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Aberrant CCND1 copies and cyclin D1 mRNA expression do not result in the production of functional cyclin D1 protein in anaplastic large cell lymphoma

Mattheos Bobos, Vassiliki Kotoula, Vassiliki Kaloutsi, Georgia Karayannopoulou, Constantine S. Papadimitriou and Ioannis Kostopoulos

Department of Pathology, Aristotle University Medical School, Thessaloniki, Greece

Summary. Scattered reports in the literature have shown that Cyclin D1 mRNA and protein may be expressed in anaplastic large cell lymphoma (ALCL). ALCLs are characterized by the presence of ALK translocations. Aberrant Cyclin D1 expression seems to promote proliferation in other types of lymphoma, while a growth promoting CCND1/TACSD1(TROP2) fusion product has also been described in tumors. Herein, we investigated 44 ALCL cases for chromosome 11 and CCND1 status (by FISH), cyclin D1 mRNA expression (by in situ hybridization and RT-PCR) and Cyclin D1 protein (by immunohistochemistry with two different monoclonal antibodies), as well as for the expression of Trop-2/GA733-1 (by immunohistochemistry). Polysomy of CCND1 (11q13) and chromosome 11 was found in 15/38 evaluated cases (39.5%). This change was specific for CD30+ neoplastic cells, as shown by double fluorescent staining. Neoplastic cells in the majority of ALCL expressed cyclin D1 mRNA (29/41 [70.7%]), in association with the presence of ALK translocations (p=0.024) and systemic, rather than cutaneous disease (p=0.021). Remarkably, however, Cyclin D1 protein was not detected in neoplastic cells (0/44 cases), neither were these found positive for Trop-2. In conclusion, aberrant copies of CCND1 / chromosome 11 may be observed in ALCL, probably as a consequence of the reported ploidy changes in these tumors. ALCL may often express cyclin D1 mRNA, which, however, does not result in the production of functional Cyclin D1 protein or Trop-2, suggesting that these proteins do not play a role in the pathogenesis of ALCL.

Offprint requests to: Ioannis Kostopoulos, MD, Department of Pathology Aristotle University Medical School, 540 06 Thessaloniki, Greece. e-mail: kostop@med.auth.gr

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Introduction

ALCL was described in the '80s by the Lennert Group (Stein et a1., 1982) as a pleiomorphic large cell lymphoma with strong membrane and Golgi associated CD30/Ki-1 antigen expression in a very high percentage of neoplastic lymphoid cells, with involvement of the paracortical region and sinuses of lymph nodes. The great majority of ALCLs show a T-cell phenotype or T-cell receptor gene rearrangement (Stein et a1., 1982, 2000; Delsol et al., 2001). Three histologic patterns of ALCL are recognised as distinctive variants: the pleiomorphic or classical, the lymphohistiocytic and the small cell variant. Clinically, ALCL include primary systemic ALCL, primary cutaneous ALCL (C-ALCL) and ALCL occurring as a secondary event in patients with lymphomatoid papulosis, mycosis fungoides, and, rarely, Hodgkin's disease (Stein et a1., 2000; Delsol et al., 2001; Greer et al., 2001).

The most characteristic and frequent genetic alteration in ALCL is the presence of the translocation t(2;5)(p23;q35), which involves the anaplastic lymphoma kinase (ALK) gene on chromosome 2 and the nucleophosmin (NPM) gene on chromosome 5, resulting in the expression of a unique chimeric 80-kD NPM-ALK protein (Rimokh et al., 1989; Kaneko et al., 1989; Mason et al., 1990; Morris et al., 1994). ALK protein expression, normal or chimeric, can be detected by immunohistochemistry using monoclonal or polyclonal antibodies. The ALK staining pattern may be cytoplasmic and nuclear or it may be restricted either to the nucleus or to the cytoplasm (Delsol et al., 2001).

The activity of a family of protein kinases known as cyclin-dependent kinases (CDKs) plays key roles in the cell-cycle control system. The activity of these kinases, which rises and falls as the cell progresses through the cycle, is controlled by a complex array of enzymes and proteins. The most important regulators of CDKs are proteins named cyclins (Alberts et al., 2002). Several cyclins (A-M, T) are expressed in mammalian cells. The D-type cyclins, D1, D2 and D3 are encoded by separate genes and are expressed in all proliferating cells (Sherr et al., 1999). In particular, Cyclin D1 interacts with its catalytic enzymes Cdk4 and Cdk6, promoting cell transition from G1 to S phase through phosphorylation of Rb family members (p107, p130) and stoichiometric association with Cip/Kip family proteins (Hunter and Pines, 1994; Sumrejkanchanakij et al., 2003; Benzeno and Diehl, 2004; Lesage et al., 2005). Rb-phosphorylation triggers the activity of E2F transcription factors, leading cells to progress through the restriction point at G1 and initiation of S phase. Although a number of studies have shown a surprising lack of correlation between increased cyclin D1 expression and increased DNA synthesis in tumors (reviewed in Fu et al., 2004), deregulation of this restriction point may occur through Cyclin D1 overexpression or through other mechanisms, such as loss of one or more pocket proteins, expression of a vital oncogene or a combination of both (Foijer et al., 2005).

Cyclin D1 has been found to be overexpressed in various human malignancies, usually as a result of CCND1 amplification. Tumors overexpressing Cyclin D1 include carcinomas of the breast, head and neck, lung (non-small cell type), uterine cervix, pancreas, colon, bladder, prostate and endometrium, as well as astrocytomas, soft tissue sarcomas and lymphomas (Zhou et al., 1998; Specht et al., 2002; Sicinska et al., 2003; Jin et al., 2004; Nimeus et al., 2004). In lymphomas, up-regulated cyclin D1 expression driven by the t(11;14)(q13;q32) translocation has been reported in mantle cell lymphoma (MCL), multiple myeloma (MM) and in cases classified as B-CLL and prolymphocytic leukaemia (Specht et al., 2002). In addition, cyclin D1 mRNA expression has been reported in ALCL (Aguilera et al., 1998; Elenitoba-Johnson et al., 2002). The gene encoding Cyclin D1 (CCND1 or BCL1 or PRAD1) is located on chromosome 11q13 and is composed of 5 exons.

Stimulated by the above literature data we decided to investigate Cyclin D1 status in ALCL at the protein, RNA and DNA level. For this purpose we recruited a series of 44 ALCL cases and investigated the presence of Cyclin D1 protein by IHC using two different monoclonal antibodies and an immunoenzymatic doublestaining method for the evaluation of staining in neoplastic cells. Furthermore, *cyclin D1* mRNA expression was investigated with in situ hybridization (ISH), RT-PCR and RQ-RT-PCR. In addition to Cyclin D1 protein investigation, we considered it necessary to exclude the presence of Trop-2 protein by IHC, because the region on cyclin D1 mRNA that was amplified by RT-PCR spanning exons 3 – 4 and detected by the ISH probes may participate in a gene fusion with TROP2 (TACSTD2, tumor-associated calcium signal transducer-2); the expression of such transcripts, as registered in Genbank accession nr. X77754 (Fornaro et al., 1995), may drive the overproduction of Trop-2 protein in tumors (Terinnoni et al., 2001). By fluorescent in situ hybridization (FISH) we investigated the status of the CCND1 gene. We also used the fluorescence immunophenotyping and interphase cytogenetics as a tool for the investigation of neoplasms (FICTION) method for the simultaneous detection of CD30 antigen with CCND1 gene copies.

