



Concomitant 1p36 deletion and *TNFRSF14* mutations in primary cutaneous follicle center lymphoma frequently expressing high levels of EZH2 protein

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Abstract

Primary cutaneous follicle center lymphoma (PCFCL) is an indolent variant of follicular lymphoma (FL) with limited information available on the genetic background of the disease. The genetic hallmark of nodal FL, the t(14;18) translocation, affecting the *BCL2* gene, is rare in PCFCL. Loss of 1p36, the most common secondary chromosomal abnormality in nodal FL, has been recently reported in 16.7% of PCFCL cases. In order to further characterize PCFCL, 21 cases were analyzed using interphase fluorescence in situ hybridization with *BCL2* break apart and 1p36/1q25 dual color probes. Sanger sequencing was used to investigate *TNFRSF14* and *EZH2* mutations and immunohistochemistry to assess *BCL2*, *EZH2* protein expressions. 1p36 deletion occurred in 22% (5/21), *BCL2* gene break in 10% (2/20) of the PCFCL cases. Mutations of the candidate tumor suppressor gene of the 1p36 region, *TNFRSF14* mutations were detected in 4/17 (23.5%) cases with 2 cases presenting with concurrent 1p36 deletion. *EZH2* hotspot mutations at Y641, A682, and A692 were not found. High *EZH2* protein expression associated with a *BCL2* negative phenotype was observed in 43% (9/21) of the cases. *BCL2* gene break or 1p36 deletion did not impact the prognosis; however, they showed association with advanced stages at diagnosis ($p = 0.016$) and a tendency with shorter event free survival ($p = 0.052$). In conclusion, 1p36 deletion co-occurs with acquired *TNFRSF14* mutations, suggesting a role of this tumor suppressor gene in the development of a subgroup of PCFCL. High *EZH2* protein expression associated with *BCL2* negative phenotype is common and might represent an ideal therapeutic target.

Keywords Primary cutaneous follicle center lymphoma · del 1p36 · *TNFRSF14* mutation · *BCL2* · *EZH2*

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Introduction

Primary cutaneous follicle center lymphoma (PCFCL) is an indolent variant of follicular lymphoma with >95% 5-year survival [1]. At diagnosis, solitary or multiple lesions, papules, plaques, or nodules are found, most often in a single region, particularly in the head and neck region or involving the trunk. Systemic dissemination occurs rarely; however, the disease often relapses. Local treatment, surgical excision, and low dose radiotherapy is highly effective with a complete remission rate approaching 100% [2]. Histologically, nodular, diffuse, or mixed growth pattern composed of centrocytes mixed with scattered centroblasts is seen [3]. Early studies emphasized the lack of *BCL2* protein expression and *BCL2*/IgH translocation in PCFCL as an important diagnostic tool to distinguish it from nodal follicular lymphoma (NFL), as it occurs in almost 90% of the systemic, nodal cases [4, 5].

Further studies observed higher frequencies of BCL2 protein expression (10–57%) and associated BCL2 translocation (10–41%) in several series of PCFCL without prognostic significance [6–9]. Recently, deletion involving the 1p36 region (del 1p36) was identified as the most frequent cytogenetic abnormality affecting 16.7% of the PCFCL cases [10]. The candidate tumor suppressor gene of this region, *TNFRSF14*, belonging to the tumor necrosis factor receptor (TNFR) superfamily, is frequently mutated in NFL; however, *TNFRSF14* mutation analysis has not been performed in PCFCL to date [11, 12]. Similarly, the mutation status and expression of the epigenetic regulator, EZH2, an attractive therapeutic target being involved in the pathogenesis of NFL, is unknown in PCFCL [13].

The aim of our study was to perform a cytogenetic, molecular, and immunohistochemical analysis, including mutation analysis of the previously unreported mutation targets *TNFRSF14* and *EZH2* in a cohort of 21 PCFCL cases and assess the clinical relevance of these findings.

Materials and methods

Patient selection

We collected and reevaluated 21 cases of PCFCL diagnosed at the Semmelweis University, Budapest between 2009 and 2017. Clinical data were collected from the medical records in the university database retrospectively. Clinical examinations included thorough physical, routine laboratory examinations (complete blood cell count, serum chemistry studies), chest X-ray, and CT scan. Peripheral blood and bone marrow involvement was investigated by flow cytometry and histology. Staging was based on the International Society for Cutaneous Lymphomas EORTC staging proposal for nonmycosis fungoides/Sézary syndrome cutaneous lymphomas. The male/female ratio was surprisingly low in our cohort, it could be due to the small number of cases, no other reason is identified.

