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Molecular Cytogenetic Analysis of Chromosomal Breakpoints in the *IGH, MYC, BCL6*, and *MALT1* Gene Loci in Primary Cutaneous B-cell Lymphomas

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Chromosomal translocations affecting the *IGH* locus and various oncogene loci are recurrent in many types of systemic B-cell lymphomas. Hardly any data exist, however, on such translocations in primary cutaneous B-cell lymphomas (PCBCL). Here, a series of 29 PCBCL was investigated by interphase fluorescence *in situ* hybridization with probes for the *IGH*, *MYC*, *BCL6*, and *MLT1* loci. None of the six follicle center cell lymphomas and nine marginal zone lymphomas showed evidence for any translocation affecting these loci. In contrast, 11 of 14 large B-cell lymphomas of the leg harbored breakpoints in at least one of the loci. Translocations involving the *MYC* locus were detected in six cases, five of them derived from a *MYC/IGH* juxtaposition and one from a translocation involving a non-*IG* gene partner. Rearrangements of the *BCL6* locus were detected in five B-cell lymphomas of the leg, and involved *IGH* (two cases), *IGL* (one case), and non-*IG* genes (two cases). This study shows that large B-cell lymphomas of the leg display a pattern of chromosomal translocations similar to their systemic counterparts whereas primary cutaneous follicle center cell lymphomas and marginal zone lymphomas lack these typical chromosomal translocations.

Key words: chromosomal translocation/FISH/primary cutaneous B-cell lymphomas
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Chromosomal translocations leading to activation of oncogenes via juxtaposition next to regulatory sequences of one of the immunoglobulin loci in 14q32 (*IGH*), 2p12 (*IGK*), or 22q11 (*IGL*) are recurrent primary genetic aberrations in various subtypes of B-cell lymphomas (Willis and Dyer, 2000; Siebert *et al*, 2001). The so-called Burkitt translocation t(8;14)(q24;q32) and its variants t(2;8)(p12;q24) and t(8;22)(q24;q11) targeting the *MYC* oncogene locus in 8q24 are the hallmark of Burkitt's lymphoma (BL) and lead to the deregulated expression of the MYC transcription factor. Breakpoints in the *MYC* locus are present in all BL. Consequently the detection of these changes is mandatory for the diagnosis of this lymphoma entity (Diebold *et al*, 2001). The translocation t(14;18)(q32;q21) leading to deregulation of the apoptosis inhibitor *BCL2* is present in approximately 90% of nodal follicular lymphomas (FL) (Nathwani *et al*, 2001). Deregulation of *BCL6* either by juxtaposition next to an *IG* locus or by promotor substitution due to a chromosomal translocation affecting band 3q27 can be detected in about 30% of systemic diffuse-large B-cell lymphomas (DLCBL) (Gatter and Warnke, 2001). BCL6 is a zinc-finger transcriptional repressor required for the development of germinal centers. Although these aberrations are associated with a certain lymphoma subtype, most of them are not specific for this entity. In addition to the examples described above, many other translocations affecting the *IGH* locus have been described to occur with much lower frequency in systemic B-cell lymphomas. Among them, a t(14;18) cytogenetically identical to that occurring in FL can lead to activation of the *MALT1* oncogene (Sanchez-Izquierdo *et al*, 2003; Streubel *et al*, 2003). This gene is also targeted by a recurrent t(11;18)(q21;q21) present in approximately 30% of systemic marginal zone lymphomas of MALT type, which leads to fusion of the *MALT1* gene with the apoptosis inhibitor-2 (*API2*) gene in 18q21 (Dierlamm *et al*, 1999; Chaganti *et al*, 2000; Ye *et al*, 2003). Overall, translocations affecting one *IG* locus are estimated to be present in at least half of the nodal B-cell non-Hodgkin lymphomas.

