Differential Expression of TRAF1 Aids in the Distinction of Cutaneous CD30-Positive Lymphoproliferations

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Lymphomatoid papulosis (LyP), primary cutaneous anaplastic large T-cell lymphoma (cALCL), and cutaneous infiltrates of systemic anaplastic large cell lymphoma (sALCL) are CD30-positive lymphoproliferative disorders of the skin that overlap clinically, histopathologically, immunophenotypically, and genetically but differ considerably in their prognosis. In particular, lesions of LyP regress spontaneously, whereas those of cALCL and sALCL persist and may progress and spread to extracutaneous sites. In contrast to patients with cALCL, LyP patients do not benefit from an aggressive radio- and/or chemotherapeutic approach. We generated a novel tumor necrosis factor receptor (TNFR)-associated factor 1 (TRAF1) antibody that recognizes a formalin-resistant epitope (Ber-TRAF1A) and investigated the expression of TRAF1, an intracellular component of TNFR signaling, in LyP and ALCL. We could show a strong TRAF1 expression in the tumor cells of most LyP cases (42/49, 84%). In contrast, tumor cells of primary and secondary cALCL revealed TRAF1 expression in only a few cases (3/41, 7%) as shown for sALCL without skin manifestation. The data indicate that TRAF1 expression reliably distinguishes LyP from primary or secondary cALCL. This might be of crucial diagnostic importance and has a strong impact on the treatment decision for patients with cALCL and LyP.

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INTRODUCTION

Primary cutaneous T-cell lymphomas (CTCLs) represent a heterogeneous group of neoplasms derived from skin-homing T-cells. Discounting classical mycosis fungoides, primary cutaneous CD30-positive lymphoproliferations are the most common entities, comprising approximately 30% of all CTCLs. This group includes lymphomatoid papulosis (LyP), primary cutaneous anaplastic large T-cell lymphoma (cALCL), and borderline cases (Macaulay, 1968; Willemze *et al.*, 2005).

The two entities have widely overlapping clinical and histomorphological features but differ in their prognosis. LyP as well as cALCL can occur at any age and present with ulcerating lesions and grouped nodules (Willemze *et al.*, 1982). A histological feature common to both diseases is the presence of atypical lymphoid CD30-positive T blasts that sometimes show the morphology of Hodgkin–Reed–Sternberg cells (Stein *et al.*, 1985). The cells of LyP and ALCL are genetically characterized by clonal T-cell-receptor gene rearrangement (Chott *et al.*, 1996; Steinhoff *et al.*, 2002). However, the two diseases differ considerably in their clinical course. Although LyP is associated with a favorable 10-year survival rate of nearly 100%, 10–20% of patients with cALCL show extracutaneous progression of the lymphoma, including involvement of the lymph nodes, bone marrow, lungs, gastrointestinal tract, or central nervous system (Beljaards *et al.*, 1993; Bekkenk *et al.*, 2000; Liu *et al.*, 2003; Shehan *et al.*, 2004; Willemze *et al.*, 2005). Approximately 10% of patients with cALCL die of lymphoma (Beljaards *et al.*, 1993; Shehan *et al.*, 2004). To avoid assigning patients to an inappropriate therapeutic regimen, for example, polychemotherapy for LyP or clinical observation alone for cALCL, it is extremely important to distinguish between the two entities during the early course of the disease. Analysis of the (2;5)-translocation exhibited by tumor cells in 50% of the primary systemic ALCL (sALCL) cases is not helpful in clarifying the differential diagnosis of LyP and cALCL, because the vast majority of cALCL cases do not carry this translocation (DeCoteau *et al.*, 1996; Herbst *et al.*, 1997). As cytotoxic molecules are expressed by the tumor cells of cALCL as well

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Abbreviations: ALCL, anaplastic large T-cell lymphoma; cALCL, cutaneous ALCL; CTCL, cutaneous T-cell lymphoma; LyP, lymphomatoid papulosis; sALCL, systemic ALCL; TNFR, tumor necrosis factor receptor; TRAF1, tumor necrosis factor receptor-associated factor 1

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as LyP, the detection of a cytotoxic immunophenotype of the tumor cells do not clarify the differential diagnosis of both diseases (Boulland *et al.*, 2000). Therefore, more refined diagnostic criteria are certainly needed.