Materials and methods

Tissue samples

Tissue material was retrieved from the archives of the Pathology Department of the Aristotle University Medical School. All specimens were formalin fixed and paraffin embedded. We retrieved 44 biopsies previously diagnosed as ALCL from 41 patients, including 2 recurrences after therapy and one case with relapse of the disease after 1 year. Archival hematoxylin and eosin (H&E) slides from each case, and slides stained for CD20, CD30/Ki-1, CD43, CD45RO, CD45RA, CD79a (Dako, Denmark), CD3 and ALK1 (Novocastra, U.K.) were reviewed. In several cases archival slides stained for CD8, CD15, CD45, EMA, CD68 (Dako), CD4, CD5 (Novocastra) and Ki67 (clone MIB1, Dako) were also available. In the majority of the cases, clonality assessment of T-cell receptor-γ (TCR-γ) rearrangement had also been performed. After reviewing all archival slides in combination with molecular assessment results, these cases were reclassified according to the latest World Health Organization criteria (Delsol et al., 2001).

Patient demographic, tumor histology, immunophenotypic and genomic data are shown in Table 1.

Tissue microarray (TMA) construction

The H&E and CD30/Ki-1 immunostained slides were used as guides. Low density TMAs were constructed with a manual microarrayer (Beecher Instruments, USA) to include 5 cores (0.6 mm diameter) from each ALCL case, as well as cores from 10 mantle cell lymphomas (MCL) as controls for cyclin D1 expression. Cores from colon and breast carcinomas were also used as controls and for block orientation.

Immunohistochemistry (IHC)

The rabbit monoclonal anti-human Cyclin D1 (clone SP4, Spring Bioscience, USA) and the mouse monoclonal anti-human Cyclin D1 (clone DCS-6, Dako) antibodies were used for the IHC detection of Cyclin D1 in 2.5 μm thick TMA and whole section slides as well.

Endothelial cells or fibroblasts served as internal controls. Cyclin D1 immunopositivity was defined as nuclear and/or cytoplasmic staining, and graded as negative (no staining or positive <10% of the cells) and as positive (>10% of the cells). The clone 162-46 (BD Pharmingen, USA) was used for investigation of the cell surface glycoprotein Trop-2/GA733-1. As an external control of the immunoreaction, a known positive control (skin, urothelial carcinoma) was used. The membrane staining intensity of protein was scored using the proposed criteria for EGFR (EGFR pharmDx, Dako). A "positive" result was defined as a test sample with greater than 1% of tumor cells stained. The conditions of pretreatment, staining and the visualization systems are shown in Table 2.

Immunoenzymatic doublestaining method for Cyclin D1 and CD30

For the doublestaining method, the Envision G/2 System/AP was used (Dako). All staining steps and the chromogenic reaction with DAB were performed as described in Table 2. After visualization of the primary antibody (Cyclin D1 SP4 or DCS-6) the slides were

Table 1. Patient demographic and tumor biologic characteristics (41 patients, 44 cases).

Case	AGE	SEX	Clinical	Immuno-	RT-PCR+FISH	IHC							
#			Classification	phenotype	t(2;5)	ALK1	Ki67 (%)	CD30	CD3	CD43	CD45RO	CD20	CD45RA
1	8	F	SYSTEMIC	T	P	P	55	P	N	P	P	N	N
2	16	M	SYSTEMIC	T	P	P	80	P	N	P	P	N	N
3	22	M	SYSTEMIC	T	P	P	75	P	P	P	P	N	N
4	27	M	SYSTEMIC	T	P	P	13	P	N	P	P	N	N
5	28	F	SYSTEMIC	T	P	P	85	P	P	P	N	N	N
6	32	M	SYSTEMIC	T	P	P	25	P	N	P	N	N	N
6A	32	M	SYSTEMIC	T	P	P	ND	P	N	P	N	N	N
7	36	M	SYSTEMIC	T	P	P	55	P	N	P	N	N	N
8	38	M	SYSTEMIC	T	N	N	96	P	N	N	P	N	N
9	40	M	SYSTEMIC	T	P	P	85	P	N	P	P	N	N
10	41	F	SYSTEMIC	T	P	N	3	P	N	P	P	N	N
11	42	M	C-ALCL	T	N	N	40	P	P	P	P	N	N
12	47	M	SYSTEMIC	T	N	P	ND	P	ND	ND	P	N	N
13	47	F	SYSTEMIC	T	P	N	93	P	P	P	P	N	N
14	47	F	C-ALCL	T	N	N	15	P	P	P	P	N	N
14B	48	F	C-ALCL	T	N	N	12	P	P	P	P	N	N
15	51	M	SYSTEMIC	T	P	N	ND	P	N	N	P	N	N
16	52	M	SYSTEMIC	T	N	N	98	P	P	P	P	N	N
17	53	M	SYSTEMIC	T	N	N	4	P	N	P	P	N	N
18	54	F	C-ALCL	T	N	N	35	P	P	P	P	N	N
19	54	F	SYSTEMIC	T	N	N	65	P	P	P	P	N	N
20	56	M	SYSTEMIC	T	N	N	35	P	P	P	P	N	N
20A	56	M	SYSTEMIC	T	N	N	40	P	P	P	P	N	N
21	57	F	SYSTEMIC	T	P	P	ND	P	N	ND	P	N	N
22	57	M	SYSTEMIC	T	P	P	ND	P	P	N	N	N	N
23	57	M	SYSTEMIC	T	N	N	67	P	N	ND	P	N	N
24	58	M	SYSTEMIC	NULL	P	N	50	P	N	N	N	N	N
25	62	M	C-ALCL	T	N	N	88	P	P	P	N	N	N
26	63	M	SYSTEMIC	T	N	N	15	P	P	P	P	N	N
27	65	M	SYSTEMIC	T	N	N	40	P	N	P	P	N	N
28	65	M	SYSTEMIC	T	P	P	4	P	N	P	P	N	N
29	67	M	SYSTEMIC	T	N	N	75	P	N	P	P	N	N
30	68	F	SYSTEMIC	T	N	N	ND	P	N	P	N	N	N
31	68	M	SYSTEMIC	T	P	N	ND	P	P	P	P	N	N
32	68	M	SYSTEMIC	T	N	N	85	P	P	P	P	N	N
33	70	F	C-ALCL	T	N	N	97	P	P	P	P	N	N
34	73	M	SYSTEMIC	T	P	P	92	P	P	P	P	N	N
35	74	M	SYSTEMIC	T	N	N	23	P	N	P	P	N	N
36	74	F	SYSTEMIC	T	N	N	28	P	N	P	P	N	N
37	74	M	C-ALCL	T	N	N	35	P	P	P	P	N	N
38	75	M	SYSTEMIC	T	N	N	75	P	N	P	P	N	N
39	75	M	C-ALCL	T	P	N	ND	P	P	P	P	N	N
40	76	M	C-ALCL	T	N	N	60	P	P	P	N	N	N
41	76	F	SYSTEMIC	T	N	N	65	P	P	P	P	N	N

A: recurrence; B: relapse; C-ALCL: cutaneous anaplastic large cell lymphoma, F: female, M: male, ND: not done.

incubated with CD30 mAb (BerH4, Dako) for 30 min. The doublestaining protocol for detection of the CD30 mAb was used according to the manufacturer's instructions. The permanent red chromogen was applied until the complex was clearly visible (16 min), the section was rinsed in tap water, counterstained with H&E for 10 sec, rinsed in tap water again, left to dry at room temperature and mounted with non-aqueous permanent mounting medium.