In contrast to systemic B-cell lymphomas only few data exist on the presence of recurrent translocations in primary cutaneous B-cell lymphomas (PCBCL). Various PCR-based studies have investigated the presence of t(14;18) involving *IGH* and *BCL2* in PCBCL but yielded contradictory results. Whereas some studies suggest absence of this transloca-

Abbreviations: BL, Burkitt's lymphoma; FISH, fluorescence *in situ* hybridization; FL, follicular lymphomas; LBCLL, large B-cell lymphomas of the leg; PCBCL, primary cutaneous B-cell lymphomas; PCFCCL, primary cutaneous follicle center cell lymphomas; PCMZL, primary cutaneous marginal zone lymphomal
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tion (Geelen *et al*, 1998; Child *et al*, 2001; Goodland *et al*, 2002) others could detect *IGH/BCL2* juxtaposition in PCBCL in a variable fraction of the cases (Cerroni *et al*, 1994; Yang *et al*, 2000; Aguilera *et al*, 2001; Mirza *et al*, 2002; Servitje *et al*, 2002). Nevertheless, it has to be taken into account that PCR-based methods for the detection of t(14;18) frequently result in positive results also in non-neoplastic tissue samples (Summers *et al*, 2001; Biagi and Seymour, 2002). For many other translocations affecting *IG* loci, PCR approaches either have not been developed or are difficult to perform because of the scattering of the molecular breakpoints or have been shown to work not reliably. Interphase fluorescence *in situ* hybridization (FISH) has been repeatedly shown to overcome these limitations and to be a robust method for the detection of promiscuous breakpoints and recurrent translocations (Siebert and Weber-Matthiesen, 1997). In order to investigate whether translocation breakpoints highly recurrent in systemic B-cell lymphomas are also present in PCBCL, we studied a series of 29 PCBCL by means of interphase FISH with assays targeting the *IGH*, *MYC*, *BCL6*, and *MALT1* loci.

Results

Classification, clinical data as well as relevant results of immunohistochemical studies of the 29 cases are presented in Table I.

Translocation breakpoints involving the IGH locus in chromosome band 14q32 All six cases of PCFCCL, all nine PCMZL, and seven of 14 LBCLL lacked molecular cytogenetic evidence for a chromosomal translocation involving the *IGH* locus (Table II). The percentage of nuclei displaying a signal constellation indicating a chromosomal breakpoint in the *IGH* locus in these lymphomas ranged from 0.5% to 3% and was, thus, far below the cutoff level. From the 14 LBCLL, seven cases contained a significant percentage of nuclei (40%–77%) with a signal constellation that did indicate a breakpoint in the *IGH* locus. Further molecular cytogenetic analyses indicated the presence of a translocation t(8;14)(q24;q32) in five and of a t(3;14)(q27;q32) in two of these seven LBCLL (Fig 1; Table II). In none of the cases with a breakpoint in the *IGH* locus a translocation t(14;18) was detected.

Translocation breakpoints involving the BCL6 locus in chromosome band 3q27 All PCFCCL and PCMZL as well as nine of 14 LBCLL lacked evidence for a breakpoint in the *BCL6* locus. The percentage of nuclei with a signal pattern indicating a *BCL6* break never exceeded 1% in these cases. Five LBCLL displayed a significant proportion of interphase nuclei (39%–75%) with a signal pattern indicating a breakpoint affecting the *BCL6* locus. These included the two cases with t(3;14)(q27;q32) described above as well as another case in which additional FISH indicated the *IGL* locus as *BCL6* translocation partner (Table II). In two LBCLL, the *IGH*, *IGL*, and *IGK* loci were excluded as *BCL6* partners. Overall, Bcl6 was expressed in nine of 22 PCBCL without *BCL6* translocation and in all five LBCLL bearing this chromosomal change. Focussing on the LBCLL group, Bcl6 was expressed in four of nine cases without *BCL6* translocation (case no. 17, 21, 25, and 26) (Tables I and II).

