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Diagnostic and prognostic markers of cutaneous lymphomas

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CHAPTER

PERSISTENT ONCOGENIC PATHWAYS DRIVE PRIMARY CUTANEOUS DIFFUSE LARGE B-CELL LYMPHOMA, LEG TYPE AND THEIR RELAPSES

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ABSTRACT

Primary cutaneous diffuse large B-cell lymphoma, leg type (PCDLBCL-LT) is an aggressive cutaneous lymphoma with a 5-year disease-specific survival (DSS) of 56%. To improve biological understanding, disease classification, and risk stratification, we aimed to gain more insight into the molecular profiles of primary and relapsed disease in PCDLBCL-LT patients. Fifty-five PCDLBCL-LT patients were analysed with targeted next-generation sequencing for 52 B-cell-lymphoma-related genes and 'triple' fluorescence *in situ* hybridization (*MYC/BCL2/BCL6*). This included 14 cases with paired analysis of diagnostic and relapse/refractory disease. The most frequent alterations were mutations in *MYD88* (72%), *CD79B* (51%), *PIM1* (36%), *TBL1XR1* (25%), and *KMT2D* (24%), loss of *CDKN2A* (65%), and *MYC* rearrangements (26%). Paired analysis showed largely identical driver alterations in relapse/refractory disease. Additionally, disease evolution was characterized by subclonal variations due to ongoing somatic hypermutation (SHM), such as in *PIM1*. Employing survival analysis, only *MYC* rearrangements showed an inferior overall survival ($p=0.045$; HR:2.23; 95%CI:1.00-4.98), DSS ($p<0.001$; HR:4.16; 95%CI:1.63-10.60), and disease-free survival ($p=0.003$; HR:2.85; 95%CI:1.39-5.86). In conclusion, this study demonstrates that *MYD88/CD79B* mutations, loss of *CDKN2A*, and *MYC* rearrangements are early events and persistent oncogenic drivers in primary and relapsed PCDLBCL-LT. In addition, disease evolution is characterized by ongoing SHM. From the prominent drivers, only *MYC* rearrangements were associated with an inferior survival. These results support the idea that activation of the NF- κ B pathway, *MYC* signaling, and *CDKN2A* loss play a critical role in pathogenesis of PCDLBCL-LT and could provide attractive targets for novel therapeutic approaches.

INTRODUCTION

Primary cutaneous diffuse large B-cell lymphoma, leg type (PCDLBCL-LT) is a rare, extranodal variant of diffuse large B-cell lymphoma (DLBCL) defined by skin localization and no extracutaneous manifestations at time of diagnosis. It is an aggressive type of primary cutaneous lymphoma with a 5-year disease-specific survival (DSS) of only 56%.¹ First-line treatment consists of immune-polychemotherapy (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone; R-CHOP). Unfortunately, immune-polychemotherapy has a high toxicity and is often poorly tolerated by this elderly and frail patient population. As such, in case a patient has a single lesion and/or poor clinical condition, local radiotherapy only can be considered.¹ Additionally, despite high complete remission rates after initial therapy, over two-thirds of the patients early relapse within months to years.²

Currently, standardized second-line and third-line treatment guidelines are lacking and no routine classifiers are available to predict patients at risk for relapsed/refractory disease. At time of diagnosis, few factors are associated with an inferior survival: multiple lesions, loss of 9p.21 (*CDKN2A/B*), and mutated *MYD88 L265P*.²⁻⁴ In 2018, our group identified an association between *MYC* rearrangements and a higher risk for disease progression and lymphoma-related death.⁵ More recently, Ducharme et al.⁶ reported that mutations in genes affecting the B-cell receptor (BCR) pathway were associated with a reduced disease-free period. However, as PCDLBCL-LT is a rare disease entity, the mentioned studies were performed in small patient cohorts and observations have not been confirmed by other studies. At present, it remains difficult to predict which patients relapse, and, given the poor outcomes, an urgent need for development of novel therapeutic approaches in patients with PCDLBCL-LT exists. Therefore, our aim was to perform a comprehensive molecular analysis of patients with PCDLBCL-LT at time of diagnosis and relapse/refractory disease, to provide insight into the molecular profile and disease evolution, and to correlate this profile with disease outcome.

