Clinicopathologic and genetic features of primary cutaneous B-cell lymphoma

Hoefnagel, J.J.

CHAPTER 6

Array-based comparative genomic hybridization analysis reveals recurrent chromosomal alterations and prognostic parameters in primary cutaneous large B-cell lymphoma

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Array-Based Comparative Genomic Hybridization Analysis Reveals Recurrent Chromosomal Alterations and Prognostic Parameters in Primary Cutaneous Large B-Cell Lymphoma

Remco Dijkman, Cornelis P. Tensen, Ekaterina S. Jordanova, Jeroen Knijnenburg, Juliette J. Hoefnagel, Aat A. Mulder, Carla Rosenberg, Anton K. Raap, Rein Willemze, Károly Szuhai, and Maarten H. Vermeer

From the Departments of Dermatology, Molecular Cell Biology, and Pathology, Leiden University Medical Center, Leiden, the Netherlands.

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Address reprint requests to C.P. Tensen, PhD, Department of Dermatology, Leiden University Medical Center, Sylvius Bldg, Wassenaarseweg 72, 2333 AL Leiden, the Netherlands; e-mail: C.P.Tensen@lumc.nl.

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ABSTRACT

Purpose
To evaluate the clinical relevance of genomic aberrations in primary cutaneous large B-cell lymphoma (PCLBCL).

Patients and Methods
Skin biopsy samples of 31 patients with a PCLBCL classified as either primary cutaneous follicle center lymphoma (PCFCL; n = 19) or PCLBCL, leg type (n = 12), according to the WHO–European Organisation for Research and Treatment of Cancer (EORTC) classification, were investigated using array-based comparative genomic hybridization, fluorescence in situ hybridization (FISH), and examination of promoter hypermethylation.

Results
The most recurrent alterations in PCFCL were high-level DNA amplifications at 2p16.1 (63%) and deletion of chromosome 14q32.33 (68%). FISH analysis confirmed *c-REL* amplification in patients with gains at 2p16.1. In PCLBCL, leg type, most prominent aberrations were a high-level DNA amplification of 18q21.31-q21.33 (67%), including the *BCL-2* and *MALT1* genes as confirmed by FISH, and deletions of a small region within 9p21.3 containing the *CDKN2A*, *CDKN2B*, and *NSG-x* genes. Homozygous deletion of 9p21.3 was detected in five of 12 patients with PCLBCL, leg type, but in zero of 19 patients with PCFCL. Complete methylation of the promoter region of the *CDKN2A* gene was demonstrated in one PCLBCL, leg type, patient with hemizygous deletion, in one patient without deletion, but in zero of 19 patients with PCFCL. Seven of seven PCLBCL, leg type, patients with deletion of 9p21.3 and/or complete methylation of *CDKN2A* died as a result of their lymphoma.

Conclusion
Our results demonstrate prominent differences in chromosomal alterations between PCFCL and PCLBCL, leg type, that support their classification as separate entities within the WHO-EORTC scheme. Inactivation of *CDKN2A* by either deletion or methylation of its promoter could be an important prognostic parameter for the group of PCLBCL, leg type.

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INTRODUCTION

In the WHO–European Organisation for Research and Treatment of Cancer (EORTC) classification, primary cutaneous B-cell lymphoma (CBCL) with the histology of a diffuse large B-cell lymphoma are classified as either primary cutaneous follicle center lymphoma (PCFCL) or primary cutaneous large B-cell lymphoma, leg type (PCLBCL, leg type).[1] PCFCL generally presents with skin lesions confined to a limited area on the head or the trunk, rarely disseminate to extracutaneous sites, and has an excellent prognosis (5-year survival > 95%).[1-4] Histologically, these PCFCLs represent a spectrum with variable proportions of (small) centrocytes and centroblasts and sometimes a follicular growth pattern in early lesions to diffuse infiltrates of generally large centrocytes (large cleaved cells) in tumorous lesions.[2,4-7] The neoplastic B-cells consistently express BCL-6, but generally not BCL-2 and MUM1/IRF4.[5,8-11]

In the EORTC classification, PCLBCL of the leg was included as a separate entity, histologically characterized by confluent sheets of centroblasts and immunoblasts and, in contrast to the group of PCFCL, strong expression of BCL-2 and MUM1 proteins.[3,9,10,12,13] Characteristically, these lymphomas present with skin tumors on the (lower) legs in elderly patients. As compared to the group of

PCFCLs, these lymphomas more frequently disseminate to extracutaneous sites and have a poorer prognosis (5-year survival ≈ 50%).[3,12,13] Recent studies suggested that cases with a similar morphology, phenotype, and prognosis may sometimes arise at sites other than the leg.[3] In the WHO-EORTC classification, the term PCLBCL, leg type, was therefore proposed for both lesions on the legs and similar lesions at other skin sites.[1]

Recent studies have started to evaluate the genetic mechanisms involved in the development and progression of these lymphomas. Gene expression profiling suggested that PCFCL and PCLBCL, leg type, have gene expression profiles similar to that of germinal center B-cell–like and activated B-cell–like diffuse large B-cell lymphoma, respectively, and revealed MUM1 expression as a valuable diagnostic marker for the latter group.[10] Previous studies using classical comparative genomic hybridization (CGH) and fluorescence in situ hybridization (FISH) demonstrated a higher number of chromosomal aberrations in PCLBCL, leg type, compared with PCFCL and demonstrated that translocations involving c-*MYC*, *BCL6*, and *IgH* genes are found only in PCLBCL, leg type.[14,15] Taken together, these studies provided further support for the view that PCFCL with a diffuse infiltration of large centrocytes and PCLBCL, leg type, are distinct types of CBCL. However, they did not provide parameters allowing differentiation between PCLBCL, leg type, with a good or poor prognosis.

In the present study, 19 biopsy samples of PCFCL and 12 of PCLBCL, leg type, were investigated for chromosomal aberrations using an array-based CGH. As compared with classical CGH, genomic DNA-chip hybridization provides a comprehensive, genome-wide analysis at a high spatial resolution. The aim of this study was to further define chromosomal aberrations in PCFCL and PCLBCL, leg type, and in particular to find differences between PCLBCL, leg type, with a favorable and unfavorable prognosis.