Recently, we and others have shown that tumor necrosis factor receptor (TNFR)-associated factor 1 (TRAF1) is differentially expressed in classical Hodgkin's lymphoma and sALCL (Dürkop *et al.*, 1999, 2003; Zapata *et al.*, 2000; Murray *et al.*, 2001; Siegler *et al.*, 2003; Rodig *et al.*, 2005). As a member of the TRAF family, TRAF1 is involved in the intracellular signal transduction of TNFR family members without a death domain, for example, CD30 (Dürkop *et al.*, 1992; Dempsey *et al.*, 2003). Although little is known about the function of TRAF1, it is accepted that TRAF1 is expressed mainly in activated lymphocytes and that it is strongly induced by NF-κB (Baker and Reddy, 1998). Therefore, cells with high NF-κB activity generally express high levels of TRAF1. It could be induced by several members of the TNFR family, including CD30, which mediate their intracellular signaling through NF-κB activation leading to subsequent TRAF1 induction (Bryce *et al.*, 2006; Dürkop *et al.*, 2006).

This study intends to clarify, for the first time, the TRAF1 expression status and its possible impact in the challenging differential diagnosis of cutaneous CD30-positive lymphoproliferations. Therefore, we generated a new highly specific murine TRAF1 mAb, which recognizes a formalin-resistant epitope. Using this novel mAb, the TRAF1 expression profile of a large series of cutaneous CD30-positive lymphoproliferations was analyzed and correlated to the clinical presentation and follow-up. Our findings demonstrate that the extent of TRAF1 expression distinguishes LyP from both cALCL and sALCL. TRAF1 staining, thus, provides an additional criterion for the differential diagnosis and disease-specific treatment of the different cutaneous CD30-positive lymphoproliferations.

RESULTS

Generation of TRAF1 antibody

For immunization, we selected a unique part of the TRAF1 molecule not found in other TRAF family members or other human proteins. BALB/c mice were immunized with this recombinant TRAF1 fragment. Prerequisites for the selection of hybridomas were: secretion of an mAb that strongly binds to the TRAF1 fragment in ELISA, no reactivity with human albumin in an ELISA, selective TRAF1 binding of several cell lysates in Western blot (Figure 1), and an immunohistological pattern strongly resembling the TRAF1 expression pattern, as previously shown by *in situ* hybridization and immunohistology with a commercial available TRAF1 mAb (Dürkop *et al.*, 1999; Zapata *et al.*, 2000). One out of about 200 hybridomas fulfilled these criteria. The mAb produced by this hybridoma was selected for further investigations and was named Ber-TRAF1A.

Clinical and pathologic features of CD30-positive lymphoproliferations

The study included 90 lymphoma biopsies (49 LyP, 28 cALCL, and 13 sALCL) from 50 patients with cutaneous CD30-positive lymphoproliferations. The patients were 35 men and 15 women ranging in age from 13 to 78 years (median age 51 years). The clinical follow-up ranged from 12 to 168 months (Table 1). Characteristics of the patients are given in detail in Table 1. Biopsies were repeatedly conducted in 31 patients with LyP and 14 patients with cALCL. The diagnoses were confirmed in all cases of repeated biopsies. During the clinical course transformation of LyP to cALCL was not observed in our patients with LyP. Although spontaneous regression was a common feature of our patients with LyP, it was not detected in our 14 patients with cALCL. Extracutaneous disease was documented in 5/14 (36%) patients with cALCL during the clinical course (at least 6 months after the first diagnosis) including lymph nodes (2/14, 14%), visceral organs (2/14, 14%), and central nervous system (1/14, 7%). None of the LyP patients died because of lymphoproliferation, whereas 3/5 patients with cALCL, which developed during the clinical course of an extracutaneous involvement, died because of progression of the lymphoma.

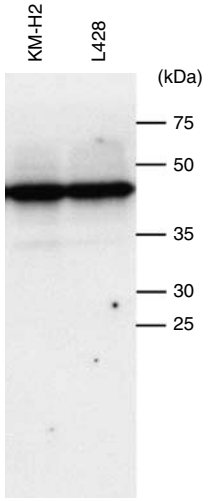

Figure 1. Western blot analysis of solubilisates of the KM-H2 and the L428 cell lines with the Ber-TRAF1A mAb. Molecular weight is indicated at the right margin.