MATERIALS AND METHODS

All patients diagnosed with PCDLBCL-LT (2000-2019) in the Leiden University Medical Center (LUMC), The Netherlands (n=56), and the University Hospitals, Leuven (UZL), Belgium (n=9), were retrospectively selected. Diagnosis was made in accordance with the current classification system of the World Health Organization (WHO)⁷ and the WHO - European Organisation for Research and Treatment of Cancer (EORTC)¹ and centrally reviewed by A.M.R.S., P.M.J., and R.W.. At time of diagnosis, presence of extracutaneous disease was excluded by standard staging procedures, consisting of a PET-CT scan or a CT-scan in combination with a bone marrow biopsy. Formalin-fixed and paraffin-embedded or fresh frozen skin biopsies of pre-treatment lesions and relapsed/refractory disease were collected from the archives of the Pathology Departments. Patients were excluded in case no tissue samples were available. Clinical characteristics were collected from the Dutch Cutaneous Lymphoma Database and from medical records. The study was performed in accordance with the Code Proper Secondary Use of Human Tissue established by the Dutch Federation of Medical Sciences and the CuraRata Biobank protocol (3.5162), and approved by the medical ethics committees of the LUMC (B19.011) and the UZL (S62445).

All cases were studied for protein expression of CD20 and/or CD79a, CD10, BCL6, MUM1, BCL2, IgM, and MYC, rearrangement status of *MYC*, and only in case of a *MYC* rearrangement for double hit status of *BCL2* and *BCL6*, with break apart rearrangement probes, as described in a previous study.⁵ The rearrangement status of 39 patients was previously reported.⁵

Genomic DNA from FFPE biopsies (n=55) was microdissected from deparaffinised 10µm sections and fully automatically isolated with the Tissue Preparation System (TPS) robot (Siemens Healthcare Diagnostics), as described previously.⁸ Genomic DNA from fresh frozen biopsies (n=7) was isolated from 25µm cryosections with the QIAamp DNA Mini Kit (Qiagen). DNA concentrations were quantified with the Qubit dsDNA HS Fluorometer (Life Technologies).

For targeted next-generation sequencing (tNGS), libraries were prepared either with the Ion Chef System (ThermoFisher Scientific) or manually for sequencing with the LYMFv1 panel. The LYMFv1 panel is a validated Ion Ampliseq panel that was developed in-house and contains 1362 amplicons, subdivided into 2 primer pools, that cover >95% of 52 B-cell lymphoma-related genes. The panel was composed based on a comprehensive review of >130 original scientific reports for frequency and clinical relevance of genetic mutations in B-cell lymphomas. This panel has a high overlap (73%) with the proposed consensus tNGS panel for mature B-cell malignancies by Sujobert et al.⁹ The generated sequencing data was aligned against the human reference genome (GRCh37/hg19) using the TMAP 5.0.7 software with default parameters and variants were called by the Torrent Variant Caller (ThermoFisher Scientific). All variants with ≥ 100 reads and a variant allele frequency of $\geq 10\%$ were functionally annotated in the Geneticist Assistant NGS Interpretive Workbench (SoftGenetics). Variants with a population frequency of $>1\%$ in the 1000 Genomes Project¹⁰, variants previously identified in sequencing runs with 'in-house' DNA samples isolated from blood of ~ 300 healthy controls, and variants in homopolymeric regions or variants with a strand bias of $>90\%$ were excluded from further analysis. The remaining variants were categorized for their pathogenicity as class 1 (benign), class 2 (likely benign), class 3 (unknown significance), class 4 (likely pathogenic), and class 5 (pathogenic).¹¹ This classification was done based on information retrieved from dbSNP¹², Clinvar¹³, Cosmic¹⁴, Alamut Focus (Version 1.0; Interactive Biosoftware, Rouen, France), and literature. In addition, a strategy was developed to further categorize all class 3 variants. This strategy is based on the CADD-Phred score¹⁵ of the variants and their notation in SIFT, Polyphen2_HDIV, LRT, and MutationTaster. Finally, all class 4 and class 5 variants, as well as all class 3 variants with a CADD-Phred score >25 , or a CADD-Phred score between 10 and 25 with ≥ 2 additional deleterious notations in SIFT, Polyphen2_HDIV, LRT, and MutationTaster were included. These variants are further referred to as 'mutations'. Quality control (QC) consisted of assessment of the coverage with a minimal count of 100 reads and a ratio of transitions versus transversions (Ts:Tv ratio) <5 . Patients were excluded in case these quality criteria were not met. The average read count per patient is listed in Supplemental Table 1.