Histology and immunohistochemistry

Histological analysis of the skin biopsies was performed on formalin-fixed, paraffin-embedded (FFPE) tissues. Twenty-nine samples of 21 patients were examined; in case of 7 patients, more than one biopsy was taken at different time points. The histological reevaluation was performed by two hematopathologist (CsJ, SZA) according to the revised WHO classification and EORTC classification [3, 14]. The main histological differential diagnosis of PCFCL are reactive follicular hyperplasia, primary cutaneous marginal zone lymphoma (PCMZL), and diffuse large B-cell lymphoma (DLBCL) with GCB phenotype or leg type. The main

pathological findings differentiating PCFCL from reactive lymphoid proliferations were dense, deep dermal, or subcutaneous infiltration of CD20 positive B-cells, clonal expression of IgG light chains, or clonal IgH rearrangement; CD10 and BCL6 expressions for the vast majority of the tumor cells together with centrocytic cytology excluded PCMZL; nodularity with expanded presence of FDC network and MUM1 negativity were the main criteria to exclude DLBCL.

The LEICA Bond Max fully automatized staining system was used for immunohistochemistry. For antigen localization, DAB polymer (DAKO Denmark) was used. The antibodies employed recognized the following antigens: CD3 (clone QBEnd10), CD20 (clone L-26), Ki67 (clone MIB-1), BCL2 (clone 124), BCL6 (clone PG.B6p), MUM1 (clone M7259), (DAKO, Glostrup, Denmark), CD21 (clone 2G9, Leica, Saint-Petersburg, Russia), and EZH2 (clone 11/EZH2, BD Transduction Lab, San Jose, USA), tonsil samples were utilized as controls.

Stained slides were scanned at $\times 20$ magnification using a Panoramic scan instrument (3D Histech, Budapest, Hungary) equipped with a Carl Zeiss objective (NA = 0.83; Carl Zeiss MicroImaging Inc., Jena, Germany). Ki67 expression was quantified with CaseViewer 2.0 (3D Histech, Budapest, Hungary), extended with QuantCenter 2.0 software. EZH2 protein expression was determined in the neoplastic follicles using a semiquantitative method, cases were considered positive if there was moderate to high intensity (2+ or 3+) staining in $> 20\%$ of the neoplastic cells. EZH2 positivity was considered high if there were $> 50\%$ positive cells in the neoplastic follicles. The cut off value for BCL2 positivity was 80%, for CD10, BCL6, and MUM1 30%.

IgH gene rearrangement analysis was carried out for clonality assessment using the BIOMED -2 Concerted Action protocol [15].

Interphase fluorescence in situ hybridization (FISH)

Interphase FISH was performed on 3- μ m-thick tissue sections using split signal FISH DNA probe for BCL2/18q21 (Vysis LSI BCL2 Dual Color Break Apart rearrangement probe, ABBOTT Molecular diagnostic, Rungis, France) and dual-color probe for 1p36/1q25 (Vysis LSI 1p36 SpectrumOrange/Vysis LSI 1q25 SpectrumGreen Dual-Color Probe, ABBOTT Molecular Diagnostic, Rungis, France) according to the manufacturer's instructions and as described before [16]. The FISH signals were visualized using a Nikon Eclipse E600 epifluorescence microscope. Image analysis was performed by using Lucia Cytogenetics image acquisition system (Laboratory Imaging, Republic of Czech). The cut-off for BCL2 split positivity and for del 1p36 assessment, lack of one orange (1p36) signal, was set at 20% counting 200 tumor cell nuclei.

Mutation analysis

DNA was extracted from formalin-fixed paraffin-embedded (FFPE) skin biopsy specimens with tumor tissue presence exceeding 80% using the QIAamp DNA FFPE Tissue Kit (Qiagen) according to the manufacturer's instructions. Bidirectional Sanger sequencing of Y641 (exon 16), A682, and A692 (exon 18) hotspots of the *EZH2* gene and the whole coding sequence of the *TNFRSF14* gene were carried out using the primers listed in Supplementary Table 1.