The results of this study show clear-cut differences in chromosomal aberrations between PCFCL and PCLBCL, leg type, which not only suggests that different pathogenetic mechanisms are involved in the development of these two types of CBCL, but also provides additional molecular support for the subdivision used in the WHO-EORTC classification. In addition, this study identified loss of 9p21.3 as a potential prognostic parameter in PCLBCL, leg type.

Table 1. Clinical Characteristics of 19 Patients With PCFCL and 12 Patients With PCLBCL, Leg Type

Case No.	Sex	Age at Diagnosis (years)	Site of Presentation	Initial Therapy	Result	Relapse	Follow-Up	
							Status	Months
PCFCL								
1	Male	55	Scalp	RT	CR	—	A−	28
2	Male	37	Scalp	RT	CR	Skin	A−	80
3	Female	72	Face	RT	CR	Skin	A−	75
4	Male	38	Scalp	CHOP/RT	CR	—	A−	22
5	Male	64	Scalp	RT	CR	Skin	A−	58
6	Male	45	Neck	RT	CR	—	A−	12
7	Male	38	Chest	RT	CR	—	A−	192
8	Male	62	Back	RT	CR	Skin + EC	D−	41
9	Male	46	Back	CHOP	CR	—	A−	52
10	Male	68	Chest + back	COP/RT	CR	Skin	A−	179
11	Female	61	Back	RT	CR	—	A−	79
12	Male	65	Chest	RT	CR	Skin + CNS	D+	102
13	Male	56	Back	RT	CR	Skin	A−	93
14	Female	80	Back	RT	CR	—	A−	22
15	Female	74	Back	RT	CR	—	A−	50
16	Male	83	Trunk	RT	CR	Skin	A−	51
17	Male	68	Back	RT	CR	—	A−	12
18	Male	25	Back	CHOP	CR	—	A−	24
19	Male	65	Penis	RT	CR	—	A−	60
PCLBCL, leg type								
20	Female	50	Leg	RT	CR	Skin	A−	62
21	Female	77	Leg	RT	CR	Skin	A−	69
22	Male	47	Leg	CHOP	CR	—	A−	56
23	Female	75	Both legs	CHOP	CR	Skin	A+	27
24	Female	80	Leg	RT	CR	—	A+	17
25	Female	75	Leg	RT	CR	Skin + EC	D+	73
26	Male	73	Leg	CHOP	CR	Skin + EC	D+	21
27	Male	69	Leg + cheek	CHOP	CR	Skin + EC	D+	35
28	Female	88	Leg	RT	CR	Skin + EC	D+	26
29	Male	89	Leg	CHOP	CR	Skin + EC	D+	12
30	Female	92	Leg	RT	PR	Skin + EC	D+	9
31	Female	76	Both legs	CHOP	CR	Skin	D+	11

Abbreviations: PCFCL, primary cutaneous follicle center lymphoma; PCLBCL, primary cutaneous large B-cell lymphoma; CHOP, cyclophosphamide, doxorubicin, oncovin, prednisone; COP, cyclophosphamide, oncovin, prednisone; RT, radiotherapy; CR, complete remission; PR, partial remission; EC, extracutaneous relapse; A−, alive with no evidence of disease; A+, alive with disease; D+, dead as a result of lymphoma; D−, dead as a result of unrelated disease.

PATIENTS AND METHODS

Patient Selection

Pretreatment skin biopsies from 31 patients with the histology of a diffuse large B-cell lymphoma were included in this study. Only cases in which large neoplastic B-cells (CD20 positive) constituted 60% or more of the total number of infiltrating cells were selected. According to the criteria of the recently published WHO-EORTC classification for cutaneous lymphomas, 19 patients were classified as PCFCL and 12 patients as PCLBCL, leg type.[1] All PCFCL showed diffuse infiltrates consisting mainly of large centrocytes, which were consistently MUM1 negative, whereas BCL-2 was expressed in only one of 19 cases (case 19). All PCLBCL, leg type, had presented with skin lesions on one or both legs, showed confluent sheets of centroblasts and immunoblasts, which strongly expressed BCL-2 (12 of 12 cases) and MUM1 (seven of seven cases). Since this group contained only patients presenting with skin lesions on one or both legs, this group will further be referred to as PCLBCL, leg type. In all 31 patients there was no evidence of extracutaneous disease at time of diagnosis as assessed by adequate staging procedures including physical examination, CBCs, computed tomography of chest and abdomen and bone marrow biopsy. The relevant clinical and follow-up data of the patients investigated are summarized in Table 1.

Genomic DNA Extraction

On average, 10 to 60 μg of genomic DNA was isolated from 25 $\times$ 20 μm frozen sections using a commercially available kit as described by the manufacturer (Genomic-tips 20/G kit; Qiagen, Hilden, Germany).

Array-Based CGH

Genome-wide analysis of DNA copy number changes of patient samples was performed using array-based CGH. Slides containing $\approx$ 3500 BACs were produced in the Leiden University Medical Center (Leiden, the Netherlands). The particular bacterial artificial chromosome (BAC) set used to produce these arrays is distributed to academic institutions by the Wellcome Trust Sanger Institute (Cambridge, United Kingdom) and contains targets spaced at $\approx$ 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. Fabrication and validation of the array, hybridization methods, and analytic procedures have been described elsewhere in detail,[16,17] whereas the clone content is available in the "Cytoview" window of the Sanger Institute mapping database site, Ensembl (http://www.ensembl.org/). Fluorescent Cy3 (tumor)/Cy5 (healthy) Log^2 ratios were classified as low copy number gain (between 0.25 and 0.75), high-level DNA amplification (> 0.75) or genomic loss (< -0.25 for hemizygosity, and < -0.75 for homozygous deletion).