High TRAF1 expression in LyP and low or absent TRAF1 expression in cALCL and sALCL

Based on immunohistological analysis, LyP cases were subdivided into 41/49 skin lesions (26 patients) of type A and 8/49 of type C (five patients, Table 2). TRAF1 expression analysis of 49 cases of LyP revealed strong cytoplasmic TRAF1 staining (41/49 cases, 84%) in atypical lymphoid cells. There was no significant difference of TRAF1 staining between LyP types A and C (Table 2). The same cases were stained for CD30. In both LyP types, nearly all CD30-positive lymphocytes express the TRAF1 protein (Figure 2). These results were supported by immunofluorescence double-staining showing colocalization of TRAF1 and CD30 in LyP type A (Figure 2).

Twenty-eight biopsies of cALCL from 14 patients were analyzed for TRAF1 expression. In contrast to LyP, cALCL

Table 1. Clinical characteristics of patients

Characteristics	LyP	cALCL	sALCL
Number	31	14	5
Sex			
Male	19	10	5
Female	12	4	0
Age (years)			
Median	45.5	65.4	43.0
Range	12–78	16–81	18–67
Extent of skin lesion			
Solitary	4 (13%)	6 (43%)	0 (0%)
Regional	12 (39%)	5 (36%)	4 (80%)
Generalized	15 (48%)	3 (8%)	1 (20%)
Involved extracutaneous sites[1]			
No extracutaneous involvement	31 (100%)	9 (64%)	0 (0%)
Lymph nodes	0 (0%)	2 (14%)	+
Visceral organs	0 (0%)	2 (14%)	2 (40%)
CNS	0 (0%)	1 (7%)	0 (0%)
Initial therapy			
Psoralen UV-A/UV-B	12 (39%)	2 (14%)	0 (0%)
Excision	5 (16%)	6 (43%)	0 (0%)
Radiotherapy	0 (0%)	2 (14%)	0 (0%)
Single agent chemotherapy (methotrexat)	6 (19%)	4 (28%)	0 (0%)
Multiagent chemotherapy	0 (0%)	0 (0%)	5 (100%)
None/topical steroids	8 (26%)	0 (0%)	0 (0%)
Follow-up (months)			
Median	43.7	37.1	43.4
Range	12–168	14–98	27–66
Clinical course			
Complete remission	14 (45%)	2 (14%)	2 (40%)
Partial remission	17 (55%)	5 (36%)	1 (20%)
Progressive disease	0 (0%)	7 (50%)	2 (40%)
Current status			
No evidence of disease	13 (42%)	2 (14%)	2 (40%)
Alive with disease	17 (55%)	9 (64%)	2 (40%)
Died of lymphoma	0 (0%)	3 (8%)	1 (20%)
Died of other cause	1 (3%)	0 (0%)	0 (0%)

Involvement of extracutaneous sites more than 6 months after diagnosis; +sALCL evolved primary in the lymph node.
cALCL, primary cutaneous anaplastic large cell lymphoma; sALCL: systemic anaplastic large cell lymphoma with secondary involvement of skin; CNS, central nervous system; LyP, lymphomatoid papulosis.

Table 2. Summary of TRAF1 immunohistochemical staining pattern

Diagnosis	*N*	TRAF1 (+)	TRAF1 (±)	TRAF1 (−)
LyP	49	41/49 (84%)	5/49 (10%)	3/49 (6%)
LyP-A	41	35/41 (85%)	3/41 (7%)	3/41 (7%)
LyP-C	8	6/8 (75%)	2/8 (25%)	0/8 (0%)
cALCL	28	1/28 (4%)	6/28 (21%)	21/28 (75%)
sALCL	13	2/13 (15%)	0/13 (0%)	11/13 (85%)
nALCL	14	2/14 (14%)	0/14 (0%)	12/14 (86%)

cALCL, primary cutaneous anaplastic large cell lymphoma; sALCL, secondary cutaneous infiltration of systemic anaplastic large cell lymphoma; nALCL, systemic anaplastic large cell lymphoma (lymph node) without cutaneous infiltration; LyP, lymphomatoid papulosis; TRAF1, tumor necrosis factor receptor-associated factor 1, (+), strong reactivity, that is, intensive and confluent staining of the entire cytoplasm in more than 80% of the tumor cells; ±, weak or moderate TRAF1 staining, that is, faint, sometimes grainy, TRAF1 staining of parts of the cytoplasm in less than 80% of the tumor cells; (−), no TRAF1 staining of tumor cells.

