Cutaneous Anaplastic Large Cell Lymphoma and Peripheral T-Cell Lymphoma NOS Show Distinct Chromosomal Alterations and Differential Expression of Chemokine Receptors and Apoptosis Regulators

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Primary cutaneous anaplastic large cell lymphoma (C-ALCL) has an indolent clinical course and favorable prognosis. On the contrary, primary cutaneous peripheral T-cell lymphoma not otherwise specified (PTL-NOS) shows aggressive clinical behavior. To identify genomic events relevant in the pathogenesis of these cutaneous T-cell lymphomas (CTCLs), we carried out array-based comparative genomic hybridization (CGH) analysis. Simultaneously, gene expression profiling was conducted to gain insight into gene expression programs associated with the different clinical behavior of these CTCLs. C-ALCL was characterized by gains on chromosome 7q and 17q and losses on 6q and 13q. PTL-NOS similarly showed gains on 7q and 17q, but was distinguished by gains on chromosome 8 and loss of a focal overlapping region on 9p21. We identified minimal common regions harboring candidate oncogenes and tumor suppressor genes in C-ALCL and PTL-NOS. Genes with a role in lymphocyte chemotaxis, apoptosis, and proliferation were overrepresented among genes differentially expressed between these lymphomas. C-ALCL showed higher expression of the skin-homing chemokine receptor genes *CCR10* and *CCR8*, which may explain the lower tendency to disseminate to extracutaneous sites. Furthermore, C-ALCL and PTL-NOS showed aberrant expression of distinct genes implicated in apoptosis and proliferation, such as *IRF4/MUM1* and *PRKCQ*, which may account for differences in clinical aggressiveness.

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INTRODUCTION

Primary cutaneous anaplastic large cell lymphoma (C-ALCL), formerly designated as primary cutaneous CD30-positive large T-cell lymphoma, is a T-cell lymphoma composed of large cells with an anaplastic, pleomorphic, or immunoblastic cytomorphology, which show expression of the CD30 receptor (Willemze *et al.*, 2005). Most patients present with solitary or localized skin tumors that have a tendency to regress spontaneously. C-ALCL has an indolent clinical behavior and rarely shows extracutaneous dissemination. This cutaneous T-cell lymphoma (CTCL) is commonly treated with radiotherapy and has an excellent prognosis with an estimated 5-year survival exceeding 90% (Bekkenk *et al.*, 2000; Liu *et al.*, 2003; Yu *et al.*, 2008). In contrast, primary cutaneous peripheral T-cell lymphoma not otherwise specified (PTL-NOS) presents with more generalized skin tumors and displays aggressive clinical behavior (Willemze *et al.*, 2005). PTL-NOS, formerly termed CD30-negative large T-cell lymphoma, frequently disseminates to extracutaneous sites and is often refractory to chemotherapeutic treatment. Patients with PTL-NOS have an estimated 5-year survival of $<15\%$ (Beljaards *et al.*, 1994; Grange *et al.*, 1999; Bekkenk *et al.*, 2003).

The genetic events involved in the pathogenesis of these cutaneous lymphomas are largely unknown. Cytogenetic analyses of C-ALCL have shown recurrent copy number alterations (CNAs) of several chromosomal regions, including gains on 6p, 7q, and 19, and losses on 6q, 9, and 18 (Boni *et al.*, 2000; Mao *et al.*, 2003; Prochazkova *et al.*, 2003; Fischer *et al.*, 2004; Zettl *et al.*, 2004). The t(2;5)(p23;q35) translocation inducing the nucleophosmin/anaplastic lymphoma kinase chimeric protein, a characteristic feature of

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Abbreviations: BAC, bacterial artificial chromosome; C-ALCL, cutaneous anaplastic large cell lymphoma; CGH, comparative genomic hybridization; CNA, copy number alteration; CTCL, cutaneous T-cell lymphoma; GO, Gene Ontology; MCR, minimal common region; PTL-NOS, peripheral T-cell lymphoma not otherwise specified; qPCR, quantitative real-time PCR; RGM, ratio of geometric mean

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nodal ALCL, is not or only rarely found in C-ALCL (DeCoteau *et al*., 1996). To date, no studies on chromosomal alterations in primary cutaneous PTL-NOS have been published.

Genetic factors responsible for the differences in clinical behavior of C-ALCL and PTL-NOS, both derived from skin-homing T cells, have not yet been resolved. Our group has previously shown that the FAS receptor is expressed by C-ALCL tumor cells, whereas expression is lost in the majority of PTL-NOS cases, suggesting differences in sensitivity to extrinsic pro-apoptotic signals (Zoi-Toli *et al*., 2000). In addition, studies have indicated that signaling through the CD30 receptor is implicated in proliferation and apoptosis of lymphoid cells in C-ALCL (Mori *et al*., 1999; Horie *et al*., 2002).

In this study, array-based comparative genomic hybridization (CGH) analysis and gene expression profiling was carried out on C-ALCL and primary cutaneous PTL-NOS tumor samples to identify chromosomal aberrations and gene expression patterns, which may contribute to a better understanding of the molecular mechanisms involved in the development and in the differential clinical behavior of these two types of CTCL.

RESULTS

Recurrent chromosomal alterations in C-ALCL and PTL-NOS with potential biological significance

Tumor biopsy samples of 11 patients diagnosed with C-ALCL and 10 patients with PTL-NOS were analyzed for numerical chromosomal alterations using array-based CGH. Clinical characteristics of the patients are shown in Table 1 and immunophenotypical characteristics of the tumor cells are provided in Table S1. In DNA isolated from all C-ALCL and PTL-NOS tumor samples, numerous chromosomal alterations were present. An overview of the cumulative array-based CGH results in a Frequency of Amplicon, Gain and Loss (FrAGL) plot is shown in for C-ALCL in Figure 1a and for PTL-NOS in Figure 1c.

Table 1. Clinical characteristics

No.	Sex	Age (years)	Extent	Therapy	Result	Site of relapse	Current status	Follow-up (months)
C-ALCL								
1	M	48	LOC	RT	CR	S; LN; LUNG	DOD	231
2	F	62	GEN	Chemo	PR	S; LN	DOD	36
3	F	74	SOL	RT	CR	S	ACR	258
4	F	45	GEN	Chemo	PR	S; LN	DOD	308
5	M	69	LOC	RT	CR	—	ACR	120
6	M	51	GEN	—	—	S	ACR	105
7	M	63	GEN	RT	PR	S	AWD	139
8	M	86	LOC	RT	CR	—	ACR	29
9	M	70	SOL	RT	CR	S	ACR	12
10	F	79	SOL	RT	CR		ACR	13
11	F	44	LOC	EXC	CR	S	AWD	62
PTL-NOS								
12	M	33	GEN	Chemo	PR	S; LN; CNS	DOD	13
13	M	65	SOL	Chemo	PR	S; CNS	DOD	8
14	F	80	GEN	Chemo	PR	S; LN	DOD	27
15	M	70	GEN	Chemo	PR	S	DOD	13
16	M	75	GEN	Chemo	PD	BLOOD	DOD	1
17	M	73	GEN	Chemo	PR	S; LN	DOD	14
18	M	58	GEN	Chemo	PD		DOD	3[1]
19	F	65	GEN	Chemo	PD	S; LN; BM	DOD	12
20	M	33	GEN	Chemo	PD	S; CNS	DOD	19
21	M	59	GEN	Chemo	PR		DOD	6[1]

ACR, alive in complete remission; AWD, alive with clinical symptoms of disease; BM, bone marrow; chemo, chemotherapy; C-ALCL, cutaneous anaplastic large cell lymphoma; CNS, central nervous system; CR, complete remission; DOD, death by disease; EXC, excision; F, female; GEN, generalized (multifocal) skin disease; LN, lymph node; LOC, localized disease; M, male; PD, progressive disease; PR, partial remission; PTL-NOS, peripheral T-cell lymphoma not otherwise specified; RT, radiotherapy; S, skin; SOL, solitary lesion.
[1]Died of therapy-related side effects.