Double-Color Interphase FISH on Nuclei Isolated From Paraffin-Embedded Tissue

The BAC clones 373L24 (covering the *c-REL* gene), 61J14 (covering the *MALT1* gene), 520K18 (covering the *BCL-2* gene), and 571M6 (covering the *CDK4* gene) were selected from the CGH-array BAC clones library (Welcome Trust Sanger Institute). These probes were labeled by standard nick translation with biotin-16-aUTP and digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, Germany). In addition, a directly labeled probe was purchased (LSI p16 (9p21)/CEP 9 dual color probe (Abbott, Hoofddorp, the Netherlands) to confirm deletion of the *CDKN2A* and *CDKN2B* gene. Isolation of intact nuclei, hybridization and immunodetection were performed as previously described.[18,19] A total of 100 nuclei were analyzed with a Leica DM-RXA fluorescence microscope (Leica, Wetzlar, Germany). Images were captured using a Cohu 4910 series monochrome CCD camera (Cohu, San Diego, CA) attached to the fluorescence microscope equipped with a PL Fluotar 100×, NA 1.30 to 0.60 objective and I3 and N2.1 filters (Leica) and Leica QFISH software (Leica Imaging Systems, Cambridge, United Kingdom).

Bisulfite Sequence Analysis

Two micrograms of patient sample–derived DNA was bisulfite converted using the EZ DNA methylation kit (Zymo Research, Orange, CA). Primers were designed to anneal to bisulfite-converted DNA as template. The following primer pairs were used for amplification; *CDKN2A* forward, 5′-AGTATTAGGAGGA-AGAAAGAGGAG-3′, *CDKN2A* reverse, 5′-TCCAATTCCCCTACAAACTCC-3′; *CDKN2B* forward, 5′- GGATAGGGGGCGGAGTTTAAGG-3′, *CDKN2B* reverse, 5′-CTCTTCCCTTCTTTCCCACGCTACTC-3′. Polymerase chain reactions and bisulfite sequence analysis were performed as previously described.[20]

RESULTS

We detected both small and whole-chromosome areas of low copy number gain (DNA copy number < 4), high-level DNA amplifications (DNA copy number ≥ 4), and deletions (DNA copy number $= 0$ or 1). In the total group of CBCL, the mean number of chromosomal aberrations was 187 (range, 44 to 649 aberrations per case).

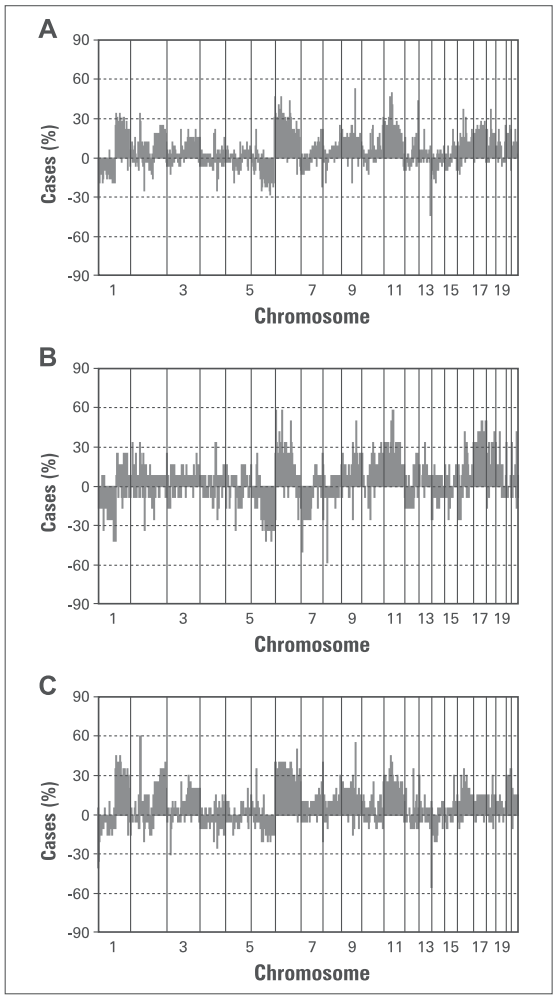

Fig 1. Genome-wide frequency of copy number alterations in (A) the complete group of primary cutaneous large B-cell lymphoma (PCLBCL), (B) the group of 19 primary cutaneous follicle center lymphoma, and (C) the group of 12 PCLBCL, leg type. Positive values represent gains, and negative values represent losses. Clones are ordered from chromosome 1 to 22 and within each chromosome according to their Wellcome Trust Sanger Institute (Cambridge, United Kingdom) mapping position.

Table 2. Most Recurrent Chromosomal Aberrations for All PCLBCL

		PCFCL (n = 19)		PCLBCL, Leg Type (n = 12)					
						D+ (n = 7)		A−/A+ (n = 5)	
Cytogenetic Band	Candidate Genes	Total	%	Total	%	No.	%	No.	%
Gain and amplification									
1q23-q25	—	10	53	2	17	1	14	1	20
2p16.1	*BCL11A, C-rel*	12	63	3	25	1	14	2	40
7q21-q22	*CDK6*	9	47	7	58	6	86	1	20
10q24.2	—	11	58	6	50	3	43	3	60
12p13.2-p13.33	*CDKN1B*	7	37	6	50	5	71	1	20
12p11.21-p11.22	—	7	37	3	25	2	29	1	20
12q13-q14	*CDK4*	10	53	10	83	6	86	4	80
14q11.2-q12	*BCL2L2*	6	32	8	67	5	71	3	60
18q21.31-q21.33	*MALT1, BCL2*	2	11	8	67	5	71	3	60
21q11.2-q22.3	*ITGB2*	7	37	0		0		0	
Loss and deletion									
6q	Complete chromosome arm	6	32	7	58	6	86	1	20
8p23.1	*BLK*	3	16	5	42	3	43	2	40
9p21.3	*MTAP, CDKN2A, CDKN2B, NSG-x*	0		8	67	6	86	2	40
14q32.33	*AKT1, IGH*	13	68	3	25	2	29	1	20

NOTE. This table lists candidate genes within a locus for which there is literature support for involvement in lymphoma.
D+, died of lymphoma; A−, alive with no evidence of disease; A+, alive with disease.