Copy-number changes were also assessed. In short, the median base coverage per amplicon was calculated and normalized using the median value of all amplicons in that sample. Samples with low read count or with a high coverage variability among amplicons in the same gene were marked so that extra care should be taken while interpreting the final results. Systematic differences between amplicons were normalized using a set of 18 libraries prepared with DNA extracted from 10 non-neoplastic tonsils. A gene was considered lost or gained if the normalized coverage of more than 2 consecutive amplicons was below or above the estimated 99% confidence intervals (CIs) of these amplicons, respectively. Copy-number analysis and visualization of results, as well as loss of heterozygosity (LOH) was done using the Next-Generation Sequence Expert (NGSE) Shiny app (<https://git.lumc.nl/druano/NGSE>).

Statistical analysis was performed using IBM SPSS statistics (version 23) and RStudio (version 1.1442). The median follow-up time was determined using the reverse Kaplan-Meier method.¹⁶ Survival was calculated from the date of first histological diagnosis to the date of death by any cause for overall survival (OS), the date of death by lymphoma for DSS, and the date of disease relapse or progression of disease or death by any cause (whichever came first) for disease-free survival (DFS). Patients without an event at the end of follow-up were censored. For OS and DFS, survival curves were plotted using the Kaplan-Meier method and compared with the Log-rank test. For DSS, cumulative incidences were estimated with the competing risk method and compared using the Gray's test, with non-lymphoma-related death considered as competing risk. In case of a statistically significant p-value (<0.05), the corresponding hazard ratios (HRs) and 95% CIs were calculated with the Cox proportional-hazards model. Based on literature, the following parameters were included in analysis: age at diagnosis (≤ 70 years vs > 70 years)², sex (male vs female), disease extension (single vs multiple lesions)², *MYD88* L265P mutations (mutant vs wild type)⁴, *CDKN2A* status (loss vs no loss)³, mutations in the BCR pathways (mutant *CD79A*, *CD79B* or *CARD11* vs wild type)⁶, *MYC* status (rearranged vs wild type)⁵, MCD (mutant *MYD88* and *CD79B* vs wild type)¹⁷, and other frequent molecular alterations in our cohort. In case the molecular profile of primary disease was not available, the profile of relapse/refractory disease was included in the survival analysis (n=5).

RESULTS

Patient characteristics

For this study, 65 patients with PCDLBCL-LT were selected. Three patients were excluded because no material was available and an additional 7 patients because the obtained sequencing data did not pass the quality control. In total, 69 skin biopsies were successfully sequenced from a total of 55 patients with PCDLBCL-LT, including paired analysis of diagnostic and relapsed/refractory disease in 14 patients (Supplemental Figure 1). The patient characteristics are presented in Table 1. The cohort consisted of 28 (51%) females and the median age at diagnosis was 78 (range, 47 to 92) years. Patients presented with single lesions in 13 of 55 (24%) cases and with multiple lesions located in 1 body region (regional disease) in 34 of 55 (62%) cases or more body regions (multifocal disease) in 8 of 55 (15%) cases.² The legs were involved in the vast majority (46/55; 84%).