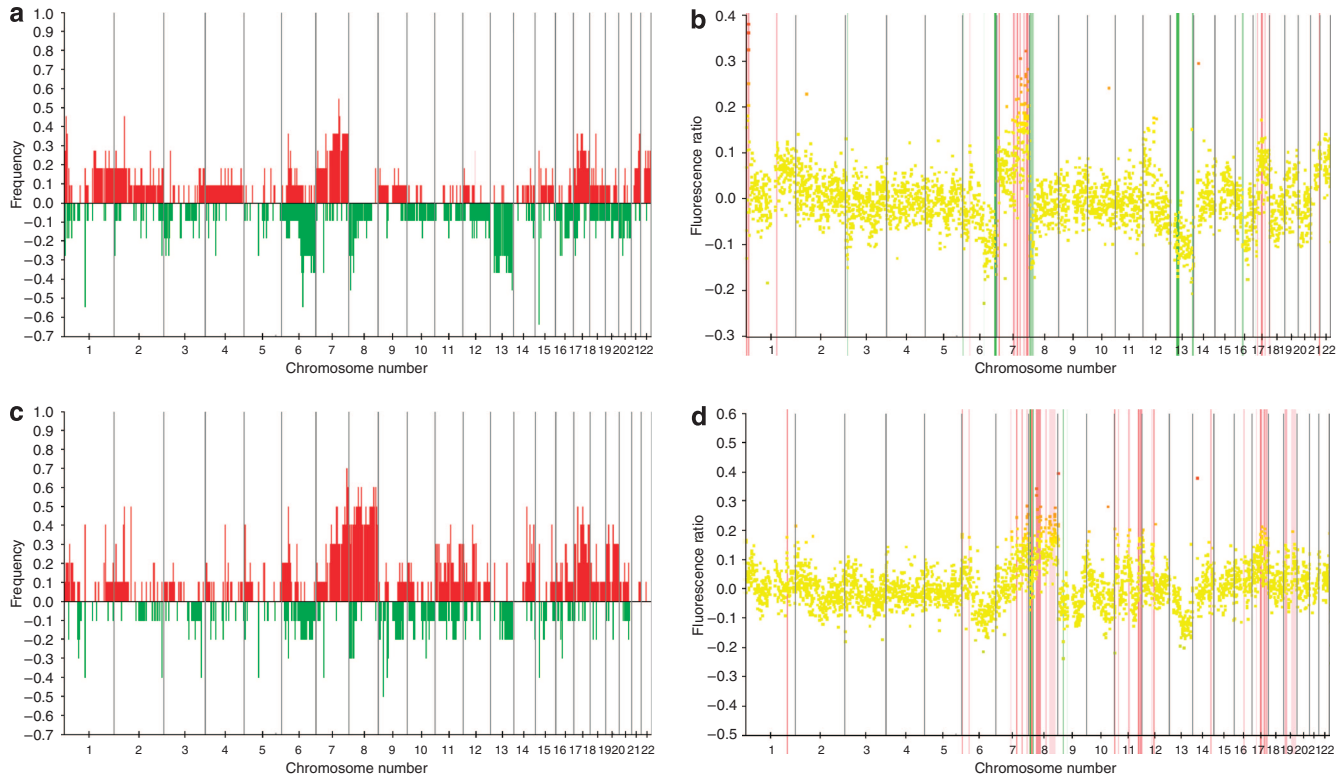


Figure 1. Visualization of the array-based CGH data using VAMP. Chromosomes are indicated on the horizontal axis; excluded are the X and Y chromosomes. Gains are depicted in red and losses in green. (**a**) Frequency of Amplicon, Gain and Loss (FrAGL) plot for C-ALCL tumor samples. (**b**) Averaged CGH pattern of C-ALCL tumor samples. MCRs with loss occurring in at least 35% of patients are indicated as green vertical lines and MCRs with gain as red vertical lines. (**c**) FrAGL plot for PTL-NOS tumor samples. (**d**) MCRs with loss occurring in at least 35% of patients indicated as green vertical lines and MCRs with gain indicated as red vertical lines in the averaged CGH pattern of PTL-NOS tumor samples.

The overall pattern of chromosomal alterations of C-ALCL is characterized by gains of large regions on chromosome 7q and 17 and losses of regions on chromosome 6q and 13. To delineate chromosomal regions harboring genes with pathobiological relevance, we determined the minimal common regions (MCRs), the smallest recurrent chromosomal region with altered probes common to the set of array-based CGH profiles (Rouveirol *et al.*, 2006). A total of 30 MCRs present in at least 35% of the patients were identified. MCRs are visualized in the averaged chromosomal pattern of C-ALCL as vertical bands in Figure 1b and are listed in Table 2. Of these recurrent MCRs with CNA, 20 represent gains of chromosomal regions and 10 correspond to losses. The most highly recurrent chromosomal alterations are gain of 7q31 and loss of 13q34 and 6q16-6q21, all affecting 45% of patients. Next, we cross-referenced the genes residing in these 30 MCRs with the Cancer Gene Census, a list of genes for which mutations have been causally implicated in cancer (Futreal *et al.*, 2004). As presented in Table 2a, the MCRs harbored 26 known oncogenes and tumor suppressor genes. The most highly recurrent MCR with gain in C-ALCL at 7q31 harbors a single oncogene, the *MET* gene that encodes the hepatocyte growth factor receptor. Three putative tumor suppressor genes, *CDC16*, *CUL4A*, and *PRDM1* reside in the loci with loss on 13q34 and 6q16-6q21.

The pattern of CNAs of PTL-NOS is predominated by gains of large regions on chromosome 7, 8, and 17 (Figure 1c). Thirty-four MCRs affecting at least 35% of patients were identified, including 30 gains and only 4 losses (Figure 1d, Table 2). The most highly recurrent MCRs with CNA are 7q36 affected by gain in 60% of patients and 7q21-7q22, 8p12-8q12, 8p21.1-8q21.3, and 8q22-8q24.2, each showing gain in 50% of patients. Forty-three known cancer-associated genes, listed in Table 2b, are located in MCRs with recurrent CNA. Although no confirmed oncogenes are located on 7q36, it harbors the *FASTK* gene encoding an anti-apoptotic kinase expressed by T cells (Simarro *et al.*, 2007). The *MYC* oncogene, previously shown to be amplified and overexpressed in Sézary syndrome and also in aggressive B-cell lymphomas, is located in the MCR with gain on 8q22-8q24.2 (Mossafa *et al.*, 2006; Vermeer *et al.*, 2008).

Similarities and differences between chromosomal alterations in C-ALCL and PTL-NOS

The cumulative patterns of chromosomal alterations of C-ALCL and PTL-NOS show many overlapping features. C-ALCL and PTL-NOS are concordant with respect to gain of large chromosomal regions on 7q and 17. These chromosomes contain several MCRS with CNA that affect both of these lymphomas, such as gains on 7q21-7q22 and

Table 2a. Minimal common regions C-ALCL

Chromosome	Cytogenetic band	CNA	Clone start	Clone end	Position start	Position end	No. of Cases affected	Genes
6	6q16-6q21	Loss	RP3-454N4	RP3-454N4	104,974,085	107,020,676	5	PRDM1*
7	7q31	Gain	RP11-328M22	RP11-126C19	110,975,058	118,629,320	5	MET
13	13q34	Loss	RP11-310D8	RP11-265C7	110,681,700	114,142,980	5	CDC16*, CUL4A*
1	1p36.2	Gain	RP11-285P3	RP11-285P3	12,462,984	15,425,500	4	
6	6q25-6q27	Loss	RP3-336G18	RP1-137D17	156,122,661	170,373,241	4	MLLT4, FGFR1OP
7	7q21	Gain	RP11-28I21b	RP5-1093O17	80,528,144	84,729,683	4	
7	7q21	Gain	RP11-212B1	RP5-1084H12	85,485,924	91,973,205	4	AKAP9
7	7q21	Gain	RP11-101N13	RP5-1145A22	92,090,477	97,314,287	4	
7	7q21-7q22	Gain	RP4-550A13	RP11-44M6	97,314,794	100,976,355	4	
7	7q32-7q34	Gain	RP11-193I17	RP11-269N18	130,792,900	139,186,224	4	TIF1
7	7q34	Gain	RP11-237G17	RP5-894A10	140,558,165	142,236,215	4	
7	7q35	Gain	RP5-819O4	RP4-811H12	144,233,517	147,084,269	4	
7	7q35-7q36	Gain	RP5-1136G13	RP4-800G7	147,259,180	149,650,854	4	
8	8p22-8p21	Loss	RP11-369E15	RP11-529P14	18,644,291	22,394,183	4	
13	13q12-13q14	Loss	RP11-550P23	RP11-351K3	29,008,462	45,700,578	4	FOXO1A, BRCA2, LHFP, LCP1
17	17q12-17q21	Gain	RP11-94L15	RP11-58O9	34,979,298	36,236,729	4	ERBB2, RARA
21	21q22	Gain	RP5-1031P17	RP1-171F15	39,702,870	41,559,383	4	
1	1p36.3	Loss	RP4-785P20	RP11-49J3	1,145,847	7,059,893	3	
1	1q21-1q23	Gain	RP1-13P20	RP11-15G16	148,424,834	161,212,962	3	NTRK1, PRCC, TPM3, SDHC, ARNT, FCGR2B, MUC1
3	3p26-3p25	Loss	RP11-167K17	RP11-329A2	6,636,703	8,869,825	3	
6	6p21.3	Gain	RP5-1077I5	RP1-93N13	32,200,774	33,467,588	3	
7	7p22-7p21	Gain	RP4-733B9	RP5-1100A7	6,234,587	12,919,070	3	PMS2
8	8p23-8p22	Loss	RP11-104F14	RP11-19N21	1,706,678	17,782,553	3	
16	16p11.2-16q11.2	Loss	RP11-274A17	RP11-274A17	32,345,186	45,067,244	3	
17	17p13	Loss	RP11-186B7	RP11-199F11	7,063,252	7,563,870	3	TP53
17	17p11.2	Gain	RP5-836L9	RP11-121A13	19,748,613	21,055,083	3	USP6
17	17q21-17q22	Gain	RP11-361K8	RP11-312B18	43,043,105	49,753,469	3	COL1A1
17	17q23	Gain	RP11-178C3	RP11-156L14	54,872,929	59,626,448	3	CLTC, NACA, BRIP1
17	17q25	Gain	RP11-87G24	GS-362-K4	72,271,167	78,774,742	3	MSF
17	17p11.2	Gain	RP1-162E17	RP1-162E17	18,114,679	19,211,694	3	

C-ALCL, cutaneous anaplastic large cell lymphoma; CNA, copy number alteration; MCR, minimal common region.
Start and end positions are delineated by the nearby borders of adjacent clones. Genes are the cancer census genes situated in the subsequent MCRs. In addition, genes indicated with an asterisk are referred to in the Results and Discussion sections.

17q21-17q25. Other MCRs in C-ALCL and PTL-NOS that have in common are gain of 6p21.3 and losses on 8p21-22 (Table 2).

However, clear differences exist between the CNA patterns of C-ALCL and PTL-NOS. Most strikingly, gains on chromosome 8 affect the majority of patients with PTL-NOS, but are almost absent in C-ALCL. On the other hand, C-ALCL tumor samples were distinguished by frequent losses on chromosome 6 and 13. A distinction of potential relevance concerns the 9p21 locus, which is not affected by loss in any of the C-ALCL patients, but is deleted in 50% of PTL-NOS patients (Figure 1c). The deleted region is quite large in most patients and contains a MCR located on 9p21.3 harboring the *CDKN2A* tumor suppressor gene.