RNA extraction from paraffin sections and RT-PCR for cyclin D1

A duplex RT-PCR protocol was performed in 8 ALCL cases, in order to validate cyclin D1 expression in ALCL, in parallel with 3 peripheral blood samples that were used as negative controls. RNA extraction was performed from paraffin sections with TRIZOL-LS (Invitrogen, UK) according to standard protocols; RT-PCR conditions were described previously (Ronchetti et al., 1999). The following primers were used in the duplex reaction: cyclin D1 mRNA target, forward, 5'-AACAGATCATCCGCAAACAC-3'; reverse, 5'-TCACACTTGATCACTCTGGA-3'; housekeeping gene (GAPDH) target, forward, 5'- ACTGGCGTCTTCACC ACCAT -3'; reverse, 5'-GGGATGATGTTCTGGAGA GC-3'.

Relative quantification (RQ) of cyclin D1 mRNA with real time PCR

cDNAs from the above mentioned 8 ALCL and 3 peripheral blood samples were normalized at 200ng/20ul reaction. Cyclin D1 RQ vs GUSB (beta glucuronidase, assay #4333767F [Applied Biosystems]) as a housekeeping gene was assessed with Taqman-MGB probes by using the inventoried assay #Hs00277039_m1 [Applied Biosystems], targeting the exon 2 – 3 junction of the NM_053056.2 (reference CCND1 mRNA). Reactions were run in an ABI7500 sequence detection system [Applied Biosystems, Biosolutions, Athens, GR] and analyzed with the SDS v1.4 software. Values corresponding to the $2^{-\Delta\Delta CT}$ method were automatically calculated by the software.

In situ hybridization for cyclin D1 mRNA

Digoxigenin (DIG)-labeled riboprobes (sense and antisense) for cyclin D1 mRNA were created by in vitro transcription (DIG RNA labeling kit, SP6/T7; Roche Hellas, GR) from the RT-PCR product mentioned above. Briefly, the 196bp product was TA-cloned (p-GEM-T easy kit; Promega, UK) and sequenced. Inserts were amplified with pUC primers (Promega), to include SP6 and T7 sites, and then submitted to in vitro transcription. For in situ hybridization, sections were deparaffinized, proteinase K digested, acetylated, and prehybridized for 2 h at 48°C. Subsequently, the probes were added, sections were incubated at 70°C for 10 min, and hybridization was performed overnight at 42°C. Stringent washings with 0.2x SSC were performed at 55°C for 30 min. Sections were then blocked for 1 h, incubated with anti-DIG-AP for 2 h, washed in TRIS/NaCl/Tween 20 buffer, and the signal was visualized with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP, all reagents from Roche). Sense and antisense probes were applied in parallel, while no-probe hybridizations were performed as an additional negative control. A Poly-T probe (BioGenex, USA) was used as a positive control for the demonstration of adequate mRNA on parallel sections. Cases were evaluated as positive, when granular perinuclear dark blue staining appeared in tumor cells.

FISH for CCND1/CEP11

The copy number of CCND1 gene locus at 11q13 and centromere, band region 11p11.11-q11, locus D11Z1 (CEP11) was estimated by FISH on interphase cells on paraffin TMA sections (4,5 μm), directly labeled with the LSI CCND1/CEP11 probe (Abbott Molecular, U.S.A.) according to the manufacturer's instructions. Hybridization signals were enumerated in a Zeiss microscope (Axioskop 2 plus HBO 100) equipped with a high quality x100 oil immersion objective, appropriate filters set (EXBP360/51 for DAPI, EX BP485/17 for FITC/spectrum green, EX BP560/18 for rhodamine/spectrum orange) and a computerized imaging system.

Table 2. Primary antibodies used in the present study.

Antibody	Source	Clone	Pretreatment/Time/Buffer	Dilution	Incubation	Visualization system
Cyclin D1 (r, mAb)	1	SP4	Trilogy, Steamer/30+30/TBS	1:45	30min	Envision+™ (1)
Cyclin D1 (m, mAb)*	2	DCS-6	ER2, Hot Plate/20min/PBS	1:125	30min	Bond™ Polymer Define Detection (4)
Trop-2/GA733-1 (m, mAb)	3	162-46	ER2, MW/30min/TBS	1:80	O/N	Super Sensitive Link-Label HRP (5)

ER2, ethylenediaminetetraacetate, pH8.8; m, mouse; mAb, monoclonal antibody; MW, microwave; O/N, overnight; PBS, phosphate buffered saline; r, rabitt; TBS, tris buffered saline; Trilogy, ethylenediaminetetraacetate, pH9.0. (1): Spring Bioscience, U.S.A.; (2): Dako, DK; (3): BD Pharmingen, U.S.A.; (4) Vision Biosystems, AUS; (5): BioGenex, U.S.A. *All the stages of IHC (deparaffinization, antigen retrieval and staining) were performed in a Bond Max autostainer (Vision Biosystems).

One hundred non-overlapping nuclei were selected randomly and scored for each tumor specimen. To define a tumor as being amplified for CCND1, the ratio of mean CCND1 gene signals to mean chromosome 11 centromere signals, counted per tumor-cell nucleus, should be greater than 2.0 (Alavi et al., 1999). In addition, tumors were classified as polysomic if more than 20% of nuclei enclosed more than two copies of the CCND1 gene (red signals) and chromosome 11 (green signals) (ratio 1) (Specht et al., 2004). Photographic images were captured with a computer-controlled digital camera and processed with a software system (FISH Imager™ METASYSTEMS, Germany). In eight cases the material was minimal (1 core). In these cases the FISH analysis was performed on whole sections.

FICTION with CD30 antibody and CCND1/CEP11 LSI probe

Fluorescence immunophenotyping and interphase cytogenetics as a tool for the investigation of neoplasms (FICTION) was performed in whole paraffin sections (4.5 μm) of ALK+ case in three steps, as previously described, with slight modifications (Martínez-Ramírez et al., 2004). Briefly, after antigen retrieval the CD30 mAb was incubated overnight at 1:30 dilution at 4°C. As a second step, incubation with fluorochrome-conjugated antibody NCL-SAM-FITC (Novocastra) at 1:20 dilution was performed for 20 min. The slides were then washed three times for 5 min in PBS 0.5%, covered with a coverslip and examined under fluorescence microscopy to verify the quality of immunofluorescence staining. As a third step, the slides were labelled with LSI CCND1/CEP11 probe as above described.

Statistical analysis

The software SPSS, version 11.5 for Windows (SPSS, Inc., Chicago, IL, U.S.A.) was used for statistical analysis. The Pearson Chi-Square correlation test (χ^2) was used for statistical significance between the variables. A P-value of less than 0.05 was considered statistically significant.

Results

FISH

FISH analysis was successful in 38 cases. None of the cases demonstrated 11q13 amplification. An increased number of CCND1 (11q13) and chromosome 11 centromeric (D11Z1) loci were found in 15 cases (14 systemic and one C-ALCL) (Table 3). In these cases the increased number of CCND1 gene locus varied from three to five signals per nucleus, indicating polysomy of 11q13 locus (Fig. 1A). Chromosome 11 and CCND1

Table 3. IHC, ISH and FISH results concerning cyclin D1 investigation in 44 ALCL cases from 41 patients.