Twenty-seven (49%) patients were systemically treated with immunopolychemotherapy, consisting of (R-)CHOP or CHOP-like regimens (n=23). In 8 of these patients, systemic treatment was combined with local radiotherapy. Another 26 patients (47%) received local treatment, consisting of radiotherapy (n=25), surgical excision (n=1), or a combination (n=1). No treatment was given in 2 of 55 (4%) patients because of spontaneous remission of a solitary lesion after biopsy (n=1) and sudden cardiac death (n=1). After initial treatment, 51 of 53 (96%) patients showed a complete response, while 2 of 53 (4%) patients had refractory/progressive disease, both after R-CHOP treatment. From the complete responders, 33 of 51 (63%) patients developed disease relapses. In second-line and/or third-line treatment, regimens were highly heterogeneous, including local radiotherapy, monotherapy with rituximab or lenalidomide, (immune-)polychemotherapy with different regimens, or autologous/allogeneic stem-cell transplantation. The median follow-up duration of all patients was 7.9 (range, 0.08 to 14.3) years. For systemically-treated patients only, the median follow-up duration was 9.1 (range, 0.7 to 14.3) years and in the subgroup of locally-treated patients this was 7.7 (range, 0.5 to 8.9) years.

Table 1. Clinical characteristics and immunophenotype of 55 patients with primary cutaneous diffuse large B-cell lymphoma, leg type

	All patients (n=55)	Systemically- treated patients (n=27) ^c	Locally-treated patients (n=26) ^c
Sex, n (%)			
Male	27 (51)	14 (52)	12 (46)
Female	28 (49)	13 (48)	14 (54)
Median age at diagnosis, years (range)	78 (47-92)	75 (47-86)	81 (59-92)
Disease localization, n (%)			
Legs	46 (84)	25 (93)	20 (77)
Other skin sites	9 (16)	2 (7)	6 (23)
Disease extension², n (%)			
Single lesion	13 (24)	4 (15)	8 (31)
Regional	34 (62)	18 (67)	15 (58)
Multifocal	8 (15)	5 (19)	3 (12)
First-line therapy, n (%)			
Local treatment	26 (47) ^a	-	26 (100)
Systemic treatment	27 (49) ^b	27 (100) ^b	-
No treatment	2 (4) ^c	-	-
Treatment response, n (%)			
Complete remission	51 (96)	25 (93)	26 (100)
Progressive disease	2 (4)	2 (7)	-
Median follow-up duration, years (range)	7.9 (0.5-14.3)	9.1 (0.7-14.3)	7.7 (0.5-8.9)
Disease relapse/ progression, n (%)	35 (66)	21 (78)	12 (46)
Median disease-free period, years (range)	1.1 (0-10.2)	1.2 (0-10.2)	1.2 (0.1-8.9)
Status at last follow-up, n (%)			
Alive	22 (40)	11 (41)	11 (42)
Died of lymphoma	21 (38)	13 (48)	7 (27)
Died of other cause	12 (22)	3 (11)	8 (31)
Survival, %			
5-year overall survival	40	45	39
5-year disease-specific survival	51	51	56
Median survival time, range (years)	3.7 (0.2-8.1)	4.0 (0.9-5.8)	3.5 (0.3-8.1)

Table 1. Clinical characteristics and immunophenotype of 55 patients with primary cutaneous diffuse large B-cell lymphoma, leg type (continued)

	All patients (n=55)	Systemically- treated patients (n=27) ^c	Locally-treated patients (n=26) ^c
Immunophenotype			
CD10	7 (13)	2 (7)	5 (19)
BCL6	32 (58)	15 (56)	16 (62)
MUM1	46 (84)	22 (81)	23 (89)
BCL2	53 (96)	27 (100)	24 (92)
IgM	50 (94) ^e	25 (96) ^d	23 (92) ^d
MYC	36 (67) ^d	18 (69) ^d	17 (65)

^aLocal therapy consisted of radiotherapy in 24 patients, surgical excision combined with radiotherapy in 1 patient, and surgical excision in 1 patient.