Response criteria and statistical analysis

The association between the different clinicopathological and immune parameters was estimated and compared using the Mann-Whitney *U* test for continuous variables. Categorical data were compared using Fisher's exact test or Mann-Whitney statistics. A *p* value of < 0.05 was considered as statistically significant. The Kaplan Meier method was used for the comparison of event free survival (EFS). Time was defined as the time elapsed from the date of diagnosis to the date of first disease recurrence or death. Time was censored for patients who had not experienced disease relapse or had not died at the time of last follow-up. Potential prognostic factors examined in our study were extent of cutaneous involvement according to the TNM classification (T1 vs T2/T3), site of cutaneous lesions, proliferation rate determined by Ki67 expression, BCL2 expression, EZH2 expression, presence of cytogenetic abnormalities (deletion of 1p36 or BCL2 break), and presence of *TNFRSF14* mutation [17]. All statistical analyses were performed using the SPSS 13.0 software).

Results

Clinical findings and follow-up

The clinical findings are summarized in Table 1. In our series of 21 patients with PCFCL, the male-to-female ratio was 4:17, with patients being exclusively Caucasian. The median age at diagnosis was 63 years (range 30–82). The clinical presentation was described as erythematous papules, plaques, or nodules. Solitary lesion (stages: T1a, b) was found in 57% of the patients (12/21), multiple lesions were present in 43% (9/21) of the patients (stages: T2a–T3b) especially on the head and neck region (11/21), and on the trunk and upper extremities (8/21) or both (2/21). The lower extremities were not involved (Fig. 1a). The patients did not have prior diagnosis of lymphoproliferative disease and any associated systemic symptoms (fever, weight loss, or night sweating). No extracutaneous disease, lymph node, or peripheral blood involvement were found at initial and within 6 months of diagnosis. Treatments

included surgical excision, radiotherapy or both, and chemotherapy for two patients. Fifteen patients responded initially with complete remission (CR) (Fig. 1b), 3 with partial remission (PR). Three patients had multiple lesions at diagnosis and surgical excision was partial. They were followed without any treatment, spontaneous remission occurred in one of them, the other two have stable disease (SD). Relapse occurred in 10 cases, extracutaneous spreading to lymph node was found in one case, who responded with CR after chemotherapy (RCHOP). At the time of the last follow-up, one patient died of pulmonary embolization after 193 months, while in CR, unrelated to the cutaneous lymphoma. All other patients are alive with a median follow-up time of 45 months (7–93), (Table 1).

Histological and immunohistochemical findings

Pure follicular infiltration pattern was observed in most of the cases (17/21), (Fig. 2a, b) Four cases presented with mixed, follicular/diffuse infiltration pattern. The predominance of small/medium sized centrocytes were seen with few mixed scattered centroblasts in 11/21 cases (grades 1 and 2) while large centrocytes and centroblasts dominated > 50% of the cells (grade 3) in 10/21 cases (Fig. 2 c, d). Results of the immunohistochemistry are summarized in Table 2. High proliferation rates above 40% were found in 10/21 cases assessed with Ki67 immunohistochemistry (Fig. 2e, f). BCL2 protein expression was observed in 10/21 cases (Fig. 2 g, h). The BCL2 positive cases tended to have lower proliferation rate than the BCL2 negative ones, but the difference was not significant (*p* = 0.198) (Table 3). BCL6 protein was expressed by the follicular B-cells in all but one case, in which BCL6 negative tumor cells were positive for CD10. EZH2 protein expression was detected in the neoplastic nodules in all BCL6 positive cases (*n* = 20) with moderate to high intensity (Fig. 2i, j). High EZH2 protein expression (> 50%) was found in 11/21 cases in the neoplastic nodules and was associated with BCL2 negative phenotype (*p* = 0.009), (Table 3). BCL2 and EZH2 expressions were independent of clinical features and outcome.

Association of the FISH findings with clinical and morphological data

Using FISH, *BCL2* gene break was found in 2/20 cases (10%, Fig. 3a), while 1p36 deletion was detected in 5/21 cases (22%, Fig. 3b). Cytogenetic abnormalities were strongly associated with stage at diagnosis: more advanced clinical stages with multiple lesions (> T1) were determined at diagnosis in 6/7 (86%) cases with cytogenetic abnormalities compared to cases without *BCL2* gene break or 1p36 deletion (3/14, 21%), (*p* = 0.016, Table 4). Other clinical features, including response to therapy and relapse rate did