	Detection method					Detection method			
	IHC	ISH		FISH		IHC	ISH		FISH
Case #	Cyclin D1	poly-T	Cyclin D1	Chr 11/11q13 (CCND1)	Case #	Cyclin D1	poly-T	Cyclin D1	Chr 11/11q13 (CCND1)
1	N	P	P	Disomy	20a	N	P	N	Disomy
2	N	P	P	Disomy	21	N	P	P	Polysomy
3	N	P	P	Polysomy	22	N	P	P	NE
4	N	P	P	Disomy	23	N	P	P	Polysomy
5	N	P	P	Polysomy	24	N	P	N	Disomy
6	N	P	P	Disomy	25	N	P	N	Disomy
6a	N	P	P	Disomy	26	N	P	N	Disomy
7	N	P	P	Polysomy	27	N	P	P	Disomy
8	N	P	P	Polysomy	28	N	P	N	Polysomy
9	N	P	P	Disomy	29	N	P	P	Polysomy
10	N	P	P	Polysomy	30	N	P	P	Polysomy
11	N	P	N	Disomy	31	N	P	P	Disomy
12	N	P	N	Disomy	32	N	P	P	Disomy
13	N	P	P	Disomy	33	N	P	P	Disomy
14	N	P	N	Disomy	34	N	P	P	Polysomy
14b	N	P	N	Disomy	35	N	P	P	NE
15	N	P	P	Disomy	36	N	P	N	Polysomy
16	N	P	P	Disomy	37	N	P	N	Disomy
17	N	P	N	Polysomy	38	N	P	P	Disomy
18	N	P	N	Polysomy	39	N	P	P	NE
19	N	P	P	Disomy	40	N	P	P	Disomy
20	N	P	N	Disomy	41	N	P	P	Polysomy

NE, not evaluable; a, recurrence; b: relapse.

Table 4. Cyclin D1 mRNA expression and polysomy 11q13 in comparison to ALK expression, ALK genetic alterations, clinical classification and primary site of involvement in the examined ALCL cases.

		Cyclin D1 mRNA, ISH expression (n=41)		11q13 status (CCND1 gene), FISH (n=38)	
		Cyclin D1+	Cyclin D1-	Polysomy	Disomy
ALK by IHC*	ALK-positive	11	2	6	6
	ALK-negative	16	5	8	12
	P value	0.55[a]		0.58[b]	
ALK genetic alterations (FISH & PCR)	Positive	16	2	7	9
	Negative	13	10	8	14
	P value	0.024		0.64	
Clinical classification	Primary systemic ALCL	26	7	14	17
	Primary cutaneous ALCL	3	5	1	6
	P value	0.021		0.13	
Primary site**	Nodal	18	4	9	12
	Extranodal	8	3	5	5
	P value	0.54[c]		0.7[d]	

P values correspond to Pearson Chi-Square test. P values <0.05 are considered significant and are indicated in bold. *: only systemic ALCL cases were included; [a]: 34 cases; [b]: 32 cases. **: only systemic ALCL cases were included; [c]: 33 cases; [d]: 31 cases.

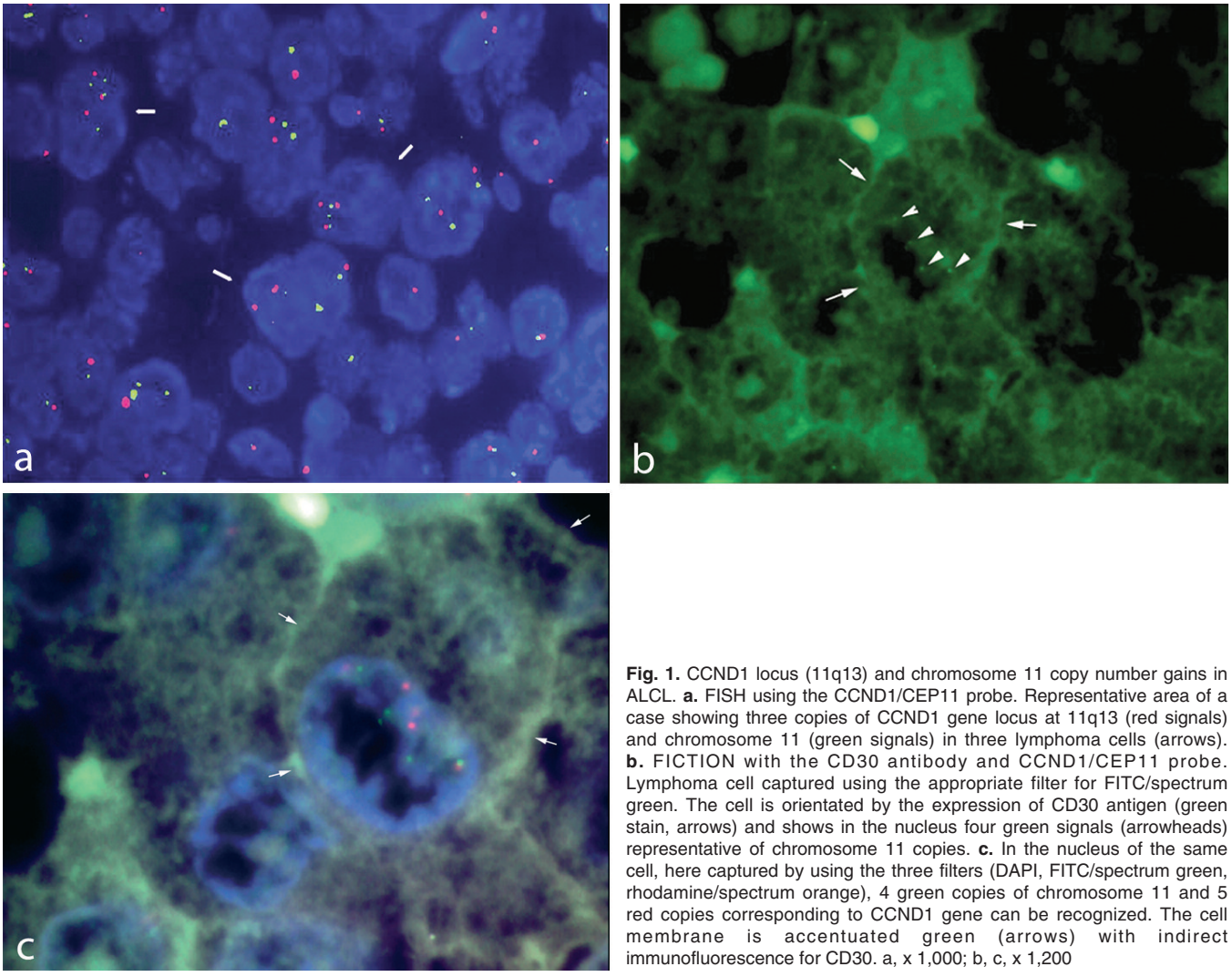

Fig. 1. CCND1 locus (11q13) and chromosome 11 copy number gains in ALCL. **a.** FISH using the CCND1/CEP11 probe. Representative area of a case showing three copies of CCND1 gene locus at 11q13 (red signals) and chromosome 11 (green signals) in three lymphoma cells (arrows). **b.** FICTION with the CD30 antibody and CCND1/CEP11 probe. Lymphoma cell captured using the appropriate filter for FITC/spectrum green. The cell is orientated by the expression of CD30 antigen (green stain, arrows) and shows in the nucleus four green signals (arrowheads) representative of chromosome 11 copies. **c.** In the nucleus of the same cell, here captured by using the three filters (DAPI, FITC/spectrum green, rhodamine/spectrum orange), 4 green copies of chromosome 11 and 5 red copies corresponding to CCND1 gene can be recognized. The cell membrane is accentuated green (arrows) with indirect immunofluorescence for CD30. a, x 1,000; b, c, x 1,200

locus status was not associated with the presence of ALK involving translocations, ALK protein expression, site of ALCL origin or disease status (Table 4). Interestingly, 10 out of 13 cases with high Ki67 index (>20% of neoplastic cells stained positive) showed polysomy 11/11q13 (p=0.048).