Gene expression patterns of C-ALCL and PTL-NOS show marked differences

To gain insight into the gene expression programs of the tumor cells that underlie the difference in clinical behavior of these entities, gene expression profiling was performed. Supervised gene expression analysis showed that 547 probe sets targeting 358 genes were significantly differentially expressed. Of these genes, 325 were relatively higher

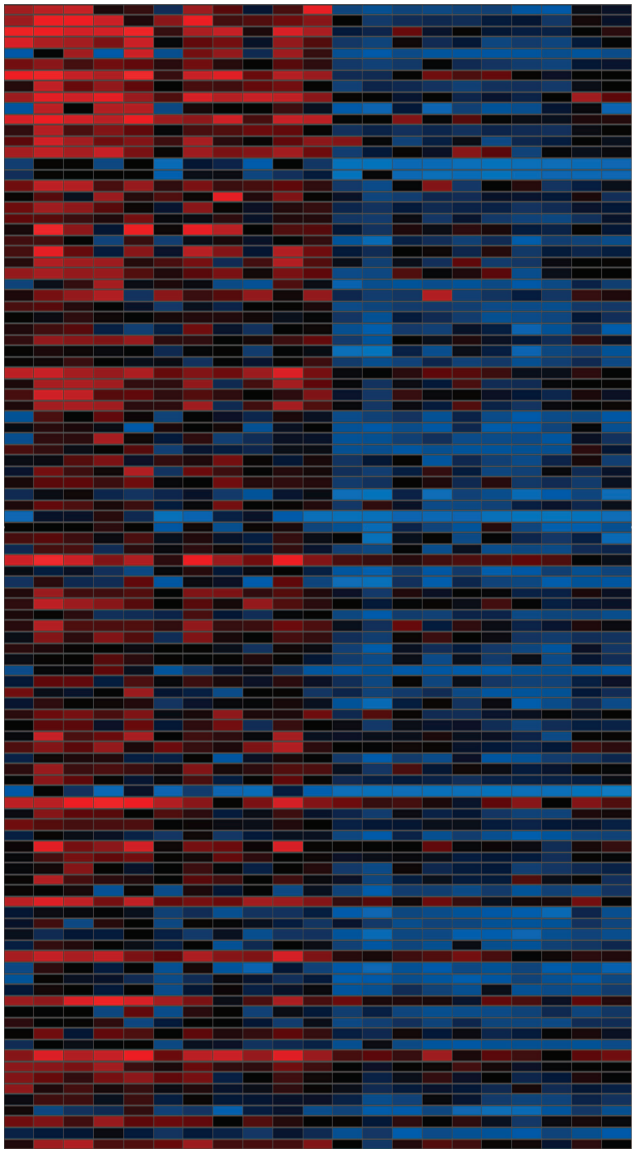
Table 2b. Minimal common regions PTL-NOS

Chromosome	Cytogenetic band	CNA	Clone start	Clone end	Position start	Position end	No. of Cases affected	Genes
7	7q36	Gain	RP4-548K24	RP4-548D19	149,838,701	151,558,264	6	FASTK*
7	7q21-7q22	Gain	RP4-550A13	RP5-1059M17	97,314,794	101,175,494	5	
8	8p12-8q12	Gain	RP11-44K6	RP11-114M5	38,488,992	61,676,214	5	FLAG1
8	8q21.1-8q21.3	Gain	RP11-34M16	RP11-120I21	82,095,683	87,610,633	5	
8	8q22-8q24.2	Gain	RP11-21E8	RP11-3O20b	101,431,725	130,562,466	5	MYC, EXT1
7	7q11.2	Gain	RP11-313P13	RP11-107L23	71,274,704	76,190,020	4	HIP1
7	7q32	Gain	RP11-128A6	RP5-999D10	127,266,861	129,658,047	4	SMO
8	8p23	Gain	GS-580-L5	RP11-104F14	0	2,817,096	4	
8	8p23-8p22	Gain	RP11-589N15	RP11-809L8	10,796,421	18,447,618	4	PCM1
9	9p13-9p12	Loss	RP11-211N8	RP11-211N8	40,774,565	46,771,375	4	
12	12q13	Gain	RP5-1057I20	RP11-302B13	46,299,979	48,258,682	4	
17	17p11.2	Gain	RP11-189D22	CTB-1187M2	17,521,462	19,614,077	4	USP6
17	17q21	Gain	RP5-1110E20	RP5-1169K15	35,920,940	41,095,565	4	BRCA1, ETV4,
17	17q21	Gain	RP11-220N20	RP11-506D12	41,565,972	46,429,569	4	COL1A1
17	17q25	Gain	RP11-478P5	RP11-313F15	69,639,765	77,988,928	4	MSF
1	1q32	Gain	RP11-383G10	RP11-534L20	202,933,682	205,087,972	3	SLC45A3
6	6p21.3	Gain	RP1-93N13	RP11-175A4	32,498,505	33,876,274	3	
8	8p23	Loss	RP3-461F17	RP11-177H2	2,304,174	11,802,558	3	
8	8p22-8p21	Loss	RP11-161I2	RP11-529P14	18,450,010	22,394,183	3	
9	9p21	Loss	RP11-149I2	RP11-149I2	21,158,465	22,479,496	3	CDKN2A-p14ARF, CDKN2A-p16(INK4a)
11	11p15	Gain	CTC-1277H1	CTC-1277H1	16,774,089	18,095,206	3	
11	11q13	Gain	RP11-126P21	RP5-901A4	66,035,373	68,072,319	3	
11	11q13	Gain	RP11-554A11	RP11-31L22	68,278,585	72,378,220	3	CCND1, NUMA1
11	11q23-11q25	Gain	RP11-35P15	RP11-215D10	116,368,601	120,790,891	3	MLL, CBL, ARHGEF12, PCSK7, BMPR1A, DDX6, PAFAH1B2
11	11q24-11q25	Gain	RP11-432I22	GS-770-G7	124,761,526	134,156,487	3	FLI1
12	12p13	Gain	GS-124-K20	RP11-264F23	0	5,163,995	3	CCND2
12	12q13	Gain	RP11-474N8	RP11-181L23	55,007,887	56,286,203	3	DDIT3
14	14q31-14q32	Gain	RP11-257P13	RP11-257P13	88,987,033	91,389,787	3	
16	16q12.1	Gain	RP11-305A7	RP11-147B17	47,397,787	49,618,230	3	CYLD
17	17q22_27	Gain	RP11-372K20	RP11-112J9	49,922,957	52,616,475	3	HLF
17	17q22-17q23	Gain	RP11-567L7	RP11-332H18	54,280,512	57,301,021	3	CLTC, NACA, BRIP1
17	17q23-17q24	Gain	RP11-156L14	RP11-89H15	58,038,015	61,192,768	3	
19	19p13.3-19p13.1	Gain	RP5-859H16	RP11-943H6	5,138,666	19,023,191	3	ELL, TPM4, BRD4, LYL1, MLLT1
19	19q12-19q13.4	Gain	CTC-325L16	GS-325-I23	36,979,850	63,806,651	3	ERCC2, BCL3, AKT2, CEBPA, CIC, TFPT, ZNF331

CNA, copy number alteration; MCR, minimal common region; PTL-NOS, peripheral T-cell lymphoma not otherwise specified.
Start and end positions are delineated by the nearby borders of adjacent clones. Genes are the cancer census genes situated in the subsequent MCRs. In addition, genes indicated with an asterisk are referred to in the Results and Discussion sections.

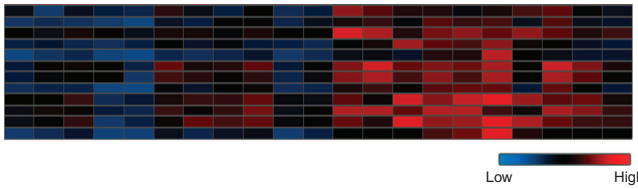
expressed in C-ALCL and 33 showed higher expression in PTL-NOS. A heatmap, indicating gene expression intensities across the samples, showing the 91 most differentially expressed genes with a ratio of geometric means (RGM) exceeding 2.5 is depicted in Figure 2a and b. To illustrate the expression of these 91 genes in benign $CD4^+$ T cells, we