mostly showed only weak TRAF1 expression (6/28 biopsies, 21%) or no TRAF1 expression at all (21/28 biopsies, 75%). The tumor cells of 1/28 cALCL case (4%) were strong TRAF1-positive. Similar results were obtained for the cutaneous manifestation of sALCL, wherein 11/13 specimens (85%) were TRAF1-negative and only 2/13 biopsies were TRAF1-positive (15%). Neither the TRAF1-positive cALCL case nor both TRAF1-positive sALCL cases exhibit a (2;5)-translocation. This indicates a similar TRAF1 induction status in sALCL and cALCL. TRAF1 expression pattern was comparable with archival lymph node biopsies, obtained from 14 patients with sALCL without skin manifestation (eight ALCL with t(2;5), six ALCL without t(2;5); Table 2). The TRAF1 expression was significantly higher in LyP than in primary cALCL and sALCL ($P<7\times10^{-7}$). Neither cALCL nor sALCL showed a significant difference of TRAF1 expression with regard to their t(2;5) status. There was no significant difference in the TRAF1 staining of patients with multiple biopsies.

Interestingly, in all subtypes of cutaneous CD30-positive lymphoproliferations the surrounding CD30-negative cells showed complete absence of TRAF1 staining, enabling a clear differentiation and interpretation of the CD30-positive tumor cells (Figure 2).

The commercial available TRAF1 mAb H3 was compared in 22 cases of CD30-positive lymphoproliferations with the Ber-TRAF1A mAb by immunohistology. In principle, both mAbs generated the same staining pattern in 10 cases of cALCL, four cases of sALCL, six cases of LyP type A, and two cases of LyP type C, but the Ber-TRAF1A mAb seems to better differentiate between low and high expression levels of TRAF1 than the H3 mAb.

DISCUSSION

Primary CD30-positive cALCL and LyP show many overlapping clinical, histological, immunophenotypic, and genetic features. Owing to the difficult differential diagnosis, the