Table 1 Clinical characteristics of the PCFCL cohort

Case	Sex	Age	Stage	Localization	Therapy	Relapse (EFS, months)	Disease outcome (follow-up, months)
1	F	75	T2b	Head and neck	Surgical excision + CVP	Yes*(9)	CR (99)
2	F	30	T1a	Head and neck	Surgical excision	Yes (47)	CR (56)
3	F	52	T2a	Head and neck	Radiotherapy	No	CR (14)
4	F	63	T1a	Head and neck	Radiotherapy	No	CR (44)
5	F	67	T1a	Head and neck	Surgical excision	No	CR (55)
6	F	48	T1a	Trunk	Surgical excision	No	CR (47)
7	M	40	T2a	Head and neck	Radiotherapy	No	CR (40)
8	F	68	T2a	Head and neck	Surgical excision	Yes (6)	PR (19)
9	M	42	T1a	Trunk	Surgical excision	Yes (8)	SD (16)
10	F	49	T1a	Trunk	Radiotherapy	Yes (78)	DUD (193)
11	F	66	T2a	Trunk	Radiotherapy	Yes (18)	SD (18)
12	F	63	T1a	Head and neck	Surgical excision	Yes (5)	CR (6)
13	F	56	T2b	Trunk	Surgical excision	Yes (34)	CR (64)
14	F	69	T3a	Head and neck, and upper extremities	No	No	SD (24)
15	F	68	T2a	Head and neck	Surgical excision	No	SD (7)
16	F	59	T1b	Upper extremities	Surgical excision + radiotherapy	Yes (23)	PR (31)
17	M	70	T1b	Head and neck	Surgical excision	No	PR (14)
18	F	63	T1a	Trunk	Surgical excision + radiotherapy	No	CR (91)
19	F	52	T1a	Trunk	Surgical excision	Yes (33)	SD (68)
20	F	67	T1a	Head and neck	Surgical excision	No	CR (80)
21	M	82	T3b	Head and neck; trunk and upper extremities	R + CVP	No	CR (12)

*Systemic lymph node dissemination detected by *PET-CT* CR = complete remission, CVP = cyclophosphamide + vincristine + prednisolone, DUD = died of unrelated cause, EFS = event-free survival, F = female, M = male, PCFCL = primary cutaneous follicle center lymphoma, PR = partial remission, R = rituximab, SD = stable disease

not show any difference between the two groups. Only event-free survival (EFS) showed a tendency to be shorter in the group with cytogenetic abnormalities; however, it did not reach statistical significance ($p = 0.052$). Ki67

expression tended to be higher in cases with cytogenetic aberrations compared to the negative ones (5/7, 71% vs 5/14, 36%, $p = 0.183$), with similar BCL2 protein expression in the two groups (Table 3).

Fig. 1 Clinical picture of a primary cutaneous follicle center lymphoma (PCFCL) with 1p36 deletion (case 1). **a** Patient presenting with multiple erythematous papules and nodules on the head. **b** Complete remission of the tumor 8 months after diagnosis treated with radiotherapy