Low copy number gains (n = 108) were more frequent than losses (n = 68) and high-level DNA amplifications (n = 11). Recurrent chromosomal alterations, being detected in more than 30% of the total group of 31 patients, included low copy number gains at 12q13-12q14 (62%), 10q24.2 (54%), and 7q21-7q22 (50%), and losses at 6q (41%). Because sex-mismatched DNA was used as

Table 3. Overview of Chromosomal Alterations and Methylation Status for Individual Cases

Case No.	Diagnosis	Follow-Up		Low Copy Number Gains (No.)							
		Status	Months	1q23-q25	7q21-q22	10q24.2	12p13.2-p13.33	12p11.21-p11.22	12q13-q14	14q11.2-q12	21q11.2-q22.3
1	PCFCL	A−	28	3	3	3	3	3	4		3
2	PCFCL	A−	80						4		
3	PCFCL	A−	75		3	3			4		
4	PCFCL	A−	22	3	3	3		3	4		3
5	PCFCL	A−	58			3				3	
6	PCFCL	A−	12				3	3	4		
7	PCFCL	A−	192	3	3	3	3	3	4	3	3
8	PCFCL	D−	41	3	3	3	3	3	4		3
9	PCFCL	A−	52	3							3
10	PCFCL	A−	179						4		
11	PCFCL	A−	79	3	3						
12	PCFCL	D+	102	3	3						
13	PCFCL	A−	93			3				3	
14	PCFCL	A−	22				3				
15	PCFCL	A−	50			3				3	
16	PCFCL	A−	51			3				3	
17	PCFCL	A−	12	3		3	3	3	4		3
18	PCFCL	A−	24	3	3		3	3	4		3
19	PCFCL	A−	60	3		3					
20	PCLBCL-leg	A−	62			3			4	3	
21	PCLBCL-leg	A−	69	3		3	3	3	4	3	
22	PCLBCL-leg	A−	56								
23	PCLBCL-leg	A+	27			3			4	3	
24	PCLBCL-leg	A+	17		3				4		
25	PCLBCL-leg	D+	73	3	3	3	3		4	3	
26	PCLBCL-leg	D+	21		3		3		4	3	
27	PCLBCL-leg	D+	35		3				4	3	
28	PCLBCL-leg	D+	26		3		3	3	4	3	
29	PCLBCL-leg	D+	12			3			4		
30	PCLBCL-leg	D+	9		3		3				
31	PCLBCL-leg	D+	11		3	3	3	3	4	3	

(continued on following page)

reference in the hybridization reaction, Xp and Xq abnormalities could not be identified.

Analysis of the two clinical entities, PCFCL (n = 19) and PCLBCL, leg type (n = 12), revealed clear differences in patterns of common chromosomal aberrations (Fig 1A-1C).

The mean number of alterations per patient in the PCFCL group was 184 (range, 52 to 649) with 109 low copy number gains (range, 35 to 353), 10 high-level DNA amplifications (range, 0 to 31), and 66 losses (range, 12 to 265). The most frequent findings in PCFCL (present in > 30% of cases) were low copy number gains at 1q23-1q25 (53%), 7q21-q22 (47%), 12p11.21-22 (37%), and 21q11.2-21q22.3 (37%); high-level DNA amplifications of chromosome 2p16.1 (63%), and deletion of a small region at chromosome 14q32 (68%).

In PCLBCL, leg type, the mean number of alterations per patient was 193 (range, 44 to 508) with 108 low copy number gains (range, 19 to 329), 13 high-level DNA amplifications (range, 0 to 37), and 72 losses (range, 20 to 179). The most frequent findings (present in > 30% of cases) were low copy number gains at 14q11.2-14q12 (67%), 12q13-12q14 (83%), 7q21-q22 (58%), and 12p13.2-12p13.33 (50%); a high-level DNA amplification at chromosome 18q21.31-18q21.33; and deletions over a large region in chromosome 6q14.1-6q27 (58%) and 8p23.1 (42%), as well as a small deletion at chromosome 9p21.3 (67%).

When investigating the chromosomal aberrations in more detail, we detected several oncogenes and/or tumor suppressor genes to be present in the altered chromosomal regions. Details on chromosomal aberrations for the complete groups, as well as individual cases, are listed in Tables 2 and 3.

Low Copy Number Gain of Chromosome 12q13-12q14

Duplication of 12q13-12q14 was frequently detected both in the group of PCFCL (53%) and in the group of PCLBCL, leg type (83%). Because alteration of this region is often present in B-cell malignancies we investigated the region in more detail. The region of duplication on chromosome 12q was located at 55.0-65.0 Mb and included the *CDK4* gene. Using FISH, we confirmed *CDK4* gene duplication in patients with gains at 12q13-12q14 (Fig 2).

High-Level DNA Amplification of Chromosome 2p16.1

In PCFCL, we identified amplification corresponding to the BAC clones RP11-440P5 and RP11-373L24, representing a small region on chromosome 2p16.1, as the most common recurrent high-level DNA amplification in 12 (63%) of 19 cases. This amplification

Table 3. Overview of Chromosomal Alterations and Methylation Status for Individual Cases (continued)

Case No.	High-Level Amplifications (No.)		Deletions (type)				Methylation (type)	
	2p16.1	18q21.31-q21.33	6q	8p23.1	9p21.3	14q32.33	$p16^{INK4A}$	$p15^{INK4B}$
1	4+	4	Hemizygous			Hemizygous		
2								
3	4+			Hemizygous		Hemizygous		
4	4+	4	Hemizygous	Hemizygous		Homozygous	Monoallelic	
5								
6	4+					Homozygous		
7	4		Hemizygous	Hemizygous		Hemizygous		
8	4+		Hemizygous			Homozygous		
9	4+		Hemizygous			Homozygous		
10	4							
11						Hemizygous		
12								
13						Hemizygous		
14	4							
15								
16	4					Hemizygous		
17	4+		Hemizygous			Hemizygous		
18	4					Hemizygous		
19						Homozygous		
20				Hemizygous				
21	3	4			Hemizygous			
22					Hemizygous			
23	4	4						
24		4+	Hemizygous	Hemizygous		Hemizygous	Monoallelic	
25	4	4+	Hemizygous	Hemizygous			Biallelic	
26		4	Hemizygous		Homozygous			
27			Hemizygous		Hemizygous	Hemizygous	Monoallelic	
28		4+	Hemizygous	Hemizygous	Homozygous			
29			Hemizygous		Homozygous	Hemizygous		
30		4	Hemizygous	Hemizygous	Homozygous			
31		4+			Homozygous			