a



RGM	Cluster	Gene name	% Gain C-ALCL	% Loss PTL-NOS
9.435	1	chemokine receptor 10	27	0
8.689	2,4	tumor necrosis factor receptor superfamily, member 8	45	0
7.994		ankyrin repeat domain 57	9	0
7.362		ankyrin repeat domain 57	9	0
6.689		G protein-coupled receptor 64	NA	NA
6.005		musculin (activated B-cell factor-1)	0	0
5.902	4,5	interferon regulatory factor 4	0	0
5.889		tropomodulin 1	9	20
5.844		Jun dimerization protein p21SNFT	22	0
5.386		nucleosomal binding protein 1	NA	NA
5.132		septin 10	9	0
4.98		tropomodulin 1	9	20
4.949		ring finger protein 157	18	0
4.866	1	chemokine receptor 7	36	0
4.69		phospholipase D family, member 5	22	0
4.679		interleukin 23 receptor	0	0
4.581		BIC transcript	18	0
4.267		histone 1, H1c	0	10
4.264	1	contactin associated protein 1	27	0
4.105		septin 10	9	0
4.034		fibulin 5	0	0
3.997		immunoglobulin heavy constant mu	9	0
3.976		deafness, autosomal dominant 5	18	0
3.965		hepatoma-derived growth factor, related protein 3	9	0
3.939		protein kinase (cAMP-dependent, catalytic) inhibitor alpha	0	0
3.906		adenylate cyclase 1	18	0
3.881		CDNA FLJ35091 fis, clone PLACE6005786	NA	NA
3.808		chromosome 4 open reading frame 24	9	0
3.797		WD repeat and FYVE domain containg 3	9	10
3.793		Coronin, actin binding protein, 2A	18	20
3.732	2	Junction-mediating and regulatory protein	0	0
3.702		Protein kinase (cAMP-dependent, catalytic) inhibitor alpha	0	0
3.542		chromosome 14 open reading frame 145	0	0
3.535	2	ring finger and FYVE -like domain containing 1	18	0
3.527		heptoma-derived growth factor, related protein 3	9	0
3.506	2	transmembrane protein 23	0	0
3.505		hepatoma-derived growth factor, related protein 3	9	0
3.483		PHD finger protein 16	0	0
3.356		kelch repeat and BTB (POZ) domain containing 11	0	20
3.296		wingless-type MMTV integration site family, member 5B	9	0
3.28		Transcribed locus	0	0
3.263		neuromedin U	9	0
3.258		similar to 2010300C02Rik protein	NA	NA
3.239		egf-like module containing, mucin-like, hormone receptor-like 1	9	0
3.236		Chromosome 8 open reading frame 70	0	0
3.218		protein tyrosine phosphatase, receptor type, N polypeptide 2	27	0
3.19		keratin 7	9	0
3.19		Transcribed locus	10	0
3.169		chromosome 13 open reading frame 32	0	20
3.164		chromosome 20 open reading frame 42	0	0
3.158		enhancer of zeste homolog 2	36	0
3.157		hepatic leukemia factor	18	0
3.147		Vasohibin-2	22	0
3.098		Clone DPDP-3 dental pulp-derived protein 3	18	0
3.086	2	TNF receptor-associated factor 1	9	0
3.046		coronin, actin binding protein, 2A	18	20
3.036		Yes-associated protein 1, 65kDa	0	20
3.032		neurocalcin delta	0	0
3.027		CDC14 cell division cycle 14 homolog B	10	20
3.023		echinoderm microtubule associated protein like 1	9	0
2.977		nucleolar protein 4	10	0
2.966		chromosome 20 open reading frame 42	0	0
2.951		solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	9	0
2.938		zinc finger CCCH-type containing 12D	0	20
2.892	2	Huntingin interacting protein 1	27	0
2.892		protein tyrosine phosphatase, receptor type, N polypeptide 2	27	0
2.881	2	phorbol-12-myristate-13-acetate-induced protein 1	9	0
2.859		elongation factor, RNA polymerase II, 2	9	0
2.813		hepatoma-derived growth factor, related protein 3	9	0
2.773		Yes-associated protein 1, 65kDa	0	20
2.758		elongation factor, RNA polymerase II, 2	0	10
2.758	6	branched chain aminotransferase 1m cytosolic	9	0
2.758		chromosome 3 open reading frame 15	9	0
2.758		similar to preproneuropeptide B	27	10
2.748		SMARCA2	9	20
2.747		Yes-associated protein 1, 65kDa	0	20
2.74	2	phorbol-12-myristate-13-acetate-induced protein 1	9	0
2.726		protein kinase C, delta binding protein	0	0
2.701		chromosome 8 open reading frame 70	0	0
2.7		ATP-binding cassette sub-family G (WHITE). member 2	9	20
2.7		cytidine monophosphate-N-acetylneuraminic acid hydroxylase	0	11
2.699		TRAF-interacting protein with a forkhead-associated domain	18	0
2.696	2	junction-mediating and regulatory protein	0	0
2.683		MRNA; cDNA DKFZp761L1121 (from clone DKFZp761L1121)	9	10
2.682		CNDA FLJ46701 fis, clone TRACH3014063	9	0
2.678		Transmembrane 6 superfamily member 1	9	0
2.671		SMARCA2	9	20
2.668		solute carrier family 35, member F3	18	0
2.652		Transmembrane protein 56	0	0
2.643	1	chemokine receptor 8	0	0
2.618	6	branched chain aminotransferase 1, cytosolic	9	0
2.607		xylosyltransferase I	0	0
2.594		F-box protein 17	9	0
2.582		sorting nexin 7	0	0
2.581		interleukin 23 receptor	0	0
2.58		SEC11-like 3	9	0
2.577		SMARCA2	9	20
2.574		inositol 1,4,5-triphosphate receptor, type 1	0	0
2.542		LanC lantibiotic synthetase component C-like 2	18	0
2.532	6	G protein-coupled receptor 132	9	0
2.527		solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	9	0
2.511		tight junction protein 2	9	10
2.503		Human immunodeficiency virus type I enhancer binding protein 3	0	0
2.5		EGFR-coamplified and overexpressed protein	18	0

b



RGM	Cluster	Gene name	% Loss C-ALCL	% Gain PTL-NOS
0.384	2,6	cyclin-dependent kinase inhibitor 2C (p18)	0	10
0.363		GTPase, IMAP family member 1	0	70
0.35		isocitrate dehydrogenase 2 (NADP+), mitochondrial	0	10
0.349		integrin, beta 2	9	20
0.332	3,4,5	protein kinase C, theta	9	20
0.331		GTPase, IMAP family member 6	0	50
0.329		GTPase, IMAP family member 6	0	50
0.328		GTPase, IMAP family member 1	0	70
0.326		FYN binding protein	0	10
0.313		GTPase, IMAP family member 4	0	50
0.303		FYN binding protein	0	10
0.272	3,4,5	protein kinase C, theta	9	20

c

Cluster	Name	Enrichment score	*P*-value
1	C–C chemokine receptor activity	1.79	7.91E–05
2	Apoptosis	1.65	0.011865
3	Positive regulation of lymphocyte proliferation	1.55	0.011155
4	Positive regulation of cytokine biosynthetic process	1.38	0.017689
5	Lymphocyte activation	1.1	0.057936
6	G1/S transition of mitotic cell cycle	1.1	0.015366

made use of published transcriptome data acquired using the same microarray platform (Piccaluga *et al.*, 2007). A heatmap illustrating transcript abundance can be found in Figure S1. Expression of the majority of genes differentially expressed between C-ALCL and PTL-NOS is low in benign $CD4^+$ T cells. A complete list of the differentially expressed genes can be found in Table S3. Genes with the relatively highest expression in C-ALCL are *CCR10* and *TNFRSF8/CD30*. PTL-NOS showed higher expression of several genes including *Protein Kinase C theta* (*PRKCQ*), *Fyn binding protein*, and several *GIMAP* genes.

As CNAs contribute to oncogenesis by altering the expression of resident genes, we assessed the possible relationship between altered expression and the presence of gains and losses of specific chromosomal regions in these lymphomas. For the most significantly differentially expressed, the frequency of gain or loss of the corresponding chromosomal region in the two lymphomas was evaluated. As apparent in Figure 2, many genes relatively overexpressed in C-ALCL were located in a chromosomal region that was frequently affected by gain in C-ALCL (*TNFRSF8/CD30*, *CCR7*, and *CCR10*), or less often in a region with loss in PTL-NOS (*TMOD1*). Conversely, the *GIMAP 1*, *4*, and *6* genes, relatively overexpressed in PTL-NOS, reside in chromosomal regions commonly showing gain in this malignancy. The number of samples per diagnostic group was insufficient to carry out a comprehensive integrative analysis by correlating expression of each individual gene to the presence of CNA of the corresponding chromosomal region.

Pathway analysis shows that C-ALCL and PTL-NOS differ in particular with respect to expression of genes involved in chemotaxis, apoptosis, and lymphocyte proliferation

Next, to interpret the comparative gene expression data and to extract information regarding biological processes and signaling pathways that may distinguish these lymphomas, we carried out knowledge-based pathway analysis. Gene Ontology (GO) analysis of the most differentially expressed genes showed significant enrichment of six GO clusters; these are depicted in Figure 2c with their respective *P*-values. Genes belonging to these enriched clusters are marked in Figure 2a and b. The lymphoma types, C-ALCL and PTL-NOS in particular, differed in expression of gene clusters with a role in chemokine receptor activity (*CCR10*, *CCR7*, *CNTNAP1*, *CCR8*), apoptosis (*TNFRSF8/CD30*, *JMY*, *RFFL*, *TMEM23/SGMS1*, *TRAF1*, *HIP1*, *PMAIP1*, *CDKN2C/p18*) and lymphocyte proliferation (*PRKCQ*). This result is consistent with the observed difference in extracutaneous dissemination and treatment resistance of these CTCLs.

Quantitative real-time PCR and immunohistochemistry confirm gene expression results

To validate the results of gene expression analysis, transcript abundance of selected genes was measured using quantitative real-time PCR (qPCR) in the C-ALCL and PTL-NOS tumor samples, as well as in $CD4^+$ T cells from healthy individuals used as benign reference (Figure 3). Genes with a role in chemotaxis (*CCR10*, *CCR7*), apoptosis (*TNFRSF8/CD30*, *TRAF1*), T-cell activation, and proliferation (*IRF4/MUM1*, *PRKCQ*) were analyzed. In addition, expression levels of the cytokine receptor gene *IL23R* and of the oncogenic Polycomb gene *EZH2* were quantified. qPCR analysis confirmed significant differential expression found in microarray-based gene expression analysis of all tested genes. Expression levels differed between C-ALCL and PTL-NOS with fold changes ranging from 3.8 (*TRAF1*) to 131.5 (*CCR7*). The genes encoding the chemokine receptors CCR10 and CCR7 appeared to be selectively expressed in tumors of C-ALCL patients. Moreover, the expression of *IRF4/MUM1*, a transcription factor that regulates T-cell apoptosis, was markedly higher in C-ALCL than in PTL-NOS or $CD4^+$ T cells. Expression of *TRAF1*, encoding a protein that relays signals from *TNFRSF8/CD30*, was significantly higher in C-ALCL than in PTL-NOS. Conversely, transcript levels of *PRKCQ* were higher in PTL-NOS than in C-ALCL and $CD4^+$ T cells (fold change of 10.9 and 4.6, respectively). The *PRKCQ* gene is exclusively expressed by T cells and functions as a downstream target of the T-cell receptor, relaying signals required for activation and survival after stimulation by antigen (Bertolotto *et al.*, 2000; Villalba *et al.*, 2001; Hayashi and Altman, 2007). Abundant expression of *PRKCQ* has been noted previously in T-cell leukemias and lymphomas (Villalba and Altman, 2002). For these reasons, we considered it a prime candidate oncogene in PTL-NOS. As oncogenes, in particular kinases, can be activated through activating mutations in addition to overexpression, we carried out analysis of the coding region of *PRKCQ* for mutations potentially resulting in constitutive activity of its kinase domain. This failed to show any mutations in the included PTL-NOS tumor samples (data not shown).

In addition, the expression of *IRF4/MUM1* and *TRAF1* genes was investigated on the protein level using immunohistochemistry. Stainings were carried out on samples included in this study for genomic profiling as well as on additional C-ALCL cases not included in this study. Staining of more than 50% of the neoplastic T cells for *IRF4/MUM1* and *TRAF1* genes was observed in 31 of 31 (100%) and 26 of 31 (84%) cases of C-ALCL, but not in any of the 15 PTL-NOS cases. Results of exemplary stainings are shown in Figure 4.

Figure 2. Comparative analysis of gene expression profiles of 11 C-ALCL and 10 PTL-NOS tumor samples. Heatmap depicting gene expression intensities of significantly differentially expressed genes (SAM algorithm, false discovery rate 0.1). Values are visualized according to the scale bar that represents the difference in expression relative to the mean expression. Red represents high, black represents intermediate, and blue represent low expression. (**a**) Genes showing higher expression in C-ALCL compared with PTL-NOS with a ratio of geometric means (RGM) higher than 2.5. (**b**) Genes expressed at lower levels in C-ALCL than in PTL-NOS with a RGM <0.4. The last two columns show the percentage of C-ALCL and PTL-NOS cases affected by chromosomal alterations at the locus where each differentially expressed gene resides. (**c**) Results of Gene Ontology analysis, showing gene clusters involved in the biological processes for which significant enrichment was discovered. Genes belonging to these clusters are indicated in a separate column ''cluster'' in panels a and b; the numbers refer to the clusters designated in panel c.