Translocation breakpoints involving the MYC locus in chromosome band 8q24 All six cases of PCFCCL, all nine PCMZL, and eight of 14 LBCLL lacked molecular cytogenetic evidence for a chromosomal translocation involving the *MYC* locus (Table II). The percentage of nuclei displaying a signal constellation indicating a chromosomal breakpoint in the *MYC* locus in these lymphomas never exceeded 2.5% and was, thus, far below the cutoff level. In six LBCLL a signal pattern indicating a chromosomal breakpoint in the *MYC* locus was detected in 48%–74% of the nuclei. These included the five LBCLL with t(8;14) described above as well as one additional LBCLL in which further FISH analyses provided evidence for a non-*IG* locus as partner. In all the five cases with t(8;14), immunohistochemical analyses detected expression of Bcl2. In contrast, expression of EBER (Epstein–Barr virus-encoded small DNA) was not detected in any of these cases and CD10 was only weakly detected in one of them (results not shown). TdT was absent in all four t(8;14)-positive LBCLL analyzed whereas only one of five cases expressed Bcl6. The proportion of cells staining positive for Ki-67 ranged between 25% and 75% in the 3 t(8;14)-positive LBCLL studied.

Translocation breakpoints involving the MALT1 locus in chromosome band 18q21 In none of the 29 PCBCL under study, FISH provided evidence for a chromosomal breakpoint affecting the *MALT1* locus. The percentage of nuclei with a signal pattern indicating a *MALT1* breakpoint never exceeds 2.5% (Table I).

Discussion

In this study, we investigated a series of 29 PCBCL for the occurrence of chromosomal breakpoints in gene loci recurrently affected in systemic B-cell lymphomas. Eleven of the 14 LBCLL cases investigated displayed signal constellations indicating the presence of a chromosomal breakpoint in *IGH*, *BCL6*, or *MYC*. In contrast, all six cases of PCFCCL and all nine cases of PCMZL were devoid of any molecular cytogenetic evidence for a breakpoint in the *IGH*, *MYC*, *BCL6*, or *MALT1* locus. Thus, with regard to the presence of chromosomal translocations LBCLL seem to significantly differ from the other two subtypes of PBCBL studied herein (11 of 14 *vs* zero of 15; $p<0.001$; Fisher's exact test).

The interphase cytogenetic aberration pattern of LBCLL resembles that of systemic DLBCL. Breakpoints affecting the *BCL6* locus were detected in five of 14 (36%) LBCLL. The incidence of *BCL6* rearrangements in systemic DLBCL is assumed to be approximately 30% (Gatter and Warnke, 2001). Remarkably, systemic DLBCL with *BCL6* breaks tend to show an increased frequency of extranodal disease (Offit *et al*, 1994). In our series, all five LBCLL with *BCL6* translocations expressed the Bcl6 protein whereas four of nine LBCLL without *BCL6* translocation were positive ($p=0.09$, Fisher's exact test) (Table I). Overall, although *BCL6* translocations did not necessarily lead to enhanced expression of the protein detectable by immunohistochem-

Table I. Clinical and immunohistochemical data from the patients studied in the present series