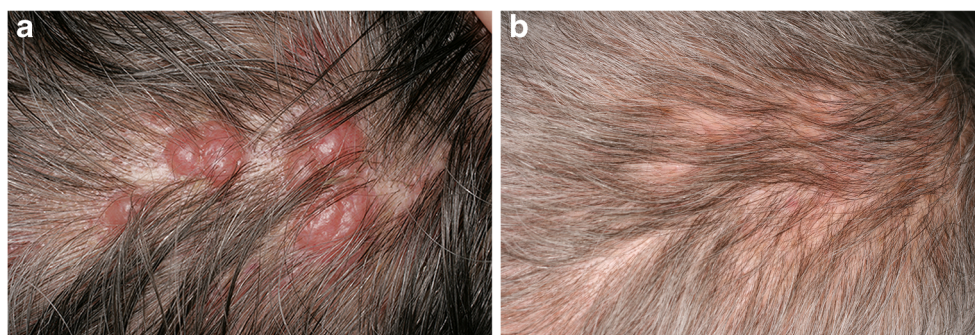
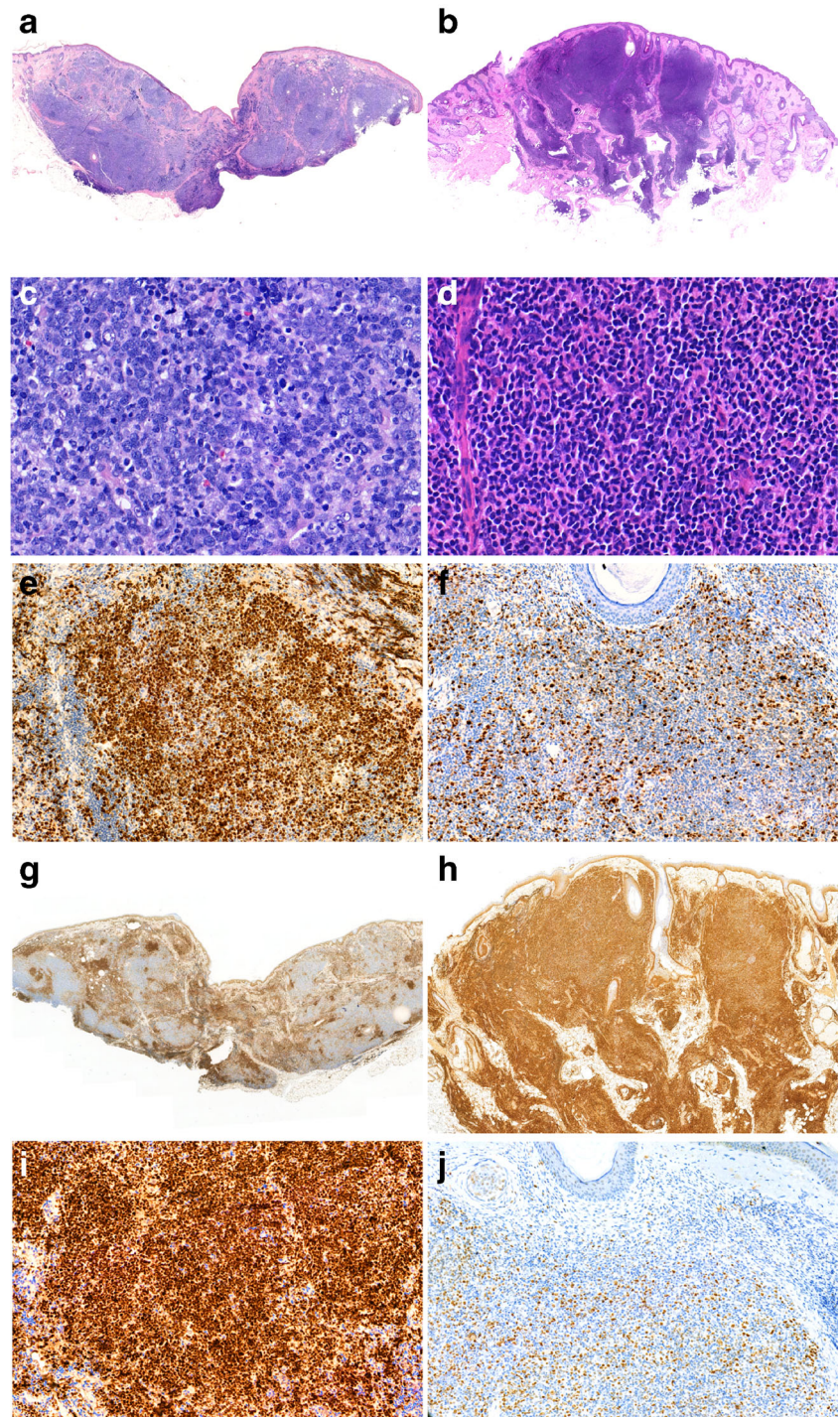


Fig. 2 Histopathologic features of PCFCLs with high-grade and low-grade morphology exhibiting different levels of Ki67, BCL2 and EZH2 protein expression. **a**, Hematoxylin-eosin (H&E) staining of the lesion (case 15) showing nodular growth pattern and high-grade morphology. **b** H&E staining of the lesion (case 2) showing nodular growth pattern and low-grade. **c** Predominance of large centroblasts and presence of scattered tingible body macrophages with apoptotic bodies in the neoplastic nodule (case 15). **d** Predominance of smaller centrocytes (case 2). **e** Immunohistochemistry (IHC) showing high Ki67 expression (> 40%) of the tumor cells (case 15). **f** IHC showing low Ki67 expression (< 40%) of the tumor cells (case 2). **g** IHC showing BCL2 negative tumor cells in the center of the nodule (case 15). **h** BCL2 protein expression of the tumor cells (case 2). **i** High EZH2 protein expression of the B-cells in the neoplastic nodule (case 15, EZH2 > 50%). **j** Low EZH2 protein expression of the B-cells in the neoplastic nodule (case 2, EZH2 < 50%)



Mutation analysis and association with clinical data, morphology, and FISH results