FICTION

This method was employed to clarify whether the observed chromosome 11 polysomy concerned neoplastic cells; it was applied in one case with strong membrane positivity for CD30 antigen on indirect immunofluorescence. FICTION analysis revealed the presence of increased red and green signals by CCND1/CEP11 probe only in neoplastic lymphoid cells (CD30+) (Fig. 1B,C).

ISH for cyclin D1 mRNA

The quality of RNA in neoplastic lymphoid cells was adequate in all tested cases (Fig. 2A). The presence of *cyclin D1* mRNA was detectable in 29/41 (70.7%) cases and was identified as a perinuclear dark blue signal. The staining intensity varied from mild (+) to strong (+++) and was confined to the neoplastic lymphoid cells, while small non neoplastic lymphocytes were negative (Fig. 2B,C). In some cases, a few fibroblasts showed mild staining. No signal was detected upon sense probe (Fig. 2D) and no-probe hybridization. MCL specimens also showed intense expression of cyclin D1 mRNA. Mild staining of tumor cells was observed in 7 cases and moderate - strong staining in 22 cases. The majority of systemic ALCLs 26/33 (79%) and three out of eight C-ALCL expressed cyclin D1 mRNA

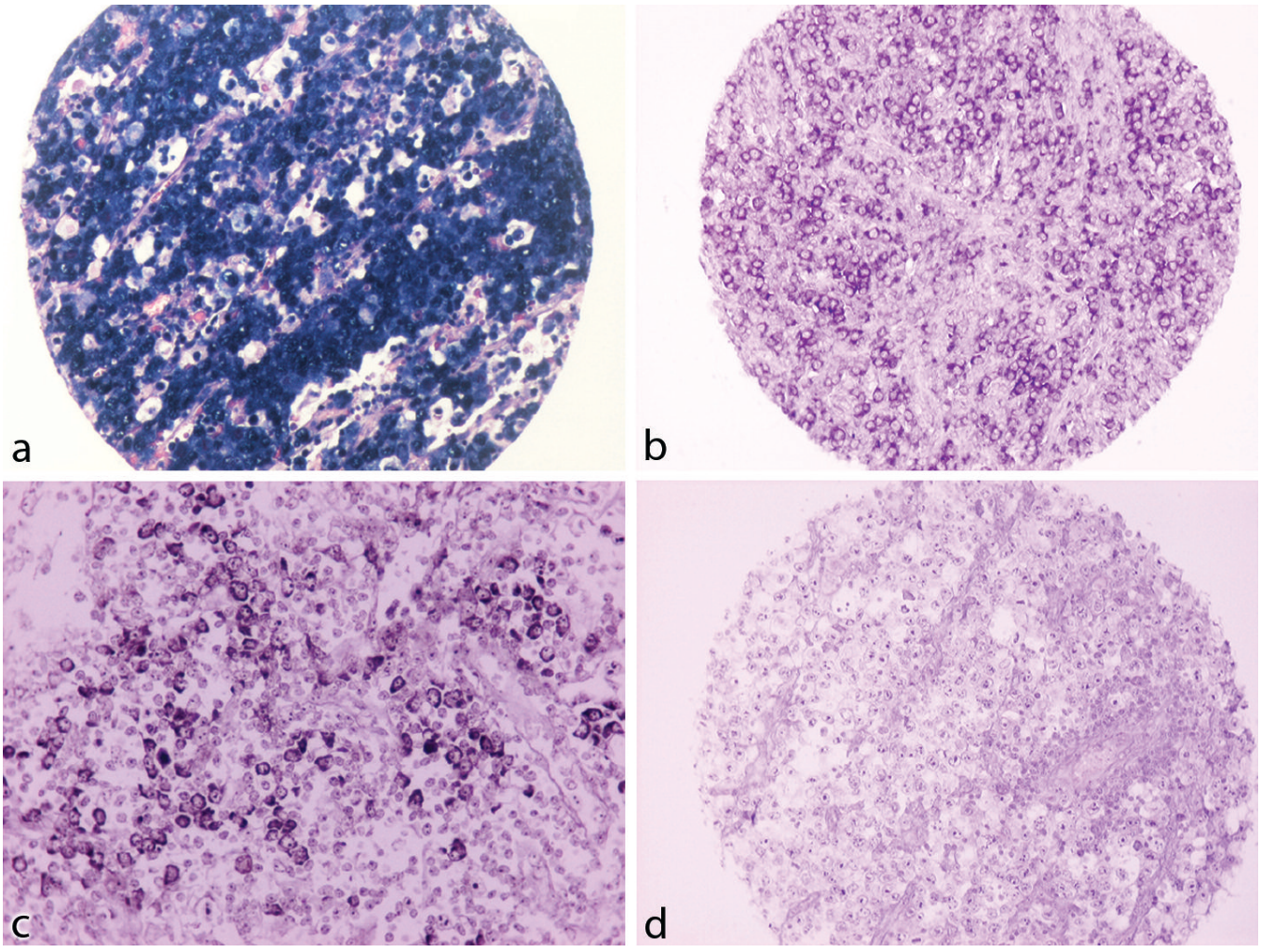

Fig. 2. In situ detection of cyclin D1 mRNA in ALCL. **a.** ALCL case with positive mRNA control; **b, c.** Strong cytoplasmic/perinuclear blue-black staining denotes presence of cyclin D1 mRNA in neoplastic lymphoid cells; **d.** Sense probe hybridization for cyclin D1. No signal was detected in lymphoma cells. a, b, d, x 200; c, x 400

(p=0.021) (Table 4). Similar results concerning cyclin D1 mRNA were found in subsequent biopsies from the same patients (Table 3). Cyclin D1 mRNA presence was cross-validated with PCR in 8 ALCL cases (Fig. 3A). Cyclin D1 relative expression was also assessed, in order to investigate for possible mRNA over-expression in comparison to non-lymphomatous controls (peripheral blood samples). Results from this assessment are shown in Fig. 3B-D. In 3 ALCL cases, relative Cyclin D1 expression values were >5, as compared to peripheral blood samples. This finding, however, did not correspond to CCND1 polysomy.

The majority of the cases with ALK translocations (16/18) expressed cyclin D1 mRNA, while almost half of ALCL-ALK negative cases expressed cyclin D1 mRNA (10/23), (p=0.024) (Table 4).

IHC for Cyclin D1 and Trop-2

The neoplastic lymphoid cells did not express Cyclin D1 protein with any of the mAbs tested, whereas the expected nuclear pattern was observed in endothelial cells, fibroblasts and basally located cells at the epidermis (basal cells and squamous cells) (Figs. 4A-D, 5C,D). In a small number of non-neoplastic cells a moderate cytoplasmic stain was observed with the DCS-6 clone (Fig. 4C,D). In rare ALCL cases scattered medium-sized cells between neoplastic lymphoid cells with round nuclei and moderate amounts of cytoplasm also showed nuclear positivity. These cells, probably histiocytes, as revealed by the double immunoenzymatic method, did not stain for the CD30 antigen. The neoplastic lymphoid cells of control MCL cases exhibited strong nuclear positivity with both tested Abs (Fig. 5A,B).

Trop-2/GA733-1 was not expressed by lymphoma cells. This protein exhibited a membraneous pattern in keratinocytes and sweat gland epithelial cells (Fig. 6A,B). Basal cells of the epidermis also showed a mild granular staining for Trop-2.