Abbreviations: PCFCL, primary cutaneous follicle center lymphoma; PCLBCL-leg, primary cutaneous large B-cell lymphoma, leg type; A−, alive with no evidence of disease; A+, alive with disease; D+, dead as a result of lymphoma; D−, dead as a result of another cause

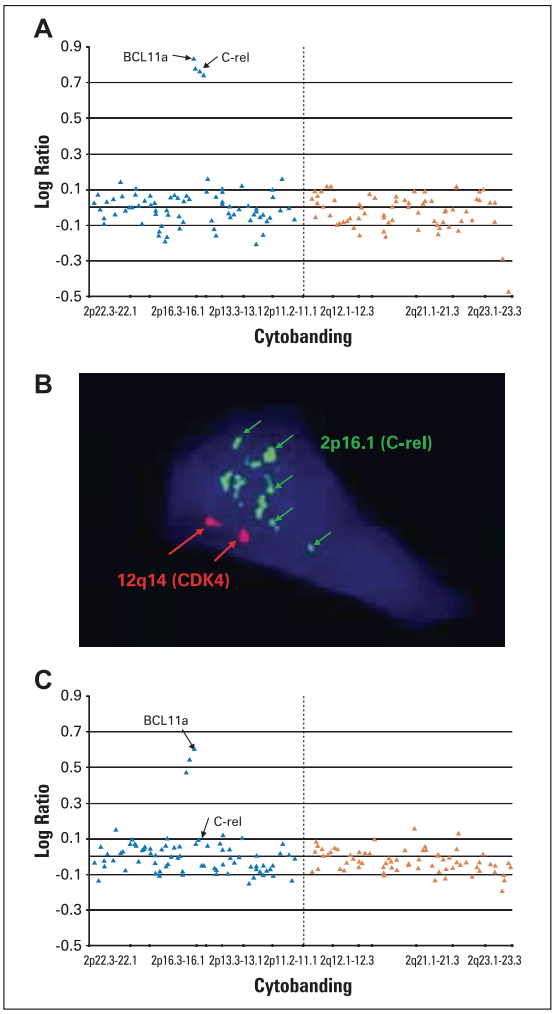

Fig 2. High-resolution analysis of chromosome 2 demonstrating (A) the frequency of gains and losses in a typical primary cutaneous follicle center lymphoma patient, (B) the amplification of *c-REL* (green) and duplication of *CDK4* (red) as shown by fluorescence in situ hybridization analysis, and (C) the selective amplification of only *BCL11A*, but not *c-REL* in a primary cutaneous large B-cell lymphoma, leg type, patient.

was centered at 60.6-61.2 Mb and included the candidate oncogenes *BCL11A* and *c-REL*. A representative individual case (case 9) demonstrating this region of amplification is depicted in Figure 2A. Using FISH, we confirmed *c-REL* gene amplifications in patients with gains at 2p16.1 (Fig 2B; case 3, a typical result for four patients analyzed). In contrast to the PCFCL patients, in the group of PCLBCL, leg type, three (25%) of 12 patients showed alteration of this region. One patient had a whole-chromosome duplication of chromosome 12, and the other two patients showed an amplification of DNA corresponding to BAC clone RP11-440P5 (containing the *BCL11A* gene) and no amplification of DNA corresponding to BAC clone RP11-373L24 (harboring the *c-REL* gene). A representative case (case 23) showing amplification of only *BCL11A* and not *c-REL* is depicted in Figure 2C.

Deletion of Chromosome 14q32

Deletion of chromosome 14q32 was detected in 13 (68%) of 19 PCFCL cases and only three (25%) of 12 PCLBCL, leg type, cases. This region could be narrowed to chromosomal band 14q32.33 containing the oncogene *AKT1*, as well as the immunoglobulin heavy chain locus.

High-Level DNA Amplification of Chromosome 18q21.31-18q21.33

High-level DNA amplification of a discrete region within 18q was observed in eight (67%) of 12 of the PCLBCL, leg type, patients, whereas low copy number gain of this region was seen in only two (11%) of 19 of the PCFCL cases. These frequent high-level DNA amplifications were centered at 54.0-60.0 Mb, a region containing the candidate oncogenes *MALT1* (54.5 Mb, clone RP11-61J14) and *BCL-2* (58.9 Mb, clone RP11-28F1). In the PCLBCL, leg type, cases the 18q21 amplification involved mostly both *BCL-2* and *MALT1* (five cases; 42%), but was restricted occasionally to either *BCL-2* (one case; 8%) or *MALT1* (two cases; 17%). Representative individual cases (cases 25, 26, 28, and 30) demonstrating examples of these DNA copy number alterations are shown in Figure 3A-3D. FISH analysis confirmed *BCL-2* and *MALT1* amplifications in the PCLBCL, leg type, tumors with gains of 18q21.31 and 18q21.33 (Fig 3E; case 24 is a representative result for four patient biopsies analyzed).

Deletion of Chromosome 9p21.3

Within the PCLCBL, leg type, group, a common minimally altered region in chromosome arm 9p (clone RP11-149I2) was identified in eight (67%) of 12 patients. All eight patients demonstrated (partial) loss of 9p21.3 (21.9-22.0 Mb) encompassing the *CDKN2A* ($p14/p16^{INK4A}$) and *CDKN2B* ($p15^{INK4B}$), *MTAP*, and *NSG-x* genes. A representative individual case (case 29) demonstrating this specific deletion is shown in Figure 4A. Deletion of the *CDKN2A* gene was also determined using FISH analysis for those cases showing 9p21.3 loss by array CGH and for which paraffin embedded biopsy material was available ($n = 3$). FISH confirmed that genomic losses lower than -0.75 (as detected by array-based CGH; cases 29 and 30) were indeed in agreement with homozygous (biallelic) deletion, whereas losses lower than -0.25 correspond to hemizygosity (Fig 4C; case 27). Interestingly, five of five PCLBCL, leg type, patients with a biallelic microdeletion within chromosome 9p21.3 died as a result of the lymphoma, as compared with one of six PCLBCL, leg type, patients without this deletion.