Case number	Gender/age (y) at diagnosis	Diagnosis		Site of presentation	Immunohistochemistry			First therapy	Status	Follow-up time (mo)
		EORTC	WHO		CD10	Bcl6	Bcl2			
1	M/58	PCFCCL	DLBCL	Head	ND	Pos	Neg	RT	A+	24
2	F/74	PCFCCL	FL	Head	ND	ND	Neg	RT	A+	18
3	M/59	PCFCCL	FL	Back	ND	Pos	Neg	RT	A0	18
4	F/61	PCFCCL	FL	Head	ND	Pos	Neg	RT	A0	21
5	F/54	PCFCCL	DLBCL	Trunk/gluteal	ND	Pos	Pos	CT	A+	168
6	M/58	PCFCCL	FL	Trunk	ND	Pos	Neg	EX	A0	32
7	M/34	PCMZL	PCMZL	Arms	ND	Neg	Pos	EX	A+	78
8	M/47	PCMZL	PCMZL	One leg	ND	Neg	Pos	EX	A+	62
9	M/59	PCMZL	PCMZL	Head	ND	Neg	Pos	EX	A+	27
10	M/63	PCMZL	PCMZL	Arm	ND	Neg	Neg	RT	A0	24
11	F/38	PCMZL	PCMZL	Trunk	ND	Neg	Neg	RT	A0	13
12	M/35	PCMZL	PCMZL	Head	ND	Neg	Pos	Rit	A+	18
13	M/44	PCMZL	PCMZL	Trunk	ND	Neg	Pos	RT	A0	32
14	F/64	PCMZL	PCMZL	Two legs	ND	ND	ND	EX/AB	A0	22
15	M/65	PCMZL	PCMZL	Trunk	ND	Neg	Neg	EX	A0	24
16	F/53	LBCLL	DLBCL	One leg	Neg	Neg	Pos	CT	A+	120
17	F/73	LBCLL	DLBCL	One leg	Neg	Pos	Pos	RT	DD	18
18	F/77	LBCLL	DLBCL	One leg	Neg	Pos	Pos	RT	A0	51
19	M/69	LBCLL	DLBCL	One leg	Neg	Neg	Pos	RT	DD	35
20	F/82	LBCLL	DLBCL	One leg	Neg	Pos	Pos	RT	DD	38
21	M/47	LBCLL	DLBCL	Two legs	Neg	Pos	Pos	CT/RT	A0	24
22	M/65	LBCLL	DLBCL	Two legs	Neg	Neg	Pos	RT	A+	60
23	M/70	LBCLL	DLBCL	Two legs	Neg	Neg	Pos	IFN	A+	28
24	F/80	LBCLL	DLBCL	One leg	ND	Pos	Pos	Ex	A0	23
25	F/74	LBCLL	DLBCL	One leg	Neg	Pos	Pos	CT	A0	12
26	F/84	LBCLL	DLBCL	One leg	Neg	Pos	Pos	CT	A0	19
27	M/89	LBCLL	DLBCL	One leg	Neg	Pos	Pos	CT	DD	12
28	F/90	LBCLL	DLBCL	One leg	Neg	Pos	Pos	RT	DD	29
29	M/79	LBCLL	DLBCL	One leg	−/+ (weak)	Neg	Pos	RT	DD	16

PCFCCL, primary cutaneous follicle center cell lymphoma; PCMZL, primary cutaneous marginal zone lymphoma; LBCLL, large B-cell lymphoma of the leg; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; M, male; F, female; ND, not done; pos, positive; neg, negative; A+, alive with disease; A0, alive without disease; DD, dead from disease; Ex, excision; CT, polychemotherapy; RT, radiotherapy; IFN, interferon α; AB, antibiotics, Rit, Rituximab.

istry, there was a significant correlation between Bcl6 expression and presence of *BCL6* translocations in PCBCL (five of five with translocation *vs* nine of 22 without translocation; $p = 0.04$, Fisher's exact test). It would be interesting to know whether at all point mutations in *BCL6* might be related to the protein expression status in PCBCL (Lossos *et al*, 2002). Translocations affecting the *IGH* locus were detected in seven of 14 (50%) of the LBCLL and, thus, also in a similar frequency to systemic DLBCL. Strikingly, the most frequent translocation partner of the *IGH* locus was the *MYC* gene in 8q24. A signal pattern indicating t(8;14)(q24;q32) was detected in five of 14 LBCLL. One additional LBCLL carried a break in the *MYC* locus not involving an *IG* partner. Translocations affecting the *MYC* locus, particularly the Burkitt translocation t(8;14), are the hallmark of BL (Diebold *et al*, 2001). Nevertheless, these aberrations are not restricted to this lymphoma subtype, and recurrently appear in other systemic lymphomas like in FL transforming to high grade lymphomas (Macpherson *et al*, 1999; Akasaka *et al*, 2000). All five LBCLL with a signal pattern indicating the presence of t(8;14) lacked typical Burkitt morphology. Moreover, in contrast to BL these LBCLL expressed strongly Bcl2 but lacked CD10 as well as Bcl6 in four of five cases (Table I). The proliferation activity

Table II. Chromosomal aberrations in the PCBCL studied herein detected by interphase FISH