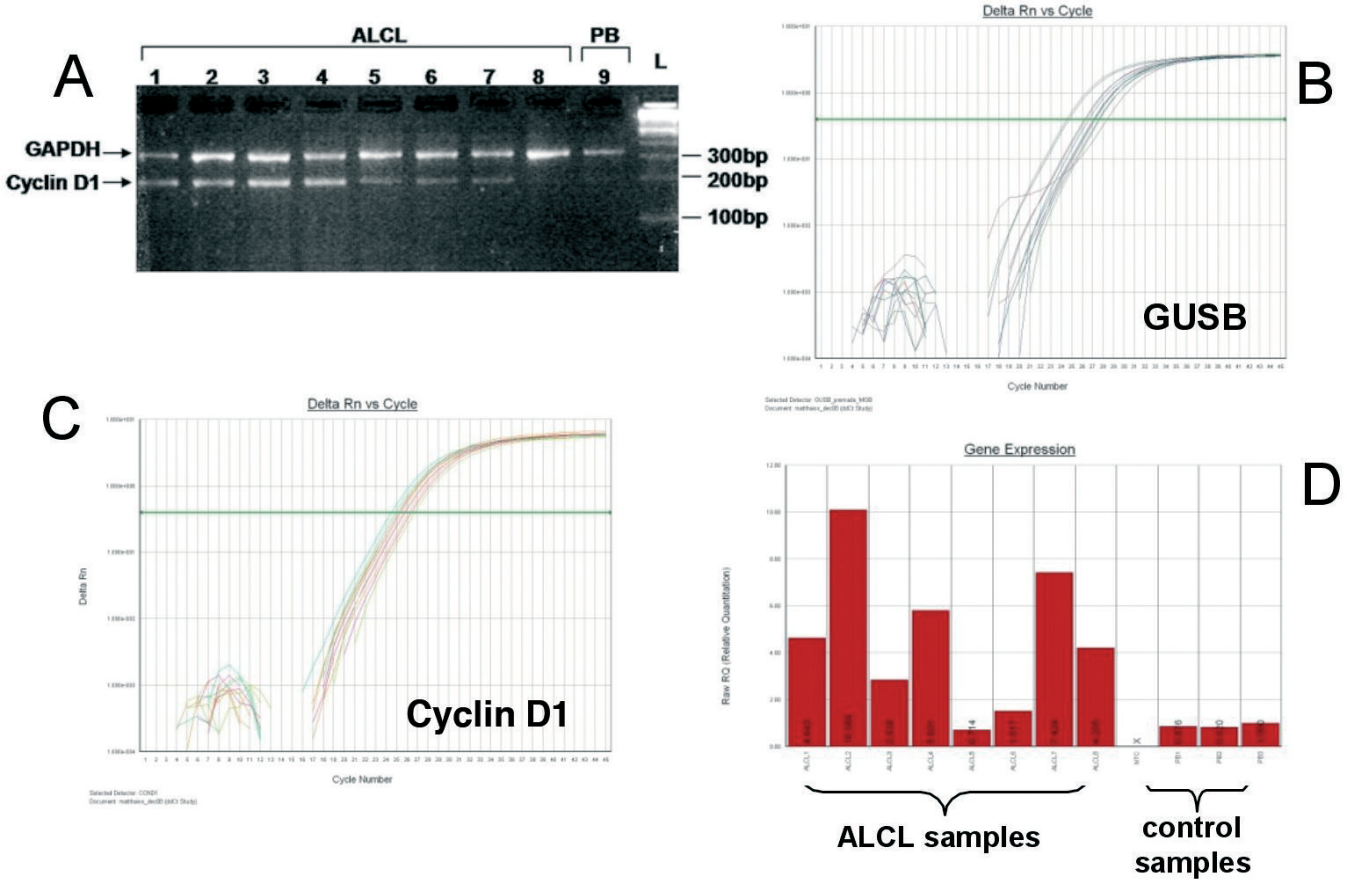

Fig. 3. A. Verification of cyclin D1 expression in ALCL with duplex PCR and relative quantification (RQ) of cyclin D1 mRNA with real time PCR. Positive (lanes 1 – 7) and negative (lane 8) ALCL cases are shown. PB: peripheral blood sample, negative for cyclin D1. L: 100bp DNA ladder. **B and C.** Amplification curves for the housekeeping gene (GUSB) and Cyclin D1 mRNA. **D.** Relative amplification plot with one of the peripheral blood samples (PB) as the calibrator. Note that in 3 ALCL cases, relative Cyclin D1 expression values were >5, as compared to peripheral blood samples.

The immunohistochemical, ISH and FISH results are presented in Table 3.

Discussion

Besides several reciprocal and complex translocations of the ALK gene and cytogenetic alterations of the NPM gene (Drexler et al., 2000), a variety of additional genetic alterations has been reported for ALCL (Ott et al., 1998; Cataldo et al., 1999; Mao et al., 2003; Zettl et al., 2004; Rassidakis et al., 2004), involving known oncogenes and tumor suppressor genes. Zettl et al. in a study using comparative genomic hybridization found a gain of chromosomal material on 11p14-pter in one ALCL/ALK- case and losses of 11q22-qter in 2 ALCL (ALK+ and ALK-). Additionally, losses of 11p were detected in 3 ALCL/ALK- cases. Ott et al using karyotype analysis reported that 2/5 primary nodal ALCLs were hyperdiploid and one was near-tetraploid. Also, in one nodal ALK+ case with complex karyotype (90-94 XXYY), polysomy of all chromosomes, including trisomy of chromosome 11, was found. Thus, in the context of the previously reported ploidy aberrations in ALCL, polysomy of chromosome 11, including the CCND1 locus, as observed in the neoplastic cells in a considerable percentage of cases in our study, was not of further surprise. This genetic material aberration occurred independently of ALK translocations and *cyclin D1* mRNA expression, while it was only marginally associated with Ki67 proliferation index. This latter marker is, however, known to be high in ALCL (Rassidakis et al., 2003), hence the corresponding statistical result may be due to the small