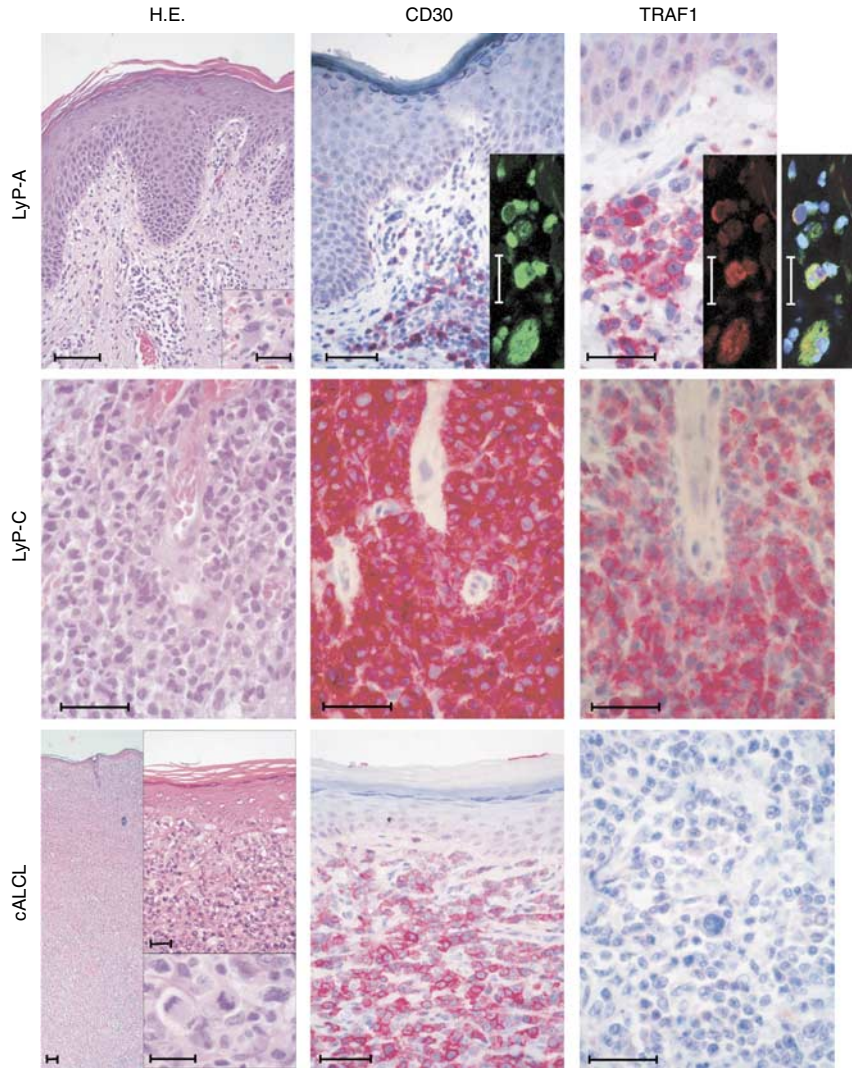

Figure 2. Immunohistological staining pattern of the Ber-TRAF1A mAb in cases of LyP type A (LyP-A), LyP type C (LyP-C), and cALCL in comparison with CD30 staining and hematoxylin–eosin staining. The immunhistological expression of TRAF1 and CD30 in LyP type A was supplemented by confocal microscopy (see insets of the CD30 and TRAF1 staining in LyP-A row). The colocalization of TRAF1 (red) and CD30 (green) expression in LyP-A is indicated in the picture at the right margin of the LyP-A row by a yellow label. Nuclei were stained by TOTO-3 (blue). Bars in the main pictures represent 50 μm, whereas the bars in the insets indicate 20 μm.

recent WHO-EORTC classification included a ''borderline group'' for cases in which a definitive distinction between cALCL and LyP cannot be done despite thorough clinical and pathological assessment. Owing to these diagnostical difficulties, LyP patients diagnosed as having a ''CD30-positive lymphoma'' are routinely treated with multiagent chemotherapy by physicians unaware of the spectrum of LyP. Unfortunately, skin relapses of LyP occur very frequently during or shortly after systemic chemotherapy. That gives the false impression of a highly aggressive T-cell lymphoma requiring even more aggressive chemotherapy (Paulli *et al.*, 1995). On the other hand, insufficient treatment of cALCL potentially leads to a fatal outcome (Shehan *et al.*, 2004). Therefore, objective criteria are needed to effectively differentiate between LyP and cALCL patients.

This study presents the first TRAF1 expression data in cutaneous CD30-positive lymphoproliferations. TRAF1 represents a possible marker to differentiate between LyP and cALCL. We developed a highly specific immunohistological routinely applicable murine mAb corresponding to a characteristic domain of the TRAF1 molecule that is not shared by other molecules, not even by those of other TRAF family members. The specificity of this TRAF1 mAb was confirmed by ELISA, Western blot analysis, and numerous immunohistological investigations. Its staining pattern is identical to the one previously described for TRAF1 expression by *in situ* hybridization (Dürkop *et al.*, 1999). Clinically and histopathologically well-defined patients with cutaneous CD30-positive lymphoproliferations were investigated to assess both the feasibility of applying TRAF1 expression and its diagnostic value in routine practice. The commercial available TRAF1 mAb H3 generated the same staining pattern as the Ber-TRAF1A mAb. However, because of better discrimination between low and high TRAF1 expression

levels, the latter mAb seems to be more suitable for the differentiation of cutaneous manifestations of ALCL and LyP.

An analysis of cutaneous CD30-positive lymphoproliferations clearly demonstrates a difference of TRAF1 expression between LyP and cALCL. TRAF1 is highly expressed in LyP. This includes LyP type C cases, which are hard to be differentiated from cALCL and cutaneous manifestations of sALCL because of the large sheets of CD30-positive tumor cells by histology and immunohistology. Double-staining immunofluorescence revealed colocalization of TRAF1 and CD30 in the LyP tumor cells, whereas TRAF1 expression was absent in the surrounding T- and B-cells. In contrast to CD30-positive cells of LyP, cALCL tumor cells were almost TRAF1-negative.