Number	Diagnosis	Breakpoints (% of cells with rearrangement)				Additional FISH analyses[a]
		IGH	*BCL6*	*MYC*	*MALT1*	
1	PCFCCL	Neg	Neg	Neg	Neg	
2	PCFCCL	Neg	Neg	Neg	Neg	
3	PCFCCL	Neg	Neg	Neg	Neg	
4	PCFCCL	Neg	Neg	Neg	Neg	
5	PCFCCL	Neg	Neg	Neg	Neg	
6	PCFCCL	Neg	Neg	Neg	Neg	
7	PCMZL	Neg	Neg	Neg	Neg	
8	PCMZL	Neg	Neg	Neg	Neg	
9	PCMZL	Neg	Neg	Neg	Neg	
10	PCMZL	Neg	Neg	Neg	Neg	
11	PCMZL	Neg	Neg	Neg	Neg	
12	PCMZL	Neg	Neg	Neg	Neg	
13	PCMZL	Neg	Neg	Neg	Neg	
14	PCMZL	Neg	Neg	Neg	Neg	
15	PCMZL	Neg	Neg	Neg	Neg	
16	LBCLL	**Pos (40.2%)**	Neg	**Pos (47.5%)**	Neg	IGH con BCL2 × 0; **IGH con MYC × 2 (50%)**
17	LBCLL	Neg	Neg	Neg	Neg	
18	LBCLL	**Pos (40.0%)**	**Pos (39.1%)**	Neg	Neg	IGH con BCL2 × 0; **IGH con BCL6 × 2 (46%)**
19	LBCLL	Neg	Neg	Neg	Neg	
20	LBCLL	Neg	**Pos (45.5%)**	Neg	Neg	IGKc sep IGKt x0, **IGLc sep IGLt × 1 (45%), IGL con BCL6 × 2 (54%)**
21	LBCLL	Neg	Neg	Neg	Neg	
22	LBCLL	**Pos (49.0%)**	Neg	**Pos (47.5%)**	Neg	IGH con BCL2 × 0; **IGH con MYC × 2 (66%)**
23	LBCLL	**Pos (45.0%)**	Neg	**Pos (53.5%)**	Neg	IGH con BCL2 × 0; **IGH con MYC × 2 (66%)**
24	LBCLL	Neg	**Pos (41.5%)**	Neg	Neg	IGKc sep IGKt x0, IGLc sep IGLt × 0
25	LBCLL	**Pos (50.5%)**	Neg	**Pos (55.5%)**	Neg	IGH con BCL2 × 0; **IGH con MYC × 2 (62%)**
26	LBCLL	Neg	Neg	**Pos (52.0%)**	Neg	IGKc sep IGKt x0, IGLc sep IGLt × 0, IGH con MYC × 0
27	LBCLL	Neg	**Pos (66.0%)**	Neg	Neg	IGKc sep IGKt x0, IGLc sep IGLt × 0
28	LBCLL	**Pos (77.0%)**	**Pos (74.5%)**	Neg	Neg	IGH con BCL2 × 0; **IGH con BCL6 × 2 (70%)**
29	LBCLL	**Pos (75.5%)**	Neg	**Pos (74.0%)**	Neg	IGH con BCL2 × 0; **IGH con MYC × 2 (84%)**

[a]Partial interphase FISH karyotypes indicating the most frequent signal pattern, described according to ISCN1995 (Mitelman, 1995). FISH, fluorescence *in situ* hybridization; PCFCCL, primary cutaneous follicle center cell lymphoma; PCMZL, primary cutaneous marginal zone lymphoma; LBCLL, large B-cell lymphoma of the leg; neg, negative; pos, positive (i.e. break apart pattern); t, telomeric; c, centromeric.

detected by Ki-67 staining was between 25% and 75% in three analyzed cases, which is below the typical level of BL, where the proliferating cell fraction reaches nearly 100% (Diebold *et al*, 2001; Nakamura *et al*, 2002). Furthermore, staining for TdT was negative in four analyzed cases and a latent EBV infection was ruled out by *in situ* hybridization with a PNA probe in all five cases. Thus, the diagnosis of a cutaneous manifestation of BL can be definitely ruled out in the LBCLL with t(8;14). Remarkably, we and others have recently shown that LBCLL carry a high load of genomic imbalances, particularly gains in 18q, 1q, 7, 12q and Xp, and losses in 6q (Mao *et al*, 2002; Hallermann *et al*, 2003), whereas classical BL mostly show rather simple karyotypes devoid of many chromosomal changes secondary to the Burkitt translocation. In summary, many LBCLL display a genotypic and phenotypic profile similar to that of systemic DLBCL. The pattern of chromosomal changes including *MYC* and *BCL6* breaks, which are supposed to be introduced in a mislead germinal center reaction, along with the presence of ongoing somatic hypermutation and a typical gene expression profile suggests at least a major subset of LBCLL to be derived from germinal center B cells.