Promoter Hypermethylation Analysis

To explore the possibility that patients with unfavorable prognosis, without biallelic loss of 9p21.3, lost expression of *CDKN2A* and/or *CDKN2B* due to promoter hypermethylation all cases were analyzed for DNA methylation, using bisulfite sequence analysis. Methylation of *CDKN2A* was detected in four patients, whereas methylation of the *CDKN2B* gene could not be demonstrated in any of the 31 cases analyzed. Two patients (cases 25 and 27) showed complete methylation of the *CDKN2A* promoter region, whereas the other two patients (cases 4 and 24) showed only partial methylation.

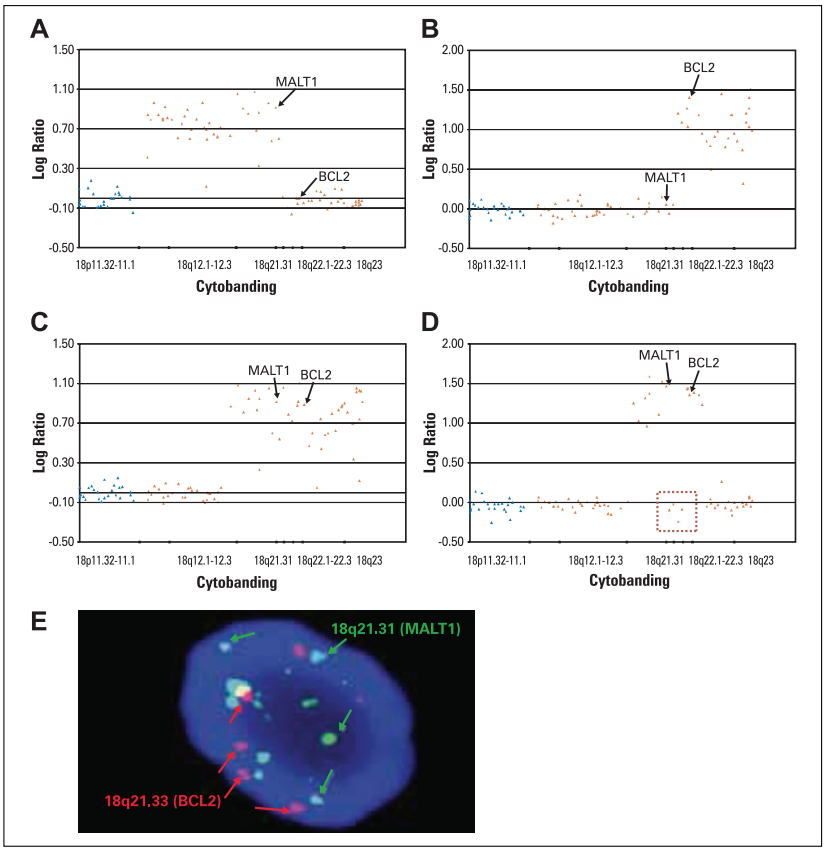

Fig 3. High-resolution analysis in primary cutaneous large B-cell lymphoma, leg type, patients showing (A) 18q21.31 (MALT1), (B) amplification of either 18q21.33 (*BCL-2*), or (C) amplification of both 18q21.31 (*MALT1*) and 18q21.33 (*BCL-2*), and (D) amplification of both 18q21.31 (*MALT1*) and 18q21.33 (*BCL-2*), although a region in between is not amplified (dotted box). (E) Fluorescence in situ hybridization analysis showing amplification of *MALT1* and *BCL-2*.

DISCUSSION

A considerable proportion of primary CBCL has the histologic appearance of DLBCL. In the EORTC classification for primary cutaneous lymphomas, most of these lymphomas were classified as primary cutaneous follicle center cell lymphoma, whereas a significant minority was classified as PCLBCL of the leg.[13] Differentiation between these two groups is considered to be important, because of differences in prognosis and first choice of treatment, radiotherapy or systemic chemotherapy, respectively. Distinction between these two types of DLBCL mainly on the basis of site of presentation has evoked much debate.[21] However, more recent clinical, histologic, immunohistochemical and molecular genetic studies have resulted in a better definition of these two types of CBCL, and confirmed that they are distinct entities.[10,22-24]

In the recently published WHO-EORTC classification, PCFCL and PCLBCL, leg type, are included as separate entities.[1] Notwithstanding, the molecular mechanisms involved in the development of these two types of CBCL are largely unresolved. Moreover, genetic abnormalities allowing differentiation between PCLBCL, leg type, patients with a good or poor prognosis have not yet been identified. In an attempt to resolve some of these issues in the present study, array-based CGH was used to define with high resolution the DNA copy number profiles of 31 patients with the histologic appearance of DLBCL, including 19 patients with PCFCL and 12 patients with PCLBCL, leg type.

The results of this study showed clear differences in chromosomal aberrations between both groups of CBCL. The most interesting chromosomal aberrations in the group of PCFCL were high-level amplifications of chromosome 2p16.1 and deletion of chromosome 14q32.33.