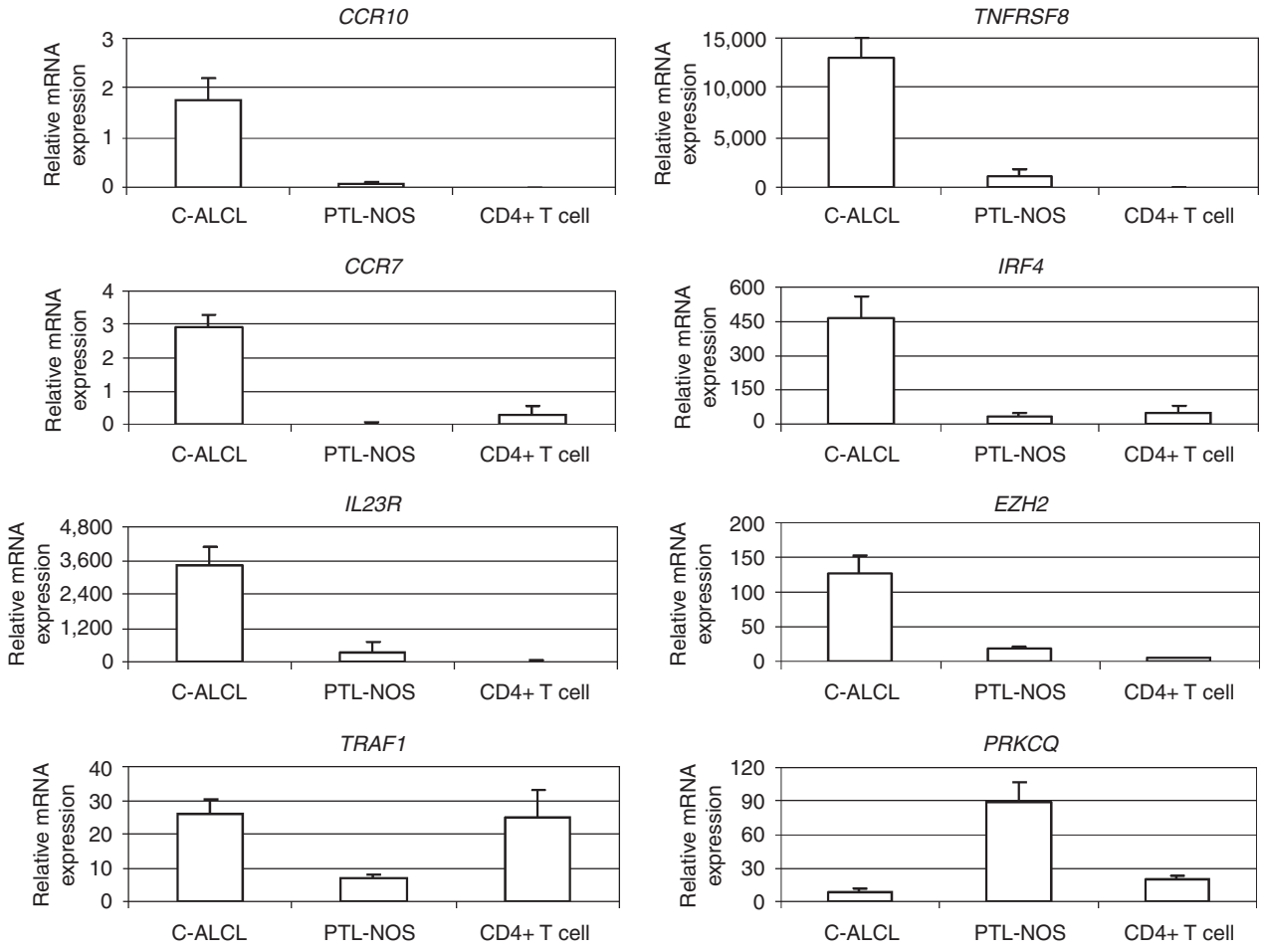


Figure 3. Expression levels of selected genes as measured by qPCR. Cumulative mRNA expression data of C-ALCL ($n=11$) and PTL-NOS ($n=10$) (mean ± SEM). The mRNA expression was measured relative to *RPS11* and *U1A* used as reference genes. Fold change for transcript levels of genes with increased expression in C-ALCL compared with PTL-NOS: *TNFRSF8*, 12.9; *IL23R*, 9.7; *EZH2*, 6.5; *IRF4*, 13.0; *CCR10*, 25.0; *CCR7*, 131.5; and *TRAF1*, 3.8. The fold change for the level of *PRKCQ* expression, overexpressed in PTL-NOS compared with C-ALCL, was 10.9.

In a report on TRAF1 expression, Assaf *et al.* (2007) described strong TRAF1 expression in only 1 of 28 (4%) C-ALCL cases. Kempf *et al.* (2008) have described *IRF4/MUM1* expression in only 2 of 10 cases of C-ALCL. Consistent with our results, more recent immunohistochemical studies have shown *IRF4/MUM1* and *TRAF1* expression in 80–100% of C-ALCL cases (Wasco *et al.*, 2008; Benner *et al.*, 2009; Feldman *et al.*, 2009).

DISCUSSION

C-ALCL and primary cutaneous PTL-NOS are two distinct types of CTCL that are both derived from skin-homing T cells, but show marked differences in clinical behavior and prognosis. Array-based CGH analysis allowed identification of recurrent chromosomal alterations harboring candidate oncogenes and tumor suppressor genes in these lymphoma types. Although both C-ALCL and PTL-NOS showed gains of large regions on chromosome 7 and 17, C-ALCL was distinguished by additional losses on chromosome 6 and 13 and PTL-NOS by gains on chromosome 8 and losses on chromosome 9. Detailed analysis of CNAs in C-ALCL showed that the most highly recurrent MCR with gain was located on 7q31 and loss on 6q16-6q21 and 13q34, each affecting 45% of the patients. The focal MCR with gain on 7q31 harbors the *MET* oncogene. *MET* amplification has previously been shown to result in its increased expression in nodal ALCL (Gogusev *et al.*, 2002) and deregulated expression has been noted in adult T-cell leukemia (Pons *et al.*, 1998; Choi *et al.*, 2007). The MCR with loss on 6q16-6q21 contains the *PRDM1/BLIMP-1* gene encoding a transcription factor that is implicated in T-cell homeostasis and differentiation. In mice lacking PRDM1/Blimp-1 activity, accumulation of $CD4^+$ T cells is observed (Kallies *et al.*, 2006; Martins *et al.*, 2006). Other cancer-associated genes located in regions with recurrent CNA with potential relevance in the pathogenesis of C-ALCL include *FOXO1A* and *BRCA2* on 13q12-13q14 (loss in 36%), *PRDM16/MEL1* on 1p36 (gain in 27%), and *TP53* on 17p13 (loss in 27% of patients). We have identified previously unreported recurrent chromosomal alterations in C-ALCL and confirmed several gains and losses found in the few studies in which conventional CGH was applied. Consistent with our results, Zettl *et al.* (2004)

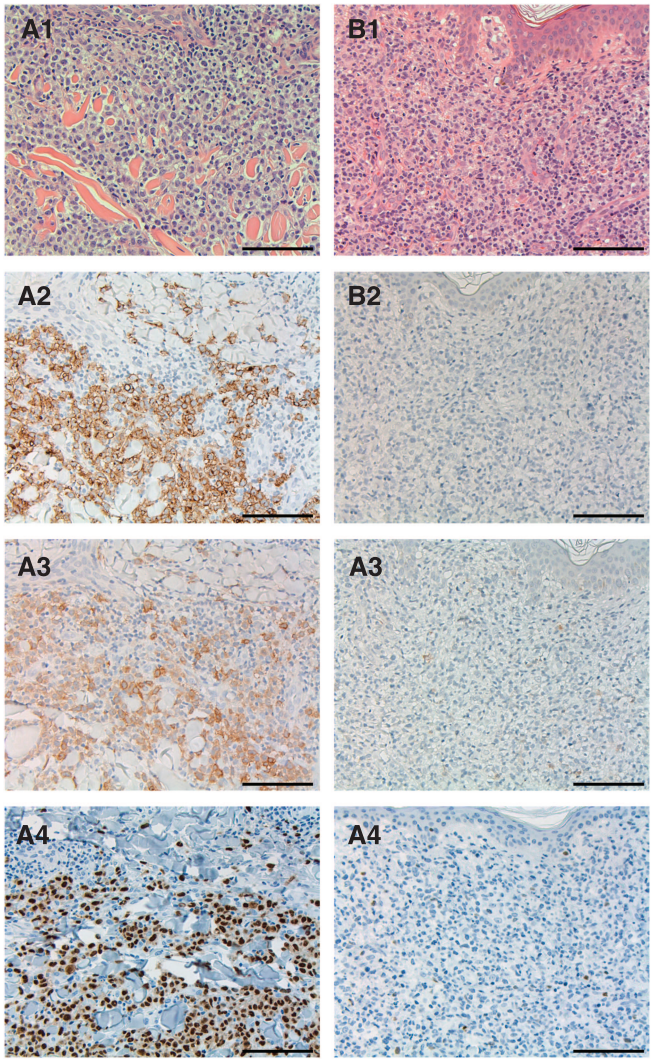


Figure 4. Immunohistochemical staining of TNFRSF8/CD30, IRF4/MUM1, and TRAF1 in C-ALCL and PTL-NOS. HE stainings showing the morphology of C-ALCL (A1) and PTL-NOS (B1) tumor cells. The tumor cells of C-ALCL strongly express TNFRSF8/CD30 (A2), TRAF1 (A3), and IRF4/MUM1 (A4). PTL-NOS tumor cells do not or only scarcely show positive staining for TNFRSF8/CD30 (B2), TRAF1 (B3), and IRF4/MUM1 (B4). Bar = 100 μm.

observed gain of regions on 7q and 6p in 2 of 11 C-ALCL samples. Mao *et al.* (2003) described gains of 1p, 5, 6, 7, 8p, and 19, showing partial overlap with our findings. In a group of seven patients with relapsing C-ALCL, Prochazkova *et al.* (2003) observed recurrent gain of regions on chromosome 9 and losses on chromosome 6 and 18. Furthermore in nodal ALCL, recurrent CNAs affecting 7q and 13q have been described (Zettl *et al.*, 2004; Salaverria *et al.*, 2008).