The clinical outcome of the LBCLL group was significantly worse than the PCFCCL/PCMZL (5 y overall survival

rate 39% *vs* 100%; p = 0.02), which is in agreement with previously published reports (Vermeer *et al*, 1996; Willemze *et al*, 1997; Grange *et al*, 2001). Nevertheless, the prognostic impact of translocations affecting *MYC* and *BCL6* in PCBCL could not be properly assessed in the present series due to the presence of translocations only in the poor prognosis group and the small sample size, which prevented multivariate analyses.

All six PCFCCL and all nine PCMZL studied herein completely lacked evidence for breakpoints in the *IGH*, *MYC*, *BCL6*, or *MALT1* locus. As the tumor cell content in all cases was more than 30% based on morphology and immunohistology, it is highly unlikely that the applied interphase FISH assays known to have a sensitivity of much less than 10% missed the translocations affecting one of these loci. Remarkably, recent comparative genomic hybridization (CGH) studies also suggested a much lower incidence of genomic imbalances in PCFCCL and PCMZL than in LBCLL (Mao *et al*, 2002; Hallermann *et al*, 2003). Thus, in agreement with the less aggressive clinical course (Vermeer *et al*, 1996; Willemze *et al*, 1997; Grange *et al*, 2001), these both subtypes of PCBCL show a much lower incidence of chromosomal instability than LBCLL. Neither recurrent chromosomal aberrations nor imbalances have so far been reported for PCFCCL and PCMZL by cytogenetic and CGH studies. By FISH the translocation t(14;18), the hallmark of systemic FL, was neither detected in the present series of PCFCCL nor in any other PCBCL studied (Kim *et al*, 2003). These findings are in agreement with several (but not all) PCR studies aiming to identify *BCL2/IGH* juxtaposition in PCBCL (Child *et al*, 2001; Goodland *et al*, 2002). Similarly, breakpoints affecting the *MALT1* locus in 18q21, which are recurrent in marginal zone lymphomas of MALT type, were not observed in any of the PCMZL investigated. This is in line with a recent study suggesting t(11;18) involving MALT1 to be absent in 27 MALT lymphomas of the skin (Ye *et al*, 2003). Streubel *et al* (2003) recently reported the presence of t(14;18) involving *MALT1* in three of 11 cutaneous MALT lymphomas. As two of theses cases were described as Ann Arbor stage IV, it remains unclear whether these cases constitute PCMZL.

So far, no recurrent chromosomal aberration has been identified in PCFCCL and PCMZL that distinguishes these lymphomas from their systemic counterparts. This contrasts with the findings in LBCLL where obviously a considerable overlap with systemic DLBCL exists with regard to the presence of recurrent genetic aberrations. Nevertheless, it has to be taken into account that also systemic FL and