High-level DNA amplification of a small region of chromosome 2p16.1 containing both the *BCL11A* and *c-REL* genes was detected in 12 (63%) of 19 PCFCL cases, but in only three of 12 PCLBCL, leg type, cases. Although in PCFCL, transcription repressor *BCL11A* is always coamplified with *c-REL*, two of three PCLBCL, leg type, cases showed amplification of only the *BCL11A*, but not of the *c-REL* gene. Amplification of *c-REL* is frequently found in nodal and extranodal DLBCL, transformed follicular lymphomas and Hodgkin's lymphoma.[25-30] In a previous study using classical CGH,[14] *c-REL* amplification was detected in only one of six PCFCL, demonstrating the superior sensitivity of the array-based CGH. These observations further support the concept that PCFCL are similar to germinal center–like DLBCL,

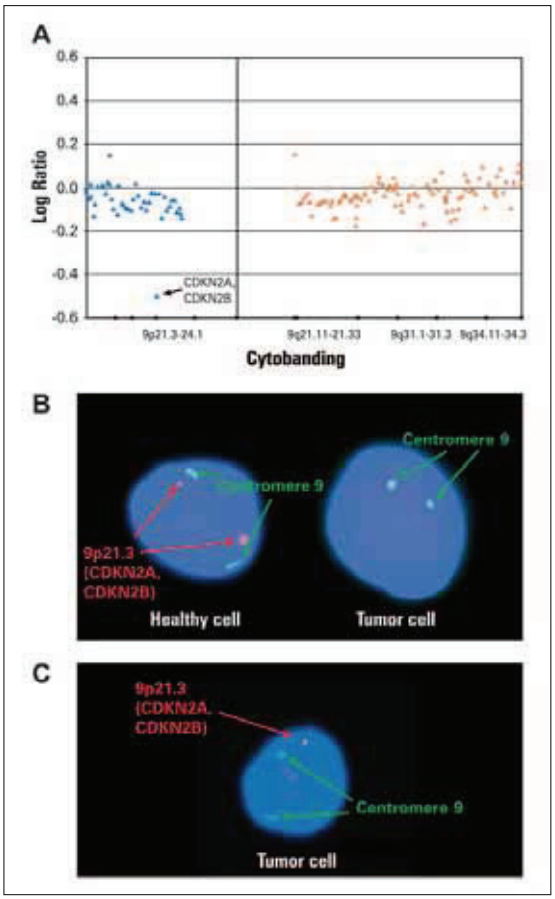

Fig 4. High-resolution analysis of representative primary cutaneous large B-cell lymphoma (PCLBCL), leg type, patients who died as a result of lymphoma showing DNA alterations involving chromosome 9. (A) Frequency of chromosome 9 gains and losses in a typical PCLBCL, leg type, patient. Fluorescence in situ hybridzation analysis showing (B) homozygous or (C) hemizygous deletion of *CDKN2A* (*p16*^{INK4A}).

because *c-REL* amplifications are characteristically found in germinal center–like DLBCL.[10,31]

Loss of chromosome 14q32.33 containing the immunoglobulin heavy chain locus was observed in 13 of 19 PCFCL patients, but in only three of 12 PCLBCL, leg type, patients. Although this region is often involved in translocations, such as t(14;18) in follicular lymphomas and a proportion of DLBCL, t(8;14) in Burkitt lymphoma and t(11;14) in mantle-cell lymphoma, previous studies demonstrated that these translocations do not occur in PCFCL.[15] The deletion of chromosome 14q32.33 in PCFCL is in agreement with previous gene expression studies from our group, demonstrating loss of expression of the immunoglobulin heavy chain mRNA as one of the most discriminating factors between both groups, occurring in PCFCL but not in PCLBCL, leg type.[10]

These results are also in line with previous immunocytochemical studies indicating that PCFCL often lacks expression of surface immunoglobulins.[4,6,7]

The most interesting observations in the group of PCLBCL, leg type, were high-level DNA amplifications of chromosome 18q21 and deletions of chromosome 9p21.3. High-level DNA amplifications of chromosome 18q21.31-q21.33 encompassing the *BCL-2* and *MALT1* genes were detected in eight (67%) of 12 PCLBCL, leg type, but in only two (11%) of 19 PCFCLs. Previous studies demonstrated that the *MALT1* and *BCL-2* gene in PCLBCL are not or are very rarely affected by translocations making this an unlikely cause for the detected DNA amplifications.[8,15,32] A more detailed analysis of these PCLBCL, leg type, patients revealed that two patients showed amplification of *MALT1* and one patient showed amplification of *BCL-2*, whereas amplification of both *MALT1* and *BCL-2* was detected in five patients. *BCL-2* gene amplification has been reported previously in nodal DLBCL with 18q21 high-level DNA amplifications as well as in 50% of primary cutaneous DLBCL.[25,33-35] However, in this latter study, no distinction was made between PCFCL and PCLBCL, leg type.

MALT1 expression may occur in B-cell non-Hodgkin's lymphoma of various histologic subtypes either through translocations involving the immunoglobulin heavy chain locus t(14;18)(q32;q21) or by genomic amplification.[36] Translocations involving *MALT1* were not detected previously in PCLBCL.[15] Our results demonstrate that 18q21 amplification occurs in eight (67%) of 12 of PCLBCL, leg type, and involve the *BCL-2* and *MALT1* genes. In five of eight patients, both the *BCL-2* and *MALT1* genes were amplified. Interestingly, we noticed that in one patient, both regions of amplification were separated by normal copy DNA. Together with the detection of selective amplification of DNA regions containing only the *MALT1* or the *BCL-2* gene in other patients, that instance demonstrates that amplification of these genes can occur as independent events affecting relatively small regions of DNA rather than gross genetic changes involving large chromosomal fragments. Our results are consistent with a recent study that showed that 18q amplifications are more common in activated B-cell–like DLBCL than in germinal center cell–like DLBCL.[37]

Deletion of a small region on chromosome 9p21.3 containing the *CDKN2A* and *CDKN2B* gene loci was detected in eight of 12 PCLBCL, leg type, patients but not in any of the PCFCL patients. Most interestingly, all seven PCLBCL, leg type, patients who died as a result of their lymphoma had a homozygous deletion of 9p21.3 (five cases), promoter hypermethylation of the *CDKN2A* gene (one case), or hemizygous deletion combined with CDKN2A hypermethylation (one case). Whereas previous studies on large groups of PCLBCL, leg type, patients suggested that a round cell morphology (as opposed to a cleaved cell morphology) and BCL-2 expression are associated with an inferior prognosis,[3] in the present study, the neoplastic B-cells in all 12 PCLBCL, leg type patients (seven of whom died as a result of their lymphoma), had a round cell morphology and strongly expressed BCL-2. The observation that loss of *CDKN2A* (*p16*^{INK4A}) was associated with inferior survival is therefore of great clinical importance, since it suggests that loss of p16^{INK4A} expression may serve as an important prognostic parameter in this group. Interestingly, loss of CDKN2B (p15^{INK4B}) expression seems to be of less importance; methylation of *CDKN2A* was observed in all cases with (partial) retention and adverse prognosis, whereas *CDKN2B* methylation could not be demonstrated.