TRAF1 is upregulated by NF-κB activation (Murray *et al.*, 2001; Dempsey *et al.*, 2003; Bryce *et al.*, 2006). Therefore, differential TRAF1 expression in LyP and ALCL could be explained by varying NF-κB activity in the tumor cells of both entities. This assumption corresponds well to the finding of low NF-κB activity in unstimulated cALCL and sALCL cell lines (Levi *et al.*, 2000; Dürkop *et al.*, 2006). Accordingly, the strong TRAF1 expression in LyP tumor cells might indicate that these cells share the high NF-κB activity with the classical Hodgkin lymphoma tumor cells (Dürkop *et al.*, 2003; Dürkop *et al.*, 2006). However, exact data about the NF-κB activity in this tumor are not available, which is at least partially due to the lack of LyP cell lines. Owing to the striking differences of TRAF1 expression between LyP and cALCL, we would speculate that the activation of TRAF1 could play a role in the mechanism(s) of the clinically obvious spontaneous regression in LyP in contrast, to the persistence and progression of tumors in cALCL, respectively. However, in contrast to our cALCL series spontaneous regression is also reported in a part of cALCL (Paulli *et al.*, 1995; Willemze *et al.*, 2005). In these cases, analysis of TRAF1 expression would be interesting. TRAF1 function is complex and not completely understood. However, it seems to be possible that TRAF1 influences the sensitivity of cells to apoptosis (Cha *et al.*, 2003; Dürkop *et al.*, 2003). Further analysis of TRAF1 expression in conjunction with the phosphorylation status of NF-κB and its inhibitors might elucidate the biology of cutaneous CD30-positive lymphoproliferations.

In conclusion, strong TRAF1 expression is almost restricted to CD30-positive cells in LyP, indicating that its overexpression in LyP is not only of pathogenetic relevance but might also be a new marker for the differential diagnosis of LyP and cALCL. Determination of the TRAF1 expression status might enable the initiation of disease-specific management in patients with cutaneous CD30-positive lymphoproliferations.

MATERIALS AND METHODS

Generation and characterization of the TRAF1 antibody

TRAF1 cDNA (acc. no. U19261) was amplified from L428 mRNA by reverse transcription PCR at nucleotide positions 418–774 using the oligonucleotide pair GAA TTC TGC CCT GGA GCA GAA CCT G/GAA TTC GCG CAA GCT CTG CTC CAG C (*Eco*RI sites are underlined). This TRAF1 cDNA-coding sequence for the amino-acid residues 140–258 was cloned into the expression vector pRSET/E (Invitrogen, Carlsbad, CA). After expression in *Escherichia coli* (BL21pLysS, Invitrogen), the recombinant protein carrying a N-terminal 6× His-tag was purified by affinity chromatography and subsequent gel filtration chromatography. BALB/c mice were immunized with the TRAF1 fragment. The TRAF1 antibody titer was determined in mouse tail-vein blood by ELISA with TRAF1 fragment-coated 96-well plates according to standard procedures (Ausubel *et al.*, 2006). After mice developed a high titer against TRAF1, spleenocytes were isolated and fused with the murine myeloma cell line NS-1 (Ausubel *et al.*, 2006). Hybridoma supernatants were harvested and tested by ELISA using TRAF1-coated and, as a negative control, albumin-coated plates. Reactivity of the TRAF1 mAbs was verified by Western blot analysis of the classical Hodgkin lymphoma cell lines L1236, L428, and KM-H2 as well as the sALCL cell line Karpas 299 (Drexler, 2001). To ensure that the new TRAF1 mAb depicts the typical TRAF1 expression pattern (Dürkop *et al.*, 1999; Zapata *et al.*, 2000; Siegler *et al.*, 2003; Bryce *et al.*, 2006), we performed immunohistological examinations of 10 tonsils and more than 200 lymphoma cases, including classical Hodgkin lymphoma, different subtypes of diffuse large B cell lymphoma, sALCL, follicular lymphoma, lymphocytic lymphoma, mantle cell lymphoma, angioimmunoblastic T-cell lymphoma, peripheral T-cell lymphoma, and marginalzone lymphoma as well as lymphoblastic T- and B-cell lymphoma. All cases were drawn from the Institute of Pathology, Charité, Campus Benjamin Franklin.