^b(Immune-)polychemotherapy consisted of a combination of rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) in 21 patients, rituximab plus cyclophosphamide, etoposide, vincristine, and prednisone (R-CEOP) in 2 patients, CHOP in 3 patients, and vincristine and prednisone (OP) in 1 patient. In 7 patients, immune-polychemotherapy was combined with local radiotherapy.

^cNo treatment was given in 2 patients due to spontaneous remission of a single lesion (n=1) and sudden cardiac death (n=1).

^dData is missing in 1 case.

^eData is missing in 2 cases.

Protein expression and rearrangement status

Nearly all cases were positive for BCL2 (53/55; 96%) and IgM (50/53; 94%), the majority expressed MUM1 (46/55; 84%), MYC (36/54; 67%), and BCL6 (32/55; 58%), and few cases expressed CD10 (7/55; 13%). In total, 14 of 53 (26%) PCDLBCL-LT patients showed a *MYC* rearrangement. An additional *BCL6* rearrangement was detected in 1 case at time of diagnosis (as described previously⁵) and in 1 case with an acquired double hit at time of relapsed disease that was initially absent. Unfortunately, the sequencing data of the diagnostic sample from this patient failed the quality control and was excluded from paired analysis. None of the *MYC*-rearranged patients had a second hit in *BCL2*.

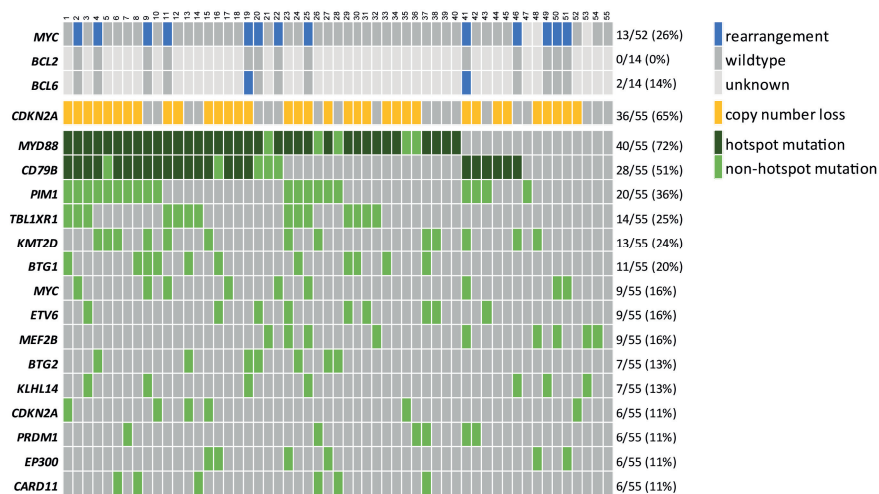


Figure 1. Oncoprint plot presenting the detected molecular alterations with an occurrence in over 10% of the 55 included patients with (PCDLBCL-LT). Our results demonstrate that activation of the NF- κ B pathway, *MYC* signaling, and loss of *CDKN2A* are the main drivers of lymphomagenesis in PCDLBCL-LT patients.