We assessed the mutation status of *TNFRSF14* by Sanger sequencing in 17 patients with available DNA samples. *TNFRSF14* mutations were detected in 4/17 (23.5%) patients with three non-sense mutations (c.35G > A; p.Trp12*) previously reported in NFL and one previously unreported

missense variant (c.157 T > G; p.Cys53Gly) targeting the extracellular domain which is likely to interfere with the ligand binding activity of TNFRSF14 [18]. Variants previously reported in the dbSNP and 1000 Genomes databases were not considered. Two cases with *TNFRSF14* mutations harbored concomitant 1p36 deletions resulting in complete loss of functional TNFRSF14. Significant impact of mutations on clinical features and disease outcome was not observed. Regarding the

Table 2 Immunophenotype and cytogenetic and molecular features

Case	CD10*	BCL6*	MUM1*	BCL2*	EZH2*	Ki67*	<i>BCL2</i> split	1p36 del	<i>TNFRSF14</i> mutation	IgH
1	+	+	–	–	70%	44%	–	+	–	N/A
2	+	+	–	+	20%	30%	–	–	–	+
3	+	+	–	+	10%	15%	–	–	–	N/A
4	+	+	–	+	50%	43%	–	–	–	+
5	+	+	–	–	60%	44%	–	–	N/A	N/A
6	+	+	–	–	40%	50%	N/A	–	c. 35G > A (p. Trp12*)	N/A
7	+	+	–	–	30%	63%	–	–	–	+
8	+	+	–	+	20%	49%	+	–	N/A	+
9	+	+	–	–	50%	22%	–	–	N/A	+
10	+	–	–	+	0	< 1%	–	–	–	N/A
11	+	+	–	–	70%	30%	N/A	–	–	–
12	+	+	–	+	40%	36%	–	–	–	+
13	+	+	–	–	90%	53%	–	+	–	+
14	+	+	–	+	80%	35%	–	+	c. 157 T > G (p. Cys53Gly); 0.995 [★] ; 0.00 [◇]	–
15	+	+	–	–	70%	89%	–	+	c. 35G > A (p. Trp12*)	N/A
16	+	+	–	+	30%	38%	–	+	–	+
17	+	+	–	–	50%	57%	–	–	–	–
18	+	+	–	–	50%	22%	–	–	–	N/A
19	+	+	–	–	50%	10%	–	–	c. 35G > A (p. Trp12*)	N/A
20	+	+	–	+	30%	20%	–	–	–	N/A
21	+	+	–	+	40%	55%	+	–	N/A	+

*Protein expression, 1p36del = 1p36 deletion, IgH=IgH rearrangement

★ PolyPhen-2 score, probably damaging; ◇ SIFT score, < 0.05 deleterious

phenotypic features, 3/4 of the mutant cases were characterized by BCL2 negativity and 3/4 demonstrated high EZH2 protein expression. (Table 2). The presence of gain of function mutations affecting the methyltransferase *EZH2* gene were

also assessed at three recurrent mutation hotspots (Y646, A682, and A692), and no mutations were detected in our PCFCL cohort.

Table 3 EZH2 and Ki67 expressions according to the BCL2 status in PCFCL

	BCL-2+ (n = 10)	BCL-2-(n = 11)	p
Ki67			
High (> 40%)	3 (30%)	7 (64%)	0.198
Low (< 40%)	7 (70%)	4 (36%)	
EZH2			
High (> 50%)	2 (20%)	9 (81%)	0.009
Low (< 50%)	8 (80%)	2 (19%)	

Discussion

The aim of the present study was to better understand the genetic background of PCFCL by performing FISH analysis of the *BCL2* break and deletion of 1p36, and mutation analysis of the *TNFRSF14* and *EZH2* genes. We report for the first time mutations of the *TNFRSF14* gene occurring simultaneously with 1p36 deletion resulting in loss of functional copy of the gene. High EZH2 protein expression was found in about half of the PCFCL cases, while EZH2 hotspot mutations was not detected in our cohort. We identified 1p36 deletion in 5/21 (22%)

