ALCL, stratified by WHO subtype and *TP63* rearrangement status, and expressed as mean ± standard deviation. n/a, not applicable (*TP63* rearrangements were not observed in ALK-positive ALCLs). (B) Percentages of tumor cell nuclei staining for p63 in ALCL, stratified by number of copies of the *TP63* locus (n=106; ALCLs with *TP63* rearrangements were excluded). (C) Percentages of tumor cell nuclei staining for p63 in ALCL subtypes with and without extra copies of *TP63*. NS, not significant. (D) Proportions of cases with extra copies or rearrangements of *DUSP22*, stratified by the presence of extra copies or rearrangements of *TP63*.

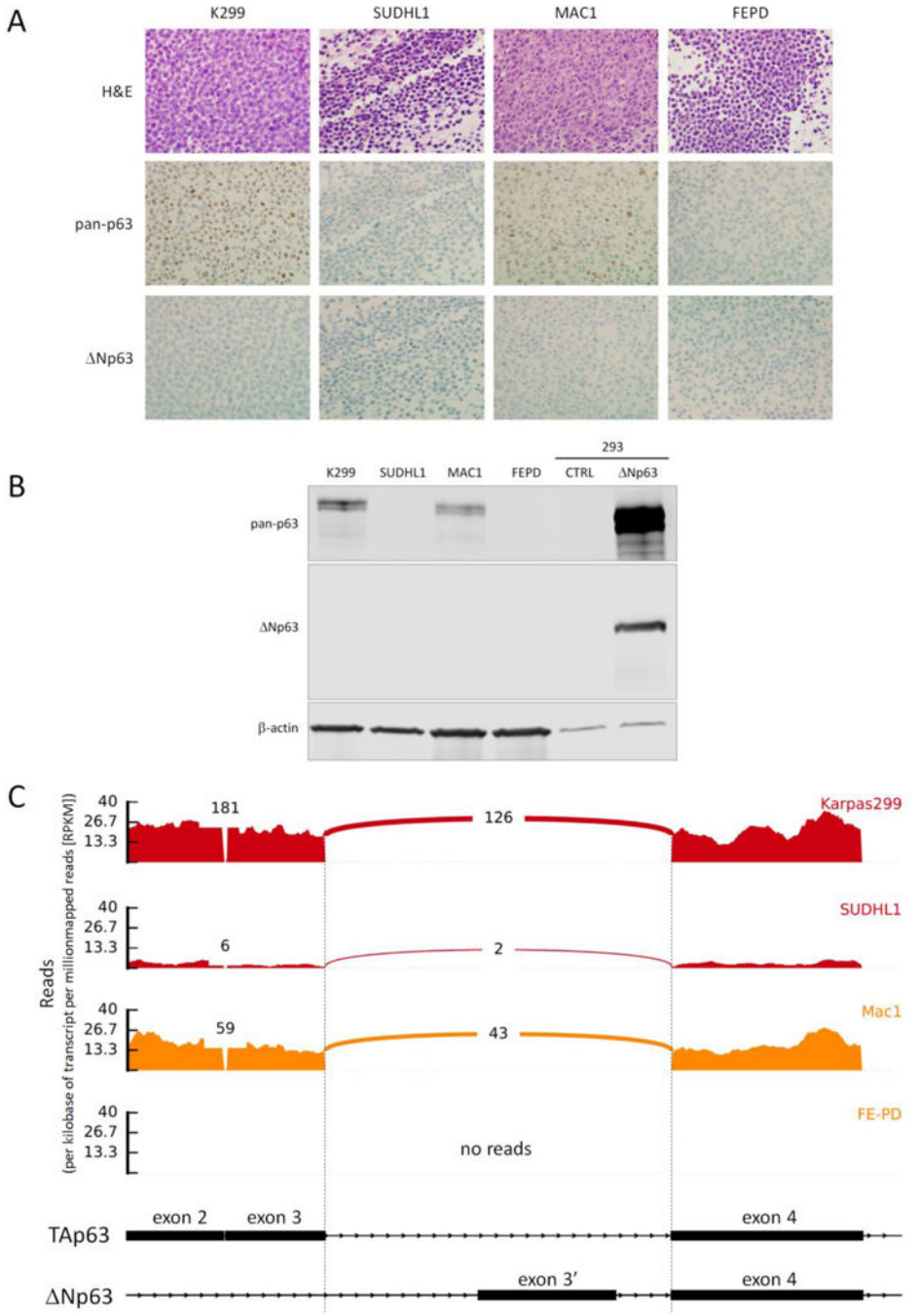

Figure 5.

p63 isoform distribution in ALCL. (A) Results of pan-p63 and ΔNp63 immunohistochemistry in sections of cell blocks prepared from ALCL cell lines. (B) Western blots of protein lysates from ALCL cell lines using antibodies to pan-p63, ΔNp63, and β-actin (loading control). The positive control for ΔNp63 is a lysate from 293 cells transfected with a ΔNp63 expression plasmid. (C) Representation of exons 2–4 of the *TP63* locus on 3q28 showing RNA sequencing data from ALCL cell lines. ALK-positive cell lines are shown in red and ALK-negative lines in orange. Both exon-level read counts and exon-

exon junctional read counts are shown. The schematic diagram is not to scale (exons are exaggerated with respect to introns to facilitate visualization).

Table 1

Demographic and genetic characteristics of ALCLs in the present study

	WHO subtype			
	ALK-positive	ALK-negative*	Primary cutaneous**	All cases
N	22	62	32	116
Age (years)				
Mean	29	62	62	56
Range	6–74	24–87	13–93	6–93
Sex				
Male	18	40	17	75
Female	4	22	15	41
Genetics				
DUSP22 rearrangement				
Present	0	19	13	32
Absent	20	43	19	82
Not done	2	0	0	2
TP63 rearrangement				
Present	0	8	2	10
Absent	22	54	30	106
Not done	0	0	0	0

Abbreviations: ALCL: anaplastic large cell lymphoma; ALK: anaplastic lymphoma kinase; WHO: World Health Organization.

* In 1 case of systemic ALK-negative ALCL the specimen available for analysis was a skin biopsy, though the patient had documented systemic disease.

** In 3 cases of primary cutaneous ALCL the specimens available for analysis were locoregional lymph nodes, though the patients had documented primary cutaneous disease.