In PTL-NOS, the most frequently affected MCR with gain was 7q36. This focal region contains the anti-apoptotic *FASTK* gene that is overexpressed in association with chromosomal gain in mycosis fungoides (Simarro *et al.*, 2007; van Doorn *et al.*, 2009). Similar to primary cutaneous PTL-NOS studied herein, tumor cells of PTL-NOS presenting in lymph nodes have been reported to be affected by recurrent gains on chromosome 7q22-qter (Zettl *et al.*, 2004), 17q11-q25, and 8q (Thorns *et al.*, 2007). A major difference between the chromosomal alterations observed in PTL-NOS and C-ALCL concerns the occurrence of gains on chromosome 8. These were highly recurrent in PTL-NOS, but did not or scarcely affect patients with C-ALCL. A MCR with gain on 8q22-8q24.2 affecting 50% of PTL-NOS patients contains the *MYC* oncogene that is amplified in patients with Sézary syndrome and aggressive B-cell lymphomas (Mossafa *et al.*, 2006; Vermeer *et al.*, 2008). Gain of chromosome 8q was previously recognized in aggressive CTCLs with shorter survival (Karenko *et al.*, 2003; Fischer *et al.*, 2004). Furthermore, we found that the 9p21 locus was affected by loss in 50% of the patients with PTL-NOS, whereas it was not deleted in any of the included C-ALCL patients. The 9p21 region contains the *CDKN2A* tumor suppressor gene, loss of which is associated with an unfavorable prognosis in patients with nodal and cutaneous lymphomas (Pinyol *et al.*, 1998). Our results contrast with those of Boni *et al.* (2000), who detected loss of 9p21 in a subset of patients with C-ALCL.

Second, gene expression analysis showed marked differences in expression patterns of gene sets in C-ALCL and PTL-NOS. In an effort to unravel these patterns of differentially expressed genes and search for biological functions and signaling pathways distinct in these lymphomas, we applied unbiased GO analysis. This showed significant enrichment for gene clusters implicated in chemokine receptor activity, apoptosis, lymphocyte proliferation, and several other biological processes. These observed differences may be associated with the differential clinical behavior of C-ALCL and PTL-NOS.

Chemokine receptors determine homing patterns of T cells and serve to mark specific T-cell subsets. C-ALCL tumor biopsies showed higher expression of the chemokine receptor genes *CCR10*, *CCR8*, and *CCR7* relative to PTL-NOS. Interestingly, the genes encoding CCR10 and CCR7 are located in chromosomal regions that are frequently affected by copy number gain in C-ALCL. CCR10 marks a subset of memory T cells with skin-homing capacity (Soler *et al.*, 2003). Binding of its ligand CCL27, which is selectively produced in the skin, recruits T cells to the cutaneous microenvironment (Homey *et al.*, 2002). CCR10 expression has been described previously in mycosis fungoides and Sézary syndrome, but not specifically in C-ALCL or PTL-NOS (Notohamiprodjo *et al.*, 2005; Fujita *et al.*, 2006; Capriotti *et al.*, 2007). Moreover, CCR8 is preferentially expressed by T cells resident in the skin (Schaerli *et al.*, 2004). The higher tendency of PTL-NOS lymphoid cells to display extracutaneous dissemination may result from lower affinity of these cells for the cutaneous microenvironment because of low expression of the skin-homing receptors *CCR10* and *CCR8*.

Unbiased comparative analysis of the gene expression programs of C-ALCL and PTL-NOS tumors showed differential expression of a gene cluster involved in apoptosis. Defective apoptosis signaling is presumed to have

an important role in the pathogenesis of C-ALCL and PTL-NOS, as the homeostasis of mature T cells from which these lymphomas are derived is predominantly governed by selective induction of cell death (Kikuchi and Nishikawa, 1997). Both C-ALCL and PTL-NOS lymphoid cells are assumed to show apoptosis impairment compared with benign $CD4^+$ T cells from which these lymphomas are derived. The occurrence of spontaneous tumor regression in a subset of patients with C-ALCL and the higher sensitivity of C-ALCL to therapy suggest that apoptosis impairment in this lymphoma type is less pronounced than in PTL-NOS. Accordingly, C-ALCL and PTL-NOS showed altered expression of different sets of apoptosis-regulatory genes. C-ALCL showed relative high expression of *IRF4/MUM1*, *TNFRSF8/CD30*, and *TRAF1* and diminished expression of *CDKN2C/p18*. qPCR analysis and immunohistochemistry confirmed increased expression of *IRF4/MUM1* and *TRAF1* by C-ALCL lymphoid cells, also relative to normal $CD4^+$ T cells. IRF4/MUM1 protects $CD4^+$ T cells against pro-apoptotic stimuli and activation-induced cell death (Lohoff *et al.*, 2004). Moreover, TRAF1, involved in the intracellular signal transduction of CD30 and other tumor necrosis factor receptors, is believed to protect T cells from apoptosis induced by various stimuli (Durkop *et al.*, 2003). Therefore, aberrant expression of IRF4/MUM1 and TRAF1 may result in increased resistance to apoptosis in C-ALCL lymphoid cells. On the contrary, PTL-NOS was characterized by high expression of *PRKCQ* and diminished expression of *FAS* and *Caspase 10*. The *PRKCQ* gene relays signals required for T-cell activation and survival after stimulation by antigen (Bertolotto *et al.*, 2000; Villalba *et al.*, 2001; Hayashi and Altman, 2007) It has been suggested that *PRKCQ* may have oncogenic activity in T-cell malignancies and may serve as a therapeutic target using Protein kinase C inhibitors (Zhou *et al.*, 1999; Villalba *et al.*, 2001; Villalba and Altman, 2002). The *PRKCQ* gene was overexpressed relative to C-ALCL as well as to normal $CD4^+$ T cells. Additional mutational analysis failed to detect activating mutations in these PTL-NOS tumor samples. Loss of expression of the FAS receptor is another mechanism through which PTL-NOS tumor cells can acquire resistance to pro-apoptotic stimuli. The *FAS* mRNA expression data from this study are consistent with previous studies showing that protein expression of the FAS receptor is lost in the majority of PTL-NOS tumor cells, whereas it is expressed in C-ALCL (Zoi-Toli *et al.*, 2000).

Taken together, C-ALCL and PTL-NOS have distinct patterns of chromosomal abnormalities, which may in part explain their different clinical behavior. We were able to identify several candidate oncogenes and tumor suppressor genes residing in MCRs with highly recurrent gains and losses in these T-cell lymphomas. The more aggressive clinical behavior of PTL-NOS may, in part, be related to chromosomal gains of regions on chromosome 8 and losses affecting 9p21, as decreased p16 and $p14^{ARF}$ expression resulting from such loss has been found to predict poor prognosis in various lymphomas (Dijkman *et al.*, 2006). Importantly, the distinct clinical behavior of these CTCLs is paralleled by differences in their gene expression programs linked to T-cell homing, apoptosis, and proliferation. Lymphoid cells of patients with C-ALCL show higher expression of skin-homing receptors, which may explain their higher affinity for the skin and lower tendency to disseminate into extracutaneous sites than PTL-NOS tumor cells. Furthermore, C-ALCL and PTL-NOS are characterized by dysregulated expression of different sets of apoptosis-regulating genes, which may account for differences in treatment resistance and tendency to progress.

MATERIALS AND METHODS

Selection of patients

Pretreatment biopsies of 11 patients with C-ALCL and 10 patients with primary cutaneous PTL-NOS were selected for this study (Table 1). In all 21 cases, the biopsies contained more than 75% malignant T cells. The immunophenotypical characteristics of the 21 cases are presented in Table S1. All patients had been retrieved from the database of the Dutch Cutaneous Lymphoma Group, reviewed by an expert panel of dermatologists and hematopathologists before entry in this database and classified using criteria of the WHO-EORTC classification for the primary cutaneous lymphomas (Willemze *et al.*, 2005). In all patients, routine staging procedures including physical examination, complete and differential blood cell counts and serum biochemical analysis, computed tomography scan of the neck, chest, and abdomen and a bone marrow biopsy had been negative. With respect to the group of primary cutaneous PTL-NOS, these cases did not meet the criteria of one of the rare subtypes of primary cutaneous PTL-NOS (aggressive epidermotropic $CD8^+$ CTCL; cutaneous gamma-delta T-cell lymphoma; $CD4^+$ small/medium pleomorphic CTCL), and none of them had a history of or concurrent patches and plaques suggesting a diagnosis of mycosis fungoides. Of 10 patients with PTL-NOS, 9 presented with generalized ulcerating tumors. CD30 staining was either completely negative (eight cases) or showed expression by a minor proportion ($<25\%$) of neoplastic T cells (cases 20 and 21). Approval for these studies was obtained from the institutional review board of the Leiden University Medical Center. Informed consent was provided according to the Declaration of Helsinki Principles.

Extraction of RNA and DNA

In all cases, DNA and RNA were isolated from the same frozen tumor biopsy sample for array-based CGH and microarray-based gene expression analysis, qPCR, and mutation analysis. RNA was extracted from $25 \times 50\,\mu m$ frozen sections using the RNeasy Kit (Qiagen, Hilden, Germany), yielding 25–60 μg total RNA. DNA was isolated from 25 frozen sections (20 μm) using the Genomic-tip 20/G kit (Qiagen), yielding 10–60 μg genomic DNA.

Array-based CGH analysis

Genome-wide analysis of CNAs was carried out using array-based CGH containing ~3,500 bacterial artificial chromosomes (BACs) produced at the Leiden University Medical Center. The particular BAC set used to produce the arrays was distributed by the Wellcome Trust Sanger Institute (Hinxton, UK) and contains large insert clones spaced at ~1 Mb density over the full genome, a set of subtelomeric sequences for each chromosome arm, and a few hundred probes selected for their involvement in oncogenesis (Knight *et al.*, 2000).