Molecular profile

The detected molecular alterations with an occurrence in over 10% of the 55 patients with PCDLBCL-LT are presented in Figure 1. The involved pathways are visualized in a schematic diagram in Figure 2. The median number of mutations per patient was 5 (range, 0 to 26). The majority of the patients (40/55; 72%) harbored a *MYD88* mutation, of which the L265P hotspot was the most frequently present (35/40; 88%). Non-L265P variants were detected in 5 of 40 (13%) patients, including S243N (n=3), an additional T84I (n=1), M232T (n=1), and Y240S (n=1). *MYD88* mutations result in activation of the NF- κ B pathway, thereby promoting cell survival and proliferation.¹⁸ Other gene mutations that result in NF- κ B activation were detected in *CD79B* in 28 of 55 (51%) patients and *CARD11* in 6 of 55 (11%) patients, both components of the B-cell receptor pathway, and mutations in *PIM1* in 20 of 55 (36%) patients, a gene involved in the affiliated JAK/STAT pathway. *CD79B* mutations comprised the hotspot Y196 variant in 23 of 28 (82%) cases. Non-Y196 variants (5/28; 18%) included splice-site variants (n=3), L199R/G40fs (n=1), and

I201fs (n=1). In 6 of 23 (26%) patients with *CD79B* Y196, additional *CD79B* non-Y196 mutations were detected, including splice-site variants (n=3) and I109, E229A, and M190fs/E208A (n=1 each). In 21 of 55 (38%) patients concurrent mutations in *MYD88* L265P and *CD79B* were detected, similar to the MCD genotype in DLBCL, as defined by Schmitz et al.¹⁷

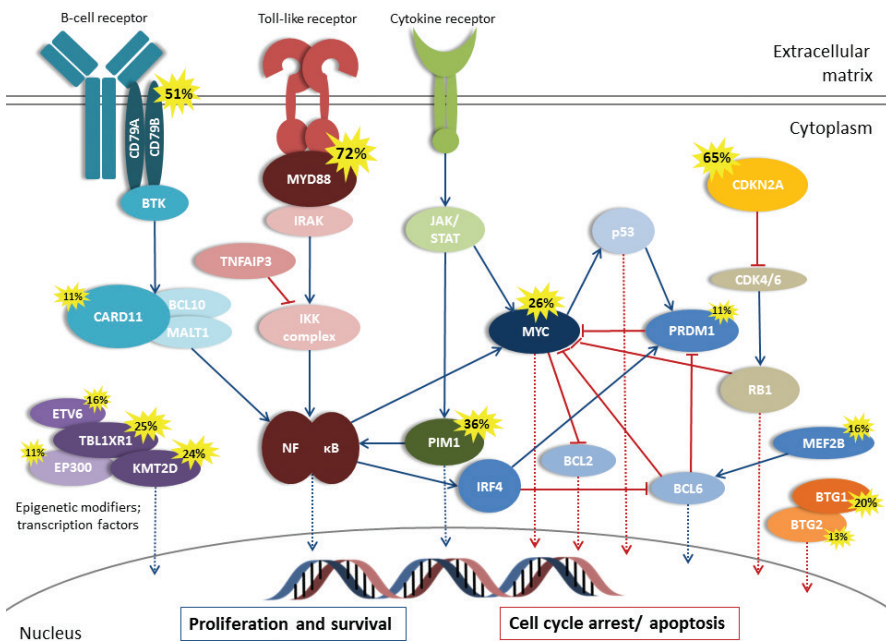


Figure 2. Schematic diagram of predominantly involved pathways in lymphomagenesis of primary cutaneous diffuse large B-cell lymphoma, leg type (PCDLBCL-LT). The central pathway that shows molecular alterations in these patients is the NF- κ B pathway, with recurrent mutations in *MYD88* (72%), *CD79B* (51%), *PIM1* (36%), and *CARD11* (11%), and the affiliated *PRDM1* gene (11%). Other commonly affected pathways are involved in cell cycle arrest/apoptosis, with *CDKN2A* loss in 65%, *MYC* rearrangements in 26%, and mutations in the transcription factors *BTG1* (20%) and *BTG2* (13%). Less frequent mutations were seen in several epigenetic modifiers, such as *TBL1XR1* (25%), *KMT2D* (24%), *ETV6* (16%), *MEF2B* (16%), and *EP300* (11%). It is likely that patients with PCDLBCL-LT will be treated with a more targeted approach in the near future, with the NF- κ B pathway, downstream *CDKN2A* and *MYC* as central targets for novel therapeutic approaches, instead of the current one-size-fits-all treatment with immune-polychemotherapy.