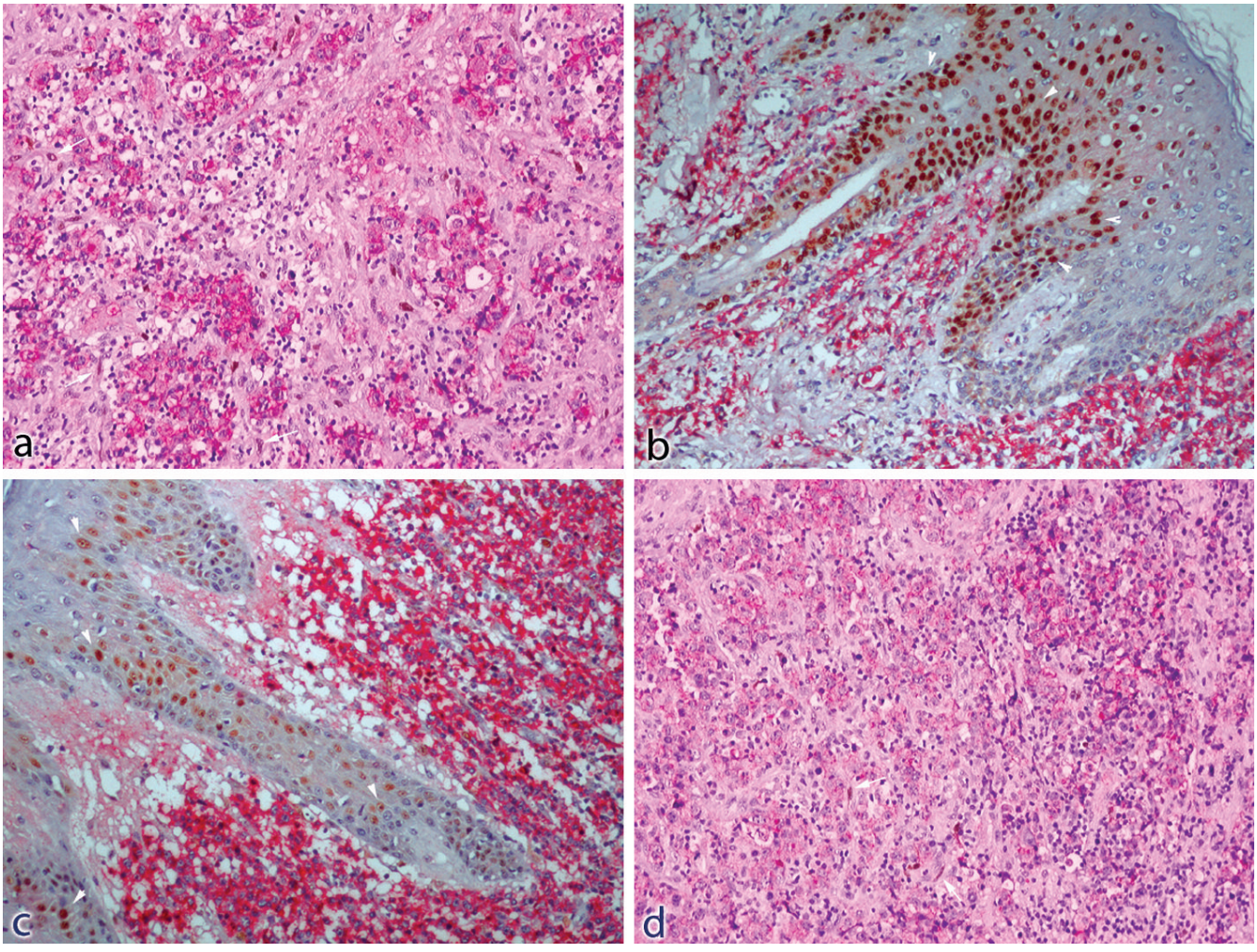

Fig. 4. Immunoenzymatic doublestaining for Cyclin D1 and CD30 in one C-ALCL case that expressed cyclin D1 mRNA. CD30 and Cyclin D1 mAb clone SP4 (**a, b**); CD30 and Cyclin D1 mAb clone DCS-6 (**c, d**). Both Cyclin D1 antibodies showed strong nuclear positivity in endothelial (arrows) and epidermal cells (arrowheads). The CD30 antigen was visualized using the alkaline phosphatase as a chromogen. x 200

number of cases in the low proliferation group. In all, the biological implications of this chromosomal copy number gain remain currently unclear.

The unexpected finding herein was that although ALCLs often express cyclin D1 mRNA, they do not seem to produce Cyclin D1 protein. Previous studies employing qualitative and quantitative fluorescence RT-PCR detected low levels of cyclin D1 mRNA in 1/2 (Aguilera et al., 1998), and 3/3 ALCL cases tested (Elenitoba-Johnson et al., 2002). Although not quantitative, ISH is considered a sensitive method for the detection of cyclin D1 mRNA and has been successfully applied in a variety of tumors, such as colon and esophageal carcinomas, astrocytomas and epitheliod sarcomas (Sallinen et al., 1999; Kristt et al., 2000; Wu et al., 2004; Lin et al., 2005). In hematologic malignancies, in situ cyclin D1 mRNA expression in lymphomas has been reported for MCL and MM (Athanasiou et al., 2001, 2003). As a method, ISH is unique to show mRNA molecules in a perinuclear aggregation, indicating that they have exited the nucleus in order to be further processed for translation. According to recently performed exhaustive BLAST searches, the perinulear signal observed with ISH in the neoplastic cells of ALCL seems specific for cyclin D1 mRNA. Thus, at least to the extent of current knowledge, cyclin D1 mRNA seems to be expressed in the majority of ALCLs. The fact that ALK translocation positive ALCL more often expressed cyclin D1 mRNA is probably related to the upregulation of cyclin D1 expression by the corresponding chimeric products, for example NPM/ALK, as shown experimentally (Wellmann et al., 1997). The association between systemic ALCL and cyclin D1 expression may also be related to ALK

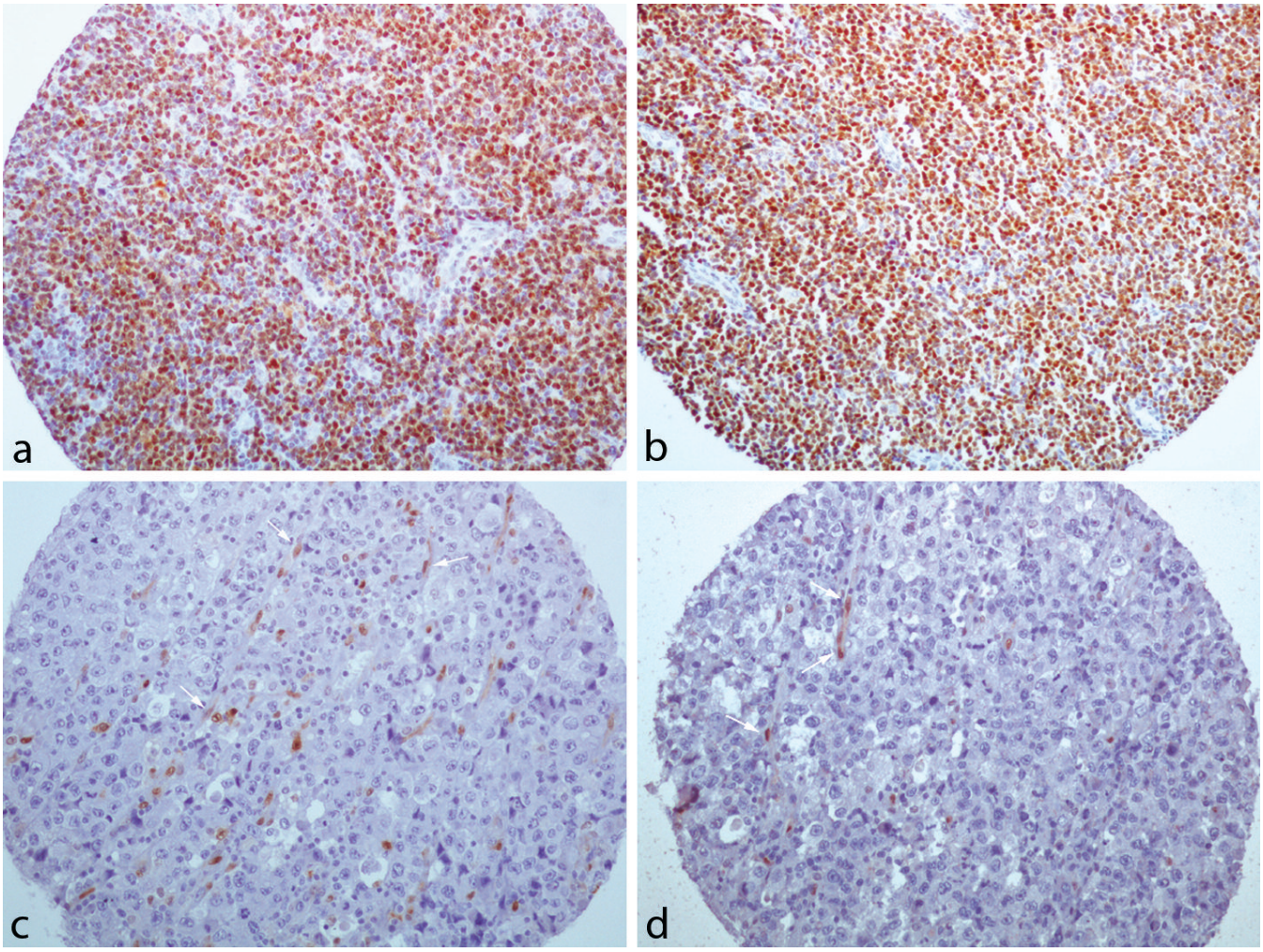

Fig. 5. Simple immunostaining with Cyclin D1 antibodies. Control MCL cases showed intense nuclear staining with both anti-cyclin D1 mAbs (**a**: clone SP4; **b**: clone DCS-6). ALCL cases were negative for Cyclin D1 with either mAb, while endothelial cells (arrows) served as endogenous controls (**c**: clone SP4; **d**: clone DCS-6). x 200

translocations that occur more often in these tumors than in cutaneous disease, as observed here in agreement with established data (Medeiros and Elenitoba-Johnson, 2007).