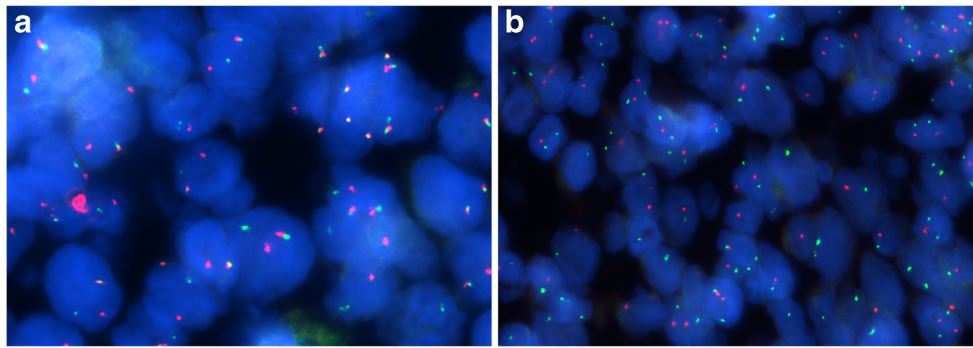


Fig. 3 FISH images of representative cases demonstrating *BCL2* break and 1p36 deletion. **a** PCFCL showing *BCL2* rearrangement using *BCL2* Dual Color Break Apart rearrangement probe, with isolated green and red

signals. **b** PCFCL demonstrating 1p36 deletion using dual-color probe for 1p36/1q25, seen with unique red signals (1p36), whereas two green signals (1q25 control) are observed in tumor cell nuclei

cases. Szablewski et al. were the first to report one case of PCFCL with 1p36 deletion in a cohort of 25 cases with 19 (76%) being *BCL2* positive and 12 (48%) harboring *BCL2* gene break [19]. A more recent manuscript from the same group described 4 cases out of 29 (16.7%) positive for 1p36 deletion, but this cohort included only 14 (48%) *BCL2* positive cases and 3 cases (11%) with *BCL2* gene break [10]. Our results are consistent with their findings, as our cohort included 10 (47%) cases positive for *BCL2*, and we identified *BCL2* gene break in 2 out of 20 cases (10%). In both cohorts, 1p36 deletion was more common than *BCL2* break: 22% compared to 10 and 16.7% compared to 11%, respectively, and the ratio of *BCL2* positive tumors was also comparable (47 vs. 48%).

These genetic abnormalities were mutually exclusive, while in NFL, 1p36 deletion was the most common secondary cytogenetic lesion besides the t(14;18) translocation. Based on this observation, FISH examination of the *BCL2* translocation and 1p36 deletion may be applied as a differential diagnostic tool to distinguish primary cutaneous and nodal FL involving the skin. Frequent 1p36 deletions and copy number neutral loss of heterozygosity were also identified in special types of NFL patients negative for t(14;18): localized FL in the inguinal region first described by Katzenberger T et al., and pediatric FL (PFL), both with localized disease and excellent prognosis [20, 21].

Cytogenetic abnormalities in our series did not show any association with morphological features and disease outcome,

Table 4 Clinical, morphological, and molecular features of cases with or without cytogenetic abnormalities

	Del 1p36 or <i>BCL2</i> break present	Without del 1p36 or <i>BCL2</i> break
Clinical data		
Number of cases	7	14
Male/female	1/6	3/11
Mean age (y)	66	55
Localization		
Head and neck	5 (71%)	8 (57%)
Another site	4 (57%)	6 (43%)
Stage		
T1	1 (14%)	11 (79%)
T2 or T3	6 (86%)	3 (21%)
Relapse rate	57%	42%
Response		
CR	3 (43%)	9 (64%)
AWD	4 (57%)	4 (29%)
DUD	—	1 (7%)
Immunohistochemistry		
BCL-2+	4 (57%)	6 (43%)
BCL-6+	7 (100%)	13 (93%)
Ki67 > 40%	5 (71%)	5 (36%)
<i>TNFRSF14</i> mutation	2 (29%)	2 (14%)

AWD = alive with disease (partial remission and stable disease), CR = complete remission, DUD = died of unrelated cause, del 1p36 = 1p36 deletion

although, relapse tended to occur in shorter time for cases with cytogenetic aberrations and the only case with systemic dissemination also carried a deletion of 1p36. Our two cases with *BCL2* break responded to therapy with CR or PR and one case showed relapse during the 12 and 19 months follow-up. Multiple lesions were found significantly more often at diagnosis in cases with *BCL2* break or 1p36 deletion. Vernadet et al. found comparable results with no significant effect of cytogenetic aberrations on disease outcome; however, CR was achieved less frequently in cases of PCFCL with cytogenetic aberrations compared to those without chromosomal abnormalities [10]. In their study, disease stage at diagnosis was not defined. *BCL2* break itself did not show association with clinical outcome in most studies on PCFCL; however, Ledard et al. observed association of extranodal dissemination with *BCL2* translocation [22]. Data on the prognostic significance of cytogenetic alterations in NFL are controversial: Cheng et al. described NFL cases with 1p36 deletions associated with poor prognosis, but others found no association with clinical outcome [11, 18].