Fabrication and validation of the array, hybridization methods, and analytical procedures have been described elsewhere in detail (Knijnenburg *et al.*, 2005). Data were analyzed using CAPweb (Institut Curie, Paris, France) and visualized using VAMP (Institut Curie, Paris, France) (La Rosa *et al.*, 2006). Copy number was classified as normal, copy number gain ($\log^2$ ratio >0.25), or genomic loss ($\log^2$ ratio < -0.25). Identified CNA of regions with copy number variations described in the Database of Genomic Variants (http://projects.tcag.ca/variation) were excluded from analysis.

Gene expression profiling

Samples and microarrays (Human Genome U133plus2.0 array, Affymetrix, Santa Clara, CA), interrogating over 47,000 human transcripts and variants, were processed according to the manufacturer's protocol as described previously (Dijkman *et al.*, 2007). The microarray images were quantified using the Genechip operating system (GCOS; v1.2 software) (Affymetrix). The 260 of 280 ratios of isolated RNA were >1.8 for all samples, as measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE), confirming RNA purity. RNA integrity was determined by gel electrophoresis, which showed two ribosomal RNA bands with the 28S rRNA band having a higher intensity than the 18S rRNA band in all cases. In addition, we used the internal controls present on the Affymetrix arrays allowing monitoring of RNA quality after hybridization. The 3′/5′ GAPDH and 3′/5′ β-actin values were within the limits recommended by Affymetrix (maximally 1.25 and 3, respectively) for all samples.

The average fluorescence intensity was determined for each microarray and then the output of each experiment was globally scaled to a target value of 200. The profiles of normal $CD4^+$ T cells were obtained from the GEO database (accession numbers GSM146182–GSM146186) previously published by Piccaluga *et al.* (2007). They obtained the $CD4^+$ T cells by positive selection using magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Normalization and variance stabilization was carried out using VSN in the R statistical software package (Huber *et al.*, 2002).

Data analysis

BAC clone and oligonucleotide probe positions were established based on Ensembl (Ensembl is a joint project between European Bioinformatics Institute (EBI), an outstation of the European Molecular Biology Laboratory (EMBL), and the Wellcome Trust Sanger Institute (WTSI), Hinxton, United Kingdom) release 44 (April 2007). Recurrent MCRs with CNA affecting at least 35% of analyzed samples were computed in CAPweb using the algorithm proposed by Rouveirol *et al.* (2006). MCRs consisting of only subtelomeric clones were not taken into consideration. The nearby borders of adjacent clones were chosen to delineate MCRs. Candidate genes with pathobiological relevance were selected by focusing on genes listed as oncogene or tumor suppressor gene in the Cancer Gene Census list (November 2008) (Futreal *et al.*, 2004). Comparative analysis of the gene expression patterns of C-ALCL and PTL-NOS was carried out using BRB-ArrayTools v3.5.0 (http://linus.nci.nih.gov/BRB-ArrayTools.html) using the significance analysis of microarray algorithm, with a false discovery rate of 0.1, performing 100 permutations. The DAVID (National Cancer Institute, Frederick, MD) bioinformatics database was used for GO enrichment analysis (Dennis *et al.*, 2003). Genes most significantly differentially expressed with a RGM higher than 2.5 or lower than 0.4 were analyzed with highest stringency for enriched GO clusters. By means of the gene location, the associated BAC clone was determined, for genes not located in the region of a BAC clone the most proximate clone spotted on the array was taken. For these BAC clones, the percentage of CNAs was calculated.

qPCR

cDNA synthesis was performed on 1 μg total RNA, after treatment with RQ1 DNase I (Promega, Madison, WI), using IScript reverse transcriptase (Bio-Rad, Veenendaal, the Netherlands), oligo$(dT)_{12-18}$, and random hexamer priming (Bio-Rad) in a final volume of 20 μl. qPCR was performed using the MyIQ Detection System and the SYBR Green Supermix (Bio-Rad). The cycle parameters for transcripts of interest and for the reference genes *U1A* and *RPS11* used for normalization were as follows: denaturing for 15 seconds at 97 °C; annealing and extension for 20 seconds at 60 °C, for 40 cycles. Primer sequences (Invitrogen, Breda, the Netherlands) are given in Table S2. Data were evaluated using MyIQ software (Bio-Rad) and the second derivative maximum algorithm, whereas confirmation of the specificity of the PCR product and standard curves were performed as previously described (Dijkman *et al.*, 2007). Freshly isolated $CD4^+$ T cells of four healthy donors were used as controls for qPCR experiments.

Mutation analysis

Mutation analysis of the coding region of the *PRKCQ* gene was carried out on cDNA from eight biopsy samples of patients with PTL-NOS. Primer sequences are listed in Table S2. The PCR fragments were purified and directly subjected to sequence reactions. The software Mutation Explorer (SoftGenetics, LLC, State College, PA) was applied for analyzing sequences and comparing with reference sequences from the NCBI database (National Center for Biotechnology Information, Bethesda, MD).

Immunohistochemistry

Immunohistochemical analysis of the protein expression of TNFRSF8/CD30, TRAF1, and IRF4/MUM1 was carried out on the 21 C-ALCL and PTL-NOS samples selected for genomic analyses. In addition, paraffin-embedded sections of an independent set consisting of 20 C-ALCL and 5 PTL-NOS samples were subjected to immunohistochemical staining. Immunostaining was performed with antibodies against TNFRSF8/CD30 (DAKO, Glostrup, Denmark), TRAF1, and IRF4/MUM1 using a standard three-step streptavidin–biotin–peroxidase-based technique after antigen retrieval with microwave heating, as described previously (Hoefnagel *et al.*, 2006). The antibodies against IRF4/MUM1 and TRAF1 were kindly provided by Professor Dr G Cattoretti (Institute of Cancer Genetics, Columbia University, New York, USA) and Professor Dr. H. Dürkop (Institute of Pathology, Charité, Berlin, Germany), respectively.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Assaf C, Hirsch B, Wagner F, Lucka L, Grunbaum M, Gellrich S *et al.* (2007) Differential expression of TRAF1 aids in the distinction of cutaneous CD30-positive lymphoproliferations. *J Invest Dermatol* 127:1898–904

Bekkenk MW, Geelen FA, van Voorst V, Heule F, Geerts ML, van Vloten WA *et al.* (2000) Primary and secondary cutaneous CD30(+) lymphoproliferative disorders: a report from the Dutch Cutaneous Lymphoma Group on the long-term follow-up data of 219 patients and guidelines for diagnosis and treatment. *Blood* 95:3653–61

Bekkenk MW, Vermeer MH, Jansen PM, van Marion AM, Canninga-van Dijk MR, Kluin PM *et al.* (2003) Peripheral T-cell lymphomas unspecified presenting in the skin: analysis of prognostic factors in a group of 82 patients. *Blood* 102:2213–9

Beljaards RC, Meijer CJ, Van der Putte SC, Hollema H, Geerts ML, Bezemer PD *et al.* (1994) Primary cutaneous T-cell lymphoma: clinicopathological features and prognostic parameters of 35 cases other than mycosis fungoides and CD30-positive large cell lymphoma. *J Pathol* 172:53–60

Benner MF, Jansen PM, Meijer CJLM, Willemze R (2009) Diagnostic and prognostic evaluation of phenotypic markers TRAF1, MUM1, bcl-2 and CD15 in cutaneous CD30-positive lymphoproliferations. *Br J Dermatol* 161:121–7

Bertolotto C, Maulon L, Filippa N, Baier G, Auberger P (2000) Protein kinase C theta and epsilon promote T-cell survival by a rsk-dependent phosphorylation and inactivation of BAD. *J Biol Chem* 275:37246–50

Boni R, Xin H, Kamarashev J, Utzinger E, Dummer R, Kempf W *et al.* (2000) Allelic deletion at 9p21-22 in primary cutaneous CD30(+) large cell lymphoma. *J Invest Dermatol* 115:1104–7

Capriotti E, Vonderheid EC, Thoburn CJ, Bright EC, Hess AD (2007) Chemokine receptor expression by leukemic T cells of cutaneous T-cell lymphoma: clinical and histopathological correlations. *J Invest Dermatol* 127:2882–92

Choi YL, Tsukasaki K, O'Neill MC, Yamada Y, Onimaru Y, Matsumoto K *et al.* (2007) A genomic analysis of adult T-cell leukemia. *Oncogene* 26: 1245–55

DeCoteau JF, Butmarc JR, Kinney MC, Kadin ME (1996) The t(2;5) chromosomal translocation is not a common feature of primary cutaneous CD30$^+$ lymphoproliferative disorders: comparison with anaplastic large-cell lymphoma of nodal origin. *Blood* 87:3437–41

Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC *et al.* (2003) DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol* 4:3

Dijkman R, Tensen CP, Jordanova ES, Knijnenburg J, Hoefnagel JJ, Mulder AA *et al.* (2006) Array-based comparative genomic hybridization analysis reveals recurrent chromosomal alterations and prognostic parameters in primary cutaneous large B-cell lymphoma. *J Clin Oncol* 24:296–305

Dijkman R, van Doorn R, Szuhai K, Willemze R, Vermeer MH, Tensen CP (2007) Gene-expression profiling and array-based CGH classify CD4+CD56+ hematodermic neoplasm and cutaneous myelomonocytic leukemia as distinct disease entities. *Blood* 109:1720–7

Durkop H, Hirsch B, Hahn C, Foss HD, Stein H (2003) Differential expression and function of A20 and TRAF1 in Hodgkin lymphoma and anaplastic large cell lymphoma and their induction by CD30 stimulation. *J Pathol* 200:229–39

Feldman AL, Law M, Remstein ED, Macon WR, Erickson LA, Grogg KL *et al.* (2009) Recurrent translocations involving the IRF4 oncogene locus in peripheral T-cell lymphomas. *Leukemia* 23:574–80

Fischer TC, Gellrich S, Muche JM, Sherev T, Audring H, Neitzel H *et al.* (2004) Genomic aberrations and survival in cutaneous T cell lymphomas. *J Invest Dermatol* 122:579–86

Fujita Y, Abe R, Sasaki M, Honda A, Furuichi M, Asano Y *et al.* (2006) Presence of circulating CCR10+ T cells and elevated serum CTACK/CCL27 in the early stage of mycosis fungoides. *Clin Cancer Res* 12:2670–5