Promoter hypermethylation of *CDKN2B* and *CDKN2A* leading to decreased expression of p15 and p16 protein was described in an earlier study on primary cutaneous B-cell lymphomas.

Although loss of p16 was described with progression of disease, no relation with prognosis was observed.[38] Our results are consistent with a recent study that showed that loss of 9p21.3 is much more common in activated B-cell–like DLBCL than in germinal center–like DLBCL, and that loss of 9p21.3 is associated with an adverse prognosis.[37]

Deletion at chromosome 9p21 is also a frequent cytogenetic abnormality in other hematologic malignancies, such as acute lymphoblastic leukemia (ALL) or pediatric ALL.[39-41] and demonstrated prognostic significance for these genetic changes.[42-44] In a recent study, Raschke et al[45] proposed that the mechanisms inducing relatively small deletions in the region containing *CDKN2A* and *CDKN2B* (as seen in our PCLBCL, leg type, patients) might be the result of exuberant processing during nonhomologous end-joining (NHEJ) DNA repair of double-strand breaks in the locus. Distinct mechanisms typically causing larger deletions, of several megabases, may be prevalent in other tumors such as bladder cancer and glioma.[45]

Another chromosomal abnormality commonly associated with poor survival is loss of chromosome 6q. However, in the present study, loss of 6q was found in not only seven of 12 PCLBCL, leg type, cases, but also in six of 19 PCFCL cases. Since these latter six patients had an excellent prognosis, it precludes usage of 6q loss as a useful prognostic marker.

In conclusion, the results of the present array-based CGH study clearly demonstrate distinct chromosomal aberrations in PCFCL and PCLBCL, leg type, which provides further support for their categorization in the WHO-EORTC classification as separate entities. The most important findings were high-level DNA amplification of 2p16.1 (*c-REL/BCL11A*) and loss of 14q32.33 in the group of PCFCL, and high-level DNA amplification of chromosome 18q21 and loss of 9p21.3 in the PCLBCL, leg type, group. Finally, although confirmation on a larger group of patients is required, our results suggest that loss of chromosome 9p21.3 might prove to be an important prognostic marker in PCLBCL, leg type.

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Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

Author Contributions

Conception and design: Remco Dijkman, Cornelis P. Tensen, Rein Willemze, Maarten H. Vermeer
Provision of study materials or patients: Juliette J. Hoefnagel, Rein Willemze, Maarten H. Vermeer
Collection and assembly of data: Remco Dijkman, Ekaterina S. Jordanova, Jeroen Knijnenburg, Aat A. Mulder, Carla Rosenberg, Anton K. Raap, Karoly Szuhai
Data analysis and interpretation: Remco Dijkman, Cornelis P. Tensen, Maarten H. Vermeer
Manuscript writing: Remco Dijkman, Cornelis P. Tensen, Rein Willemze, Maarten H. Vermeer
Final approval of manuscript: Remco Dijkman, Cornelis P. Tensen, Rein Willemze, Maarten H. Vermeer

GLOSSARY

Amplification/deletion: Chromosomal aberrations that result in amplification or deletion in regions of the chromosome. Chromosomal amplification results in a gain in copy number of genes, whereas chromosomal deletion results in gene loss from the chromosome.

Array-based CGH: Array-based comparative genomic hybridization is a method that uses microarrays to probe changes in chromosomal DNA, thereby identifying precise areas in which genetic changes occur in cancer cells.

BAC clones: Derived from a cloning system designed at cloning DNA fragments in excess of 100 to 300 kb, BAC (F-factor-based bacterial artificial chromosome) clones are a library of genomic DNA fragments that are plasmid based and easy to isolate, making them feasible for genomic analysis.

Bisulfite sequence analysis: DNA treated with sodium bisulfite prior to sequence determination converts unmethylated cytosines in DNA to deoxyruracils, leaving methylated cytosines unchanged. The pattern of methylated cytosines in the final sequence of the DNA is assessed by polymerase chain reaction using specific primers followed by sequence analysis.

Copy number gain/loss: Chromosomal amplifications or deletions that result in gains or losses, respectively, of genes in the affected regions of the chromosome. Chromosomal amplification may result in chromosomes acquiring multiple copies of genes. Chromosomal deletions may result in biallelic gene loss when deletions occur in corresponding regions of both chromosomes.

Cutaneous B-cell lymphoma (CBCL): Refers to B-cell non-Hodgkin's lymphomas with only skin lesions at the time of diagnosis. This heterogeneous group includes primary cutaneous marginal zone lymphoma, primary follicle center lymphoma (CBCL with a indolent clinical behavior) and primary cutaneous large B-cell lymphoma, leg type (CBCL with an intermediate clinical behavior).

FISH (fluorescence in situ hybridization): A test to detect *ErbB2* gene amplification in the tumors of patients who intend to receive trastuzumab therapy for breast cancer, in situ hydridization is a sensitive method that is generally used to detect specific gene sequences in tissues sections or cell preparations by hybridizing the complimentary strand of a nucleotide probe to the sequence of interest. FISH uses a fluorescence probe to increase the sensitivity of in situ hybridization.

Promoter hypermethylation: Methylation of the promoter region of a gene can lead to DNA silencing as a consequence of the inability of activating transcriptional factors to bind to the promoter region, a process important in gene transcription. In addition, repressor complexes may be attracted to sites of promoter methylation, leading to the formation of inactive chromatin structures.