Patients

The study included a total of 90 skin samples stemming from 50 clinically well-characterized patients with CD30-positive cutaneous lymphoproliferations. All patients were treated and clinically followed up by the Department of Dermatology, Charité, Berlin. The CD30-positive cutaneous lymphoproliferations were classified based on the histopathological features, supplemented by additional clinical information according to the recent WHO-EORTC classification of lymphoid neoplasms (Willemze *et al.*, 2005). This collective consisted of 31 patients with LyP, 14 patients with cALCL, and five patients presenting with a secondary skin manifestation of sALCL. Two of these sALCL cases showed a (2;5)-translocation.

LyP was defined as recurrent nodular eruptions with evidence of spontaneous regression of all lesions and histological features consistent with LyP. cALCL was defined as nodules or tumor lesions with histological features typical for cALCL and no evidence of extracutaneous manifestation at initial presentation (Willemze *et al.*, 2005). According to the amount of CD30-positive atypical cells, LyP cases were subdivided into three histological types designated as A(scattered CD30-positive cells), B (absence of CD30-positive cells), and C (sheets of CD30-positive cells histologically undistinguishable from cALCL) (Willemze *et al.*, 1982). As LyP type C histologically and immunohistologically often resembles cALCL, we verified the differential diagnosis between these entities according to the clinical findings of spontaneous regression and skin eruption. We intended to clarify the differential diagnosis between the LyP subtypes containing CD30-positive cells and cALCL. Therefore, we restricted the study to cases of LyP types A and C.

Diagnosis and staging of patients were completed after a comprehensive history and physical examination, complete blood

cell counts including peripheral blood smears for Sézary cells, CD4:CD8 ratio, a general chemistry panel including serum lactate dehydrogenase, a chest X-ray, and an ultrasound of the abdomen and peripheral lymph nodes. Suspected visceral involvement was evaluated by imaging studies or by tissue and bone marrow biopsies, when indicated. Characteristics to the patients including extent of skin lesions, occurrence of extracutaneous disease, treatments, clinical course, and outcome were recorded for each patient (Table 1). Each skin sample was investigated twice by T-cell receptor-γ PCR and Genescan analysis (Lukowsky, 2003). The consent of the patients was obtained to use their biopsies. The ethical committee of the Charité (Universitätsmedizin, Berlin) has approved the described studies. The study was conducted according to the Declaration of Helsinki Principles.

Immunohistology

All skin and lymph node samples were examined by hematoxylin–eosin staining and immunohistological staining (TRAF1 (Ber-TRAF1A and H3, latter: Santa Cruz, Heidelberg, Germany)), CD4 (1F6; Novocastra, Newcastle upon Tyne, UK), (DAKO, Glostrup, Denmark) CD3 (polyclonal CD3), CD8 (C8–144), CD30 (Ber-H2), ALK1, and ALKc (Cordell *et al.*, 1984). To unmask antigen epitopes, all sections were pretreated by high-pressure cooking (10 mM citrate buffer, pH 6.0). For TRAF1/CD30 double-fluorescence staining, TRAF1 was detected by alkaline phosphatase monoclonal anti-alkaline phosphatase method (Cordell *et al.*, 1984) and CD30 by anti-mouse Cy2 sera (Dianova, Hamburg, Germany). Nuclear staining was performed by TOTO-3 (Molecular Probes, Paisley, UK) and the fluorescence staining was evaluated by a confocal microscope (Leitz, Wetzlar, Germany).

Reactivity for TRAF1 was determined and scored independently by two pathologists. Intensity of staining for TRAF1 was scored as follows: (+) strong staining, that is, intensive and confluent staining of the entire cytoplasm in more than 80% of the tumor cells; (+/−) weak or moderate staining, that is, faint, sometimes grainy, staining of parts of the cytoplasm in less than 80% of the tumor cells; (−) no staining of tumor cells.

Statistical analysis

Statistical analysis was performed using the χ^2 test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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