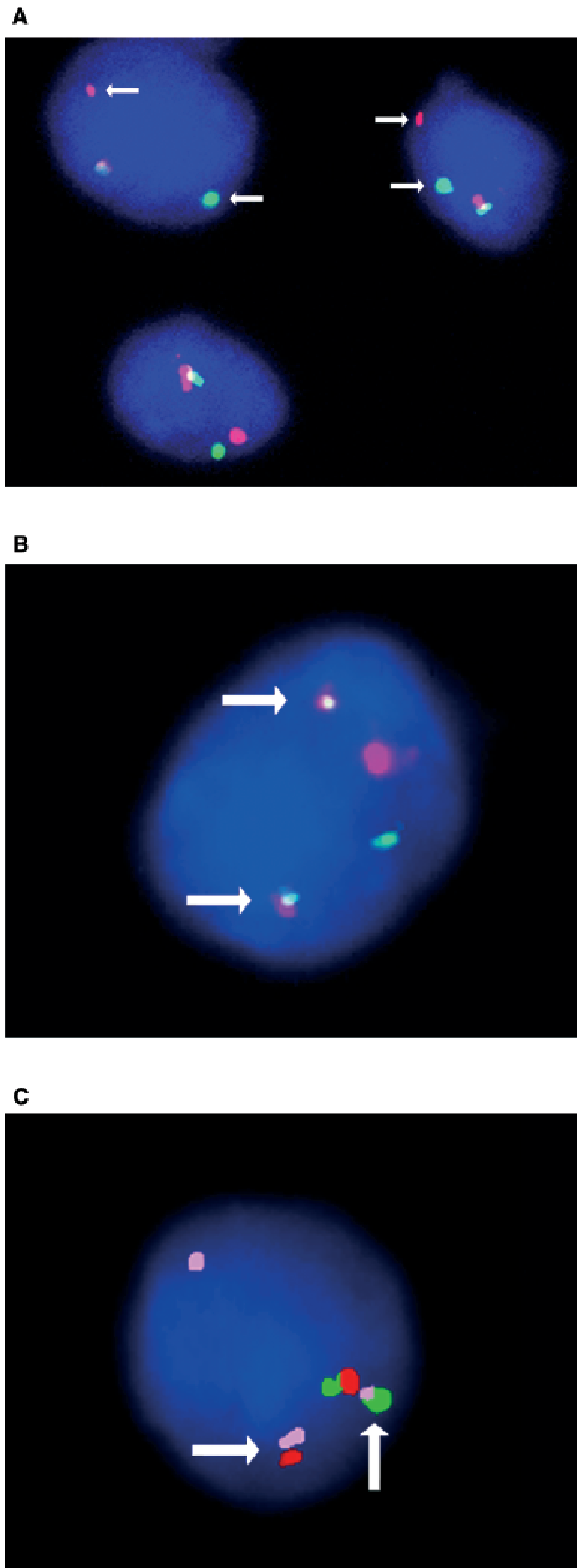

Figure 1
(*A*) Interphase nuclei from case 28 hybridized with the LSI *IGH* break apart probe (Vysis). The two nuclei at the top display a significant dissociation of the red and green signals (*arrows*) indicating the presence of a translocation affecting the *IGH*. (*B*) Interphase nucleus from case 16 hybridized with the LSI *MYC/IGH* double fusion probe (Vysis). The presence of two fused red and green signals (*arrows*) indicates that a translocation t(8;14)(q24;q32) juxtaposing the *MYC* and *IGH* loci has taken place. The isolated red and green signals point to the unrearranged *MYC* and *IGH* alleles, respectively. (*C*) False color display of an interphase nucleus from case 28 (the same as in *A*) hybridized with the triple color *BCL6/IGH* probe. A probe spanning the *BCL6* breakpoint region labeled with a blue fluorochrome (but shown in pink) was combined with the LSI *IGH* break apart probe (Vysis). The presence of two dissociated red and green signals each colocalizing with pink dots (*arrows*) indicates the occurrence of a translocation t(3;14)(q27;q32) juxtaposing the *IGH* and *BCL6* loci. One isolated pink signal and one red/green colocalization point to the unaltered *BCL6* and *IGH* alleles, respectively.

marginal zone lymphomas exist lacking the characteristic primary chromosomal changes. Future studies including gene expression profiling have to unravel whether these subgroups of systemic lymphomas are related to PCFCCL and PCMZL or whether different pathogenetic mechanisms underlay the development of these cutaneous B-cell lymphomas.

Material and Methods

Patients features Fresh frozen biopsies from 29 patients with PCBCL were collected prior to therapy for diagnostic purpose. The patients gave informed consent to the biopsies. According to the criteria of the EORTC classification (Willemze *et al*, 1997), this group included six primary cutaneous follicle center cell lymphomas (PCFCCL), nine primary cutaneous marginal zone lymphomas (PCMZL), and 14 large B-cell lymphomas of the leg (LBCLL). The classification according to the WHO is shown in Table I. At the time of diagnosis, in all cases lymphoma manifestations were restricted to the skin, as confirmed by complete staging procedures including blood analyses, computerized tomography of the chest, ultrasound of peripheral lymph nodes and the abdomen, and a negative bone marrow histology. The diagnosis PCBCL was confirmed by demonstration of clonal *IGH* gene rearrangement and/or monotypic light chain expression by immunohistochemistry. Patients features are also shown in Table I.