However, no Cyclin D1 protein was observed in the neoplastic cells of the 44 ALCLs examined here. Previous conflicting reports on Cyclin D1 protein expression in ALCL seem to reflect the specificity of the antibody used in each study. Cyclin D1 protein was detected with the 5D4 antibody (Yatabe et al., 2001), which recognizes Cyclin D1 but cross-reacts with Cyclin D2. Nuclear positivity for Cyclin D1 was also observed in 7/10 cutaneous ALCLs (Mao et al., 2006) with a polyclonal rabbit antibody that has been raised against amino acids 1-295 of Cyclin D1 (Santa Cruz, sc-753). According to the respective data sheet this antibody strongly cross-reacts with Cyclin D2 and D3. In contrast, when using the specific anti-Cyclin D1 DCS-6 (Kanavaros et al., 2001) or the more recently developed SP4 clone (Cheuk et al., 2004), no Cyclin D1 expression was found in the neoplastic cells of ALCL, in full concordance with our results in a larger series of these tumors upon using both these antibodies. The lack of Cyclin D1 protein detection in ALCL expressing the corresponding mRNA could be due to a variety of reasons, for example, genetic defects leading to altered protein structure other than the stabilizing ones in MCL (Wiestner et al., 2007), or deregulation of miRNAs responsible for cyclin D1 mRNA silencing (Sun et al., 2008). Interesting hypotheses can be raised on this issue for further investigation. However, according to our findings, Cyclin D1 protein does not seem to play a role in the pathogenesis or maintenance of ALCL.

Previous evidences suggested a role of other cyclins in ALCL proliferation. Turturro et al. showed that Cyclin D1 was absent in SUDHL-1 and KARPAS 299 cell lines derived from t(2;5)-positive anaplastic large cell lymphoma, whereas Cyclin D2 was expressed from both cell lines, and high levels of Cyclin D3 were detected only in SUDHL-1 cell line (Turturro et al., 2001). Cyclin D3 expression in the above ALCL cell lines was further straingthen by the findings of Amin et al. (2003). In the above study, Cyclin D3 downregulation and $p21^{waf1}$ upregulation was induced by STAT3 activation by JAK3 and that NPM-ALK mediated activation of STAT3 is influenced by the functional status of JAK3. Similar decrease of Cyclin D3 levels was observed after silencing of the cJun gene in Karpas 299 and SUDHL-1 cell lines (Leventaki et al., 2007). Recent study, using microarray analysis of RNA extracted from frozen tissue blocks of 14 ALCL cases, showed that Cyclin D3RNA was present on both ALCL groups of tumors, with 13-fold greater value in ALK+ than ALK- tumors as confirmed by Q-RT-PCR analysis (Thompson et al., 2005). Immunohistochemical expression of Cyclin D3 in this study was also evident in all tested tumors, with stronger staining in ALCL+ tumors.

As previously suggested (Terinnoni et al., 2001), CCND1/TROP2 fusion products are widely transcribed in cancer, autonomously upregulating the production of Trop-2, which is the only translation product in this case. Trop-2 is a type-1 transmembrane glycoprotein with a single transmembrane domain encoded by the corresponding intronless gene located on 1p32-p31 (Fornaro et al., 1995). Trop-2 acts as cell surface receptor, while phosphorylation of this molecule induces transduction of intracellular calcium signalling. Overexpression of Trop-2 protein in tumor cells may play an important role in the control of cell-cell adhesion and in tumor development (Ohmachi et al., 2006), while the same protein has recently been proposed as a

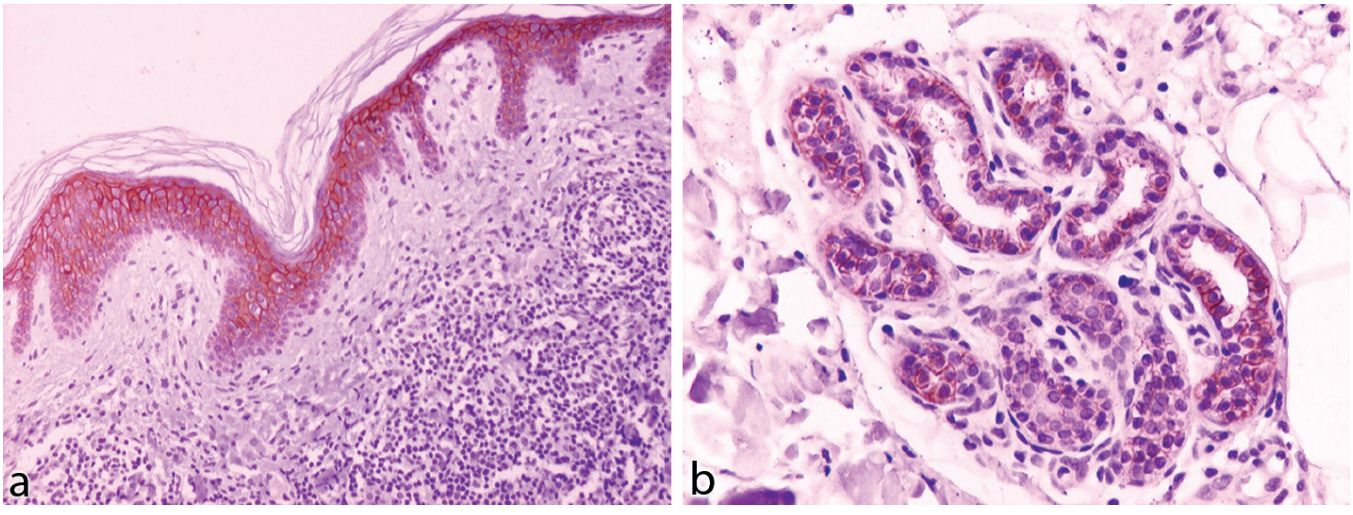

Fig. 6. Absence of TROP2 protein in ALCL. Trop-2/GA733-1 is expressed in a membraneous pattern in squamous cells of the epidermis **(a)** and epithelial cells of secretory portion of sweat glands **(b)**. The underlying cutaneous ALCL lesion is negative. x 200

therapeutic target in colon cancer (Wang et al., 2008). In ALCL, the ectopic expression of Trop-2 would potentially indicate the presence of CCND1/TROP2 fusion. However, Trop-2 was not present in the neoplastic cells, whereas keratinocytes and sweat gland epithelial cells served as adequate endogenous controls where available.

In conclusion, a chromosomal aberration concerning polysomy of CCND1 gene and trisomy/polysomy of chromosome 11 was detected in ALCLs probably as a consequence of the commonly observed polyploidy in this group of tumors. Cyclin D1 mRNA is often expressed in ALCL, mostly related to the presence of translocations involving the ALK gene and to systemic disease. However, the observed aberrations do not result in the production of functional Cyclin D1 protein, which, therefore, does not seem to play a role in the proliferation of ALCL.

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