Immunohistochemistry revealed *BCL2* expression in 48% of our cases, with a tendency to correlate with low proliferation rate and small cell morphology. This can be explained by the fact that, FLs with high *BCL2* expression gain resistance to apoptosis and can expand with low proliferation rate, while *BCL2* negative FL need high proliferation rates to expand. *BCL2* positivity ranged from 12 to 75% of the reported cases in the literature [7, 8, 10, 17, 23]. The discrepancy might be caused by using different antibodies or by the method evaluating the *BCL2* immunostaining. High frequency of *BCL2* protein expression was also observed in t(14;18) negative NFL, indicating other mechanisms than *BCL2* gene translocation for protein overexpression in the germinal center derived tumor cells. *BCL2* amplification could be one, but this abnormality has been seldom reported and we did not observe *BCL2* amplification in our cases [8].

Next, we investigated the possibility of *TNFRSF14* gene inactivation by somatic mutations in PCFCL and found 4 cases out of 17 harboring two types of mutations in the *TNFRSF14* gene. Three patients had c.35G > A; p.Trp12* mutation that was previously described by Cheung et al. in NFL [18]. We are the first to report a mutation occurring in exon 2 (c.157 T > G; p. Cys53Gly) possibly affecting the protein function. Two of the four mutant cases had concomitant 1p36 deletion leading to complete loss of functional *TNFRSF14*. The tumor suppressor role of *TNFRSF14* gene was suggested based on earlier observations on lymphoma and adenocarcinoma cell lines and was confirmed by recent studies on FL [24, 25]. High rate of partial or complete loss of functional *TNFRSF14* gene by deletion and/or somatic mutations was described in classical NFL by several authors, and *TNFRSF14* mutations were accompanied by copy number neutral loss of heterozygosity of the 1p36 locus in over 70% of mutated cases in pediatric-type FL, that is

a t(14;18) negative variant of FL with low genetic complexity and excellent clinical outcome [11, 18, 21]. Significant impact of mutations on disease outcome of our four patients with *TNFRSF14* gene was not observed.

We performed mutation analysis of the histone methyltransferase *EZH2* at three known mutation hotspots and assessed the *EZH2* protein expression using immunohistochemistry. While all cases were wild type for the hotspots analyzed, about half of the cases (52%) expressed high levels of *EZH2* protein, and this was strongly associated with *BCL2* negativity. However, we did not find correlation between *EZH2* and Ki67 expression. In contrast, *EZH2* was coexpressed with Ki67 in the neoplastic centroblasts in systemic follicular lymphoma like the non-neoplastic centroblasts in the germinal centers [26]. High *EZH2* expression was found to correlate with high proliferation rate in DLBCL and also in T-cell neoplasms, but not with *BCL2* expression [27, 28]. High *EZH2* expression was shown to be a good prognostic factor for ABC-type DLBCL in another study [29]. We did not find any correlation between the *EZH2* expression and clinical outcome in our PCFCL cohort.

In summary, findings of this study extend our knowledge on the genetic background of PCFCL, that seems to be heterogeneous. We identified for the first time somatic mutations of the *TNFRSF14* gene and/or 1p36 loss in the minority of the cases, a small minority showed *BCL2* rearrangement and other cases exist in which the genetic abnormalities have yet to be identified. FISH examination may help in the diagnostic process to identify primary cutaneous cases with 1p36 deletion negative for *BCL2* rearrangement, but this should be confirmed in a larger study group. A large subset of our cohort negative for *BCL2* protein expressed high level of *EZH2* protein, might be a target for future treatment.

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Authors' contribution MM, EK, TS, and MV have made substantial contributions to acquisition of the data. AG, BB, MV, DK, GP, and JC have made substantial contributions to analysis and interpretation of data. ÁSZ, CB, and AM made substantial contribution to conception and design of the manuscript, and ÁSZ has written the manuscript. AG, BB, MV, DK, GP, JC, and CB conducted cytogenetic, phenotypic, and biologic analyses. All authors have been involved in revising the manuscript and approved the final manuscript.

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Compliance with ethical standards

All protocols were approved by the Institutional Ethical Review Board (TUEB no. 7/2006).

Conflicts of interest The authors declare that they have no conflict of interest.

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