In addition to activation of the NF- κ B pathway, NF- κ B itself promotes plasma cell differentiation via *IRF4* and *PRDM1¹⁹*, with mutations in 4 of 55 (7%) patients and 6 of 55 (11%) patients, respectively. Also frequently affected were the epigenetic modifiers *TBL1XR1* (14/55; 25%), *KMT2D* (13/55; 24%), *MEF2B* (9/55; 16%), *ETV6* (9/55; 16%) and *EP300* (6/55; 11%), as well as the regulators of cell cycle/apoptosis *MYC* (9/55; 16%) and the tumor suppressor *CDKN2A* (6/55; 11%). Finally, the transcription factors *BTG1* (11/55; 20%) and *BTG2* (7/55; 13%) were commonly mutated. *MYC* mutations co-occurred with *MYC* rearrangements in 8 of 9 (89%) patients. In contrast, patients without a *MYC* mutation only harbored *MYC* rearrangements in 6 of 44 (14%) patients.

More often than *CDKN2A* mutations, copy-number loss of *CDKN2A* was observed (36/55; 65%). While it is difficult to reliably distinguish a hemizygous from a homozygous loss, 4 patients concomitantly presented with a *CDKN2A* mutation, suggesting hemizygous loss of *CDKN2A*. Other copy-number alterations observed were only present in few patients, including loss of *BCL7A* (6%), *CD70* (6%), and *PCLG2* (4%), and gain of *HIST1H1E* (6%) and *BCL2* (6%) (data not shown).

Figure 3. Side-by-side overview of the results of comprehensive molecular analysis of paired diagnostic and relapsed/refractory disease in 14 patients with primary cutaneous diffuse large B-cell lymphoma, leg type (PCDLBCL-LT). Oncoprint plot representing the molecular alterations of PCDLBCL-LT, demonstrating that the dominant drivers of lymphomagenesis, i.e. *MYD88/CD79B*, *CDKN2A*, and *MYC*, are early oncogenic events in disease evolution. In addition, mutations in SHM motifs were present in *TBL1XR1*, *KMT2D*, *BTG2*, *ETV6*, *MYC*, and *MYD88* (S243N variant), with an occurrence ranging from 8% to 100% of the variants per gene. Remark: In all cases with a discrepancy in the mutational profile between diagnostic and relapse/refractory disease, it was ruled out that the variants were already present in low frequency (<10%) in the diagnostic and relapse samples, respectively. Abbreviations: FU, follow-up; D+, death by lymphoma; Do, death by unrelated cause; A+, alive w disease; Ao, alive w/o disease; CR, complete remission; PD, progressive disease.

*mutation in SHM motif (WRCY-RGYW)

Disease evolution

Paired molecular analysis of diagnostic and relapsed/refractory disease was available in 14 patients (Figure 3). A conceptual representation of disease evolution is presented in Figure 4. Treatment consisted of local radiotherapy in 4 of 14 (29%) patients and immune-polychemotherapy in 10 of 14 (71%) patients, which was combined with local radiotherapy in 5 patients. Thirteen patients achieved a complete remission after initial treatment and 1 patient had refractory/progressive disease under immune-polychemotherapy. In case of treatment with local radiotherapy only, disease relapse was present in another body site in 2 patients. The median time between diagnostic and relapse/refractory disease was 14 (range, 4 to 80) months.