Futreal PA, Coin L, Marshall M, Down T, Hubbard T, Wooster R *et al.* (2004) A census of human cancer genes. *Nat Rev Cancer* 4:177–83

Gogusev J, Telvi L, Nezelof C (2002) Molecular cytogenetic aberrations in CD30$^+$ anaplastic large cell lymphoma cell lines. *Cancer Genet Cytogenet* 138:95–101

Grange F, Hedelin G, Joly P, Beylot-Barry M, D'Incan M, Delaunay M *et al.* (1999) Prognostic factors in primary cutaneous lymphomas other than mycosis fungoides and the Sezary syndrome. The French Study Group on Cutaneous Lymphomas. *Blood* 93:3637–42

Hayashi K, Altman A (2007) Protein kinase C theta (PKCtheta): a key player in T cell life and death. *Pharmacol Res* 55:537–44

Hoefnagel JJ, Mulder MM, Dreef E, Jansen PM, Pals ST, Meijer CJ *et al.* (2006) Expression of B-cell transcription factors in primary cutaneous B-cell lymphoma. *Mod Pathol* 19:1270–6

Homey B, Alenius H, Muller A, Soto H, Bowman EP, Yuan W *et al.* (2002) CCL27–CCR10 interactions regulate T cell-mediated skin inflammation. *Nat Med* 8:157–65

Horie R, Watanabe T, Morishita Y, Ito K, Ishida T, Kanegae Y *et al.* (2002) Ligand-independent signaling by overexpressed CD30 drives NF-kappaB activation in Hodgkin-Reed-Sternberg cells. *Oncogene* 21:2493–503

Huber W, von Heydebreck A, Sultmann H, Poustka A, Vingron M (2002) Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics* 18(Suppl 1): S96–104

Kallies A, Hawkins ED, Belz GT, Metcalf D, Hommel M, Corcoran LM *et al.* (2006) Transcriptional repressor Blimp-1 is essential for T cell homeostasis and self-tolerance. *Nat Immunol* 7:466–74

Karenko L, Sarna S, Kahkonen M, Ranki A (2003) Chromosomal abnormalities in relation to clinical disease in patients with cutaneous T-cell lymphoma: a 5-year follow-up study. *Br J Dermatol* 148:55–64

Kempf W, Kutzner H, Cozzio A, Sander CA, Pfaltz MC, Muller B *et al.* (2008) MUM1 expression in cutaneous CD30$^+$ lymphoproliferative disorders: a valuable tool for the distinction between lymphomatoid papulosis and primary cutaneous anaplastic large-cell lymphoma. *Br J Dermatol* 158:1280–7

Kikuchi A, Nishikawa T (1997) Apoptotic and proliferating cells in cutaneous lymphoproliferative diseases. *Arch Dermatol* 133:829–33

Knight SJ, Lese CM, Precht KS, Kuc J, Ning Y, Lucas S *et al.* (2000) An optimized set of human telomere clones for studying telomere integrity and architecture. *Am J Hum Genet* 67:320–32

Knijnenburg J, Szuhai K, Giltay J, Molenaar L, Sloos W, Poot M *et al.* (2005) Insights from genomic microarrays into structural chromosome rearrangements. *Am J Med Genet A* 132A:36–40

La Rosa P, Viara E, Hupe P, Pierron G, Liva S, Neuvial P *et al.* (2006) VAMP: visualization and analysis of array-CGH, transcriptome and other molecular profiles. *Bioinformatics* 22:2066–73

Liu HL, Hoppe RT, Kohler S, Harvell JD, Reddy S, Kim YH (2003) CD30$^+$ cutaneous lymphoproliferative disorders: the Stanford experience in lymphomatoid papulosis and primary cutaneous anaplastic large cell lymphoma. *J Am Acad Dermatol* 49:1049–58

Lohoff M, Mittrucker HW, Brustle A, Sommer F, Casper B, Huber M *et al.* (2004) Enhanced TCR-induced apoptosis in interferon regulatory factor 4-deficient CD4(+) Th cells. *J Exp Med* 200:247–53

Mao X, Orchard G, Lillington DM, Russell-Jones R, Young BD, Whittaker S (2003) Genetic alterations in primary cutaneous CD30$^+$ anaplastic large cell lymphoma. *Genes Chromosomes Cancer* 37:176–85

Martins GA, Cimmino L, Shapiro-Shelef M, Szabolcs M, Herron A, Magnusdottir E *et al.* (2006) Transcriptional repressor Blimp-1 regulates T cell homeostasis and function. *Nat Immunol* 7:457–65

Mori M, Manuelli C, Pimpinelli N, Mavilia C, Maggi E, Santucci M *et al.* (1999) CD30–CD30 ligand interaction in primary cutaneous CD30(+) T-cell lymphomas: a clue to the pathophysiology of clinical regression. *Blood* 94:3077–83

Mossafa H, Damotte D, Jenabian A, Delarue R, Vincenneau A, Amouroux I *et al.* (2006) Non-Hodgkin's lymphomas with Burkitt-like cells are associated with c-Myc amplification and poor prognosis. *Leuk Lymphoma* 47:1885–93

Notohamiprodjo M, Segerer S, Huss R, Hildebrandt B, Soler D, Djafarzadeh R *et al.* (2005) CCR10 is expressed in cutaneous T-cell lymphoma. *Int J Cancer* 115:641–7

Piccaluga PP, Agostinelli C, Califano A, Rossi M, Basso K, Zupo S *et al.* (2007) Gene expression analysis of peripheral T cell lymphoma, unspecified, reveals distinct profiles and new potential therapeutic targets. *J Clin Invest* 117:823–34

Pinyol M, Cobo F, Bea S, Jares P, Nayach I, Fernandez PL *et al.* (1998) p16(INK4a) gene inactivation by deletions, mutations, and hypermethylation is associated with transformed and aggressive variants of non-Hodgkin's lymphomas. *Blood* 91:2977–84

Pons E, Uphoff CC, Drexler HG (1998) Expression of hepatocyte growth factor and its receptor c-met in human leukemia-lymphoma cell lines. *Leuk Res* 22:797–804

Prochazkova M, Chevret E, Beylot-Barry M, Sobotka J, Vergier B, Delaunay M *et al.* (2003) Chromosomal imbalances: a hallmark of tumour relapse in primary cutaneous CD30+ T-cell lymphoma. *J Pathol* 201:421–9

Rouveirol C, Stransky N, Hupe P, Rosa PL, Viara E, Barillot E *et al.* (2006) Computation of recurrent minimal genomic alterations from array-CGH data. *Bioinformatics* 22:849–56

Salaverria I, Bea S, Lopez-Guillermo A, Lespinet V, Pinyol M, Burkhardt B *et al.* (2008) Genomic profiling reveals different genetic aberrations in systemic ALK-positive and ALK-negative anaplastic large cell lymphomas. *Br J Haematol* 140:516–26

Schaerli P, Ebert L, Willimann K, Blaser A, Roos RS, Loetscher P *et al.* (2004) A skin-selective homing mechanism for human immune surveillance T cells. *J Exp Med* 199:1265–75

Simarro M, Mauger D, Rhee K, Pujana MA, Kedersha NL, Yamasaki S *et al.* (2007) Fas-activated serine/threonine phosphoprotein (FAST) is a regulator of alternative splicing. *Proc Natl Acad Sci USA* 104:11370–5

Soler D, Humphreys TL, Spinola SM, Campbell JJ (2003) CCR4 versus CCR10 in human cutaneous TH lymphocyte trafficking. *Blood* 101:1677–82

Thorns C, Bastian B, Pinkel D, Roydasgupta R, Fridlyand J, Merz H *et al.* (2007) Chromosomal aberrations in angioimmunoblastic T-cell lymphoma and peripheral T-cell lymphoma unspecified: a matrix-based CGH approach. *Genes Chromosomes Cancer* 46:37–44

van Doorn R, van Kester MS, Dijkman R, Vermeer MH, Mulder AA, Szuhai K *et al.* (2009) Oncogenomic analysis of mycosis fungoides reveals major differences with Sezary syndrome. *Blood* 113:127–36

Vermeer MH, van Doorn R, Dijkman R, Mao X, Whittaker S, van Voorst V *et al.* (2008) Novel and highly recurrent chromosomal alterations in Sezary syndrome. *Cancer Res* 68:2689–98

Villalba M, Altman A (2002) Protein kinase C-theta (PKCtheta), a potential drug target for therapeutic intervention with human T cell leukemias. *Curr Cancer Drug Targets* 2:125–37

Villalba M, Bushway P, Altman A (2001) Protein kinase C-theta mediates a selective T cell survival signal via phosphorylation of BAD. *J Immunol* 166:5955–63

Wasco MJ, Fullen D, Su L, Ma L (2008) The expression of MUM1 in cutaneous T-cell lymphoproliferative disorders. *Hum Pathol* 39:557–63

Willemze R, Jaffe ES, Burg G, Cerroni L, Berti E, Swerdlow SH *et al.* (2005) WHO-EORTC classification for cutaneous lymphomas. *Blood* 105:3768–85

Yu JB, Blitzblau RC, Decker RH, Housman DM, Wilson LD (2008) Analysis of primary CD30+ cutaneous lymphoproliferative disease and survival from the Surveillance, Epidemiology, and End Results database. *J Clin Oncol* 26:1483–8

Zettl A, Rudiger T, Konrad MA, Chott A, Simonitsch-Klupp I, Sonnen R *et al.* (2004) Genomic profiling of peripheral T-cell lymphoma, unspecified, and anaplastic large T-cell lymphoma delineates novel recurrent chromosomal alterations. *Am J Pathol* 164:1837–48

Zhou T, Song L, Yang P, Wang Z, Lui D, Jope RS (1999) Bisindolylmaleimide VIII facilitates Fas-mediated apoptosis and inhibits T cell-mediated autoimmune diseases. *Nat Med* 5:42–8

Zoi-Toli O, Vermeer MH, De Vries E, Van Beek P, Meijer CJ, Willemze R (2000) Expression of Fas and Fas-ligand in primary cutaneous T-cell lymphoma (CTCL): association between lack of Fas expression and aggressive types of CTCL. *Br J Dermatol* 143:313–9