Immunohistochemistry Biopsy material was either split or two neighboring punch biopsies were taken from the same skin lesion. For FISH one part of the material was snap frozen in liquid nitrogen, the other part was fixed in 4% buffered formalin, embedded and cut into 2 μm sections. Besides hematoxylin/eosin and Giemsa staining, immunohistochemistry was performed. For immunohistochemistry, the sections were dewaxed in xylol (Merck, Darmstadt, Germany), rehydrated in decreasing serial dilutions of ethanol, and stained with antibodies to CD20 (clone L26, Dako, Glostrup, Denmark), Bcl2 (clone 124, Dako), and to Bcl6 (clone PG-B6p; Dako). Furthermore, part of the cases were stained with Ki-67 (clone MIB1, Dako), CD10 (clone 56C6, Novocastra, Newcastle-Upon-Tyne, UK), and TdT (code 004, LavVision/Neomarkers Fremont). The NexES IHC immunostaining device (Ventana Medical Systems, Strasbourg, France) was used in combination with an alkaline phosphatase anti-alkaline phosphatase technique (APAAP) and Fast Red as substrate according to the manufacturer's instructions (Ventana Medical Systems). When more than 50% of lymphoma cells showed a distinct staining with an antibody, the case was considered as positive.

Furthermore, latent Epstein–Barr virus infection was studied using the PNA *In Situ* Hybridization Detection Kit (code K5201, Dako) according to instructions of the manufacturer.

Interphase FISH Interphase FISH was performed without knowledge of the histopathologic diagnosis. For FISH, thick cryosections (60 μm) were mechanically disaggregated and digested in 5% pepsin solution for 5 min at 37°C. Cytospins were prepared and the cells were fixed for 10 min in 1% paraformaldehyde solution, dehydrated in graded ethanol series, and air-dried at room temperature. Directly labeled probes flanking the *IGH* (LSI *IGH* break apart probe, Vysis, Downers Grove, Illinois), *IGK*, *IGL* (break-apart probes, described in Martin-Subero *et al*, 2002b), *MYC* (LSI *MYC* break-apart probe, Vysis), *BCL6* (LSI *BCL6* break apart probe, Vysis), and *MALT1* (LSI *MALT1* break-apart probe, Vysis; and Dierlamm *et al*, 1999) loci were applied to study the occurrence of chromosomal translocations affecting those genes. The LSI *IGH/MYC*/CEP8 and LSI *IGH/BCL2* dual fusion probes (all from Vysis) were applied to assess the presence of the t(8;14) and t(14;18) chromosomal translocations, respectively. For the detection of the t(3;14) (*IGH/BCL6*) and t(3;22) (*IGL/BCL6*), the above quoted *IGH* and *IGL* break-apart probes were combined with a probe spanning the *BCL6* breakpoint region (Sanchez-Izquierdo *et al*, 2001) differentially labeled with diethyl aminomethyl coumarin (DEAC, blue fluorescence). Non-commercial probe preparation and FISH were performed according to previously published protocols (Martin-Subero *et al*, 2002a, b).

Results were evaluated using a fluorescence microscope (Axioplan or Axioskop, Zeiss, Göttingen, Germany) equipped with the appropriate filter sets and connected to a charge coupled device camera. Evaluation of at least 200 nuclei was attempted per cytospin preparation whenever possible.

Evaluation criteria and diagnostic cutoffs for the applied double-color FISH probes have been determined recently. For all these assays, the cutoffs were far below 10% applying appropriate evaluation criteria (Martin-Subero *et al*, 2003). Based on these controls and the estimated tumor cell content in the PCBCL samples studied herein (at least 30% based on morphology and immunohistology) the diagnostic cutoff was uniformly set for all probes at 10% (Montesinos-Rongen *et al*, 2002; Martin-Subero *et al*, 2002b, 2003).

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