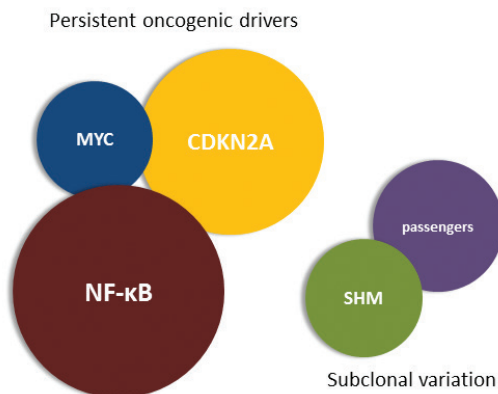


Figure 4. Conceptual representation of lymphomagenesis in patients with primary cutaneous diffuse large B-cell lymphoma, leg type (PCDLBCL-LT). Overall, our results suggest that activation of the NF-κB pathway, *MYC* signaling, and loss of *CDKN2A* are early drivers of PCDLBCL-LT and are persistently present in primary and relapsed/refractory disease. In addition, subclonal variations in disease evolution are largely characterized by passenger mutations and ongoing somatic hypermutation (SHM).

Overall, the molecular profile at time of diagnosis was predominantly similar with the profile in relapse/refractory disease. In 4 of 14 (29%) patients, the molecular profile was identical at both time points. In the other 10 patients, the profile varied slightly, with acquired mutations (n=4), lost mutations (n=3), or both (n=3). Interestingly, from the supposed driver alterations, *MYD88* mutations and *MYC* rearrangements were uniformly present in both diagnostic and relapse/refractory disease. This was also the case for the less frequently mutated genes *TBL1XR1*, *KMT2D*, *SGK1*, and *ATM*.

Acquired mutations were detected in several other genes, of which *PIM1* was most remarkable, with acquired mutations in 3 of 14 (21%) cases, ranging from 1 to 3 mutations per patient. This increase can be explained by the fact that *PIM1* is a known target of ongoing somatic hypermutation (SHM)²⁰, and indeed, these variants were all present in SHM motifs (WRCY-RGYW). Other genes with variants in SMH motifs were *TBL1XR1*, *KMT2D*, *BTG2*, *ETV6*, *MYC*, and *MYD88* (S243N variant), with frequencies ranging from 8%-100% of the variants per gene. On the other hand, for several genes, such as *BTG1*, *CARD11*, and *ETV6*, some mutations were present at time of diagnosis but were completely absent in the relapsed/refractory samples.

Table 2. Survival analysis of molecular factors in patients with primary cutaneous diffuse large B-cell lymphoma, leg type

	Overall survival		Disease-specific survival		Disease-free survival	
	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)
A. All patients (n=55)						
<i>MYC</i> ⁵ (rearranged vs WT)	0.045	2.23 (1.00-4.98)	<0.001	4.16 (1.63-10.60)	0.003	2.85 (1.39-5.86)
<i>MYD88 L265P</i> ⁴ (mutant vs WT)	0.557	-	0.453	-	0.926	-
<i>CDKN2A</i> ³ (loss vs no loss)	0.923	-	0.878	-	0.787	-
BCR pathway ⁶ (<i>CD79A</i> , <i>CD79B</i> or <i>CARD11</i> mutant vs WT)	0.474	-	0.689	-	0.958	-
B. Systemically-treated patients (n=27)						
<i>MYC</i> ⁵ (rearranged vs WT)	0.005	4.47 (1.43-13.99)	<0.001	7.51 (1.95-28.87)	<0.001	13.77 (3.59-52.85)
<i>MYD88 L265P</i> ⁴ (mutant vs WT)	0.994*	-	0.608*	-	0.425	-
<i>CDKN2A</i> ³ (loss vs no loss)	0.901*	-	0.770*	-	0.960	-
BCR pathway ⁶ (<i>CD79A</i> , <i>CD79B</i> or <i>CARD11</i> mutant vs WT)	0.607*	-	0.921*	-	0.532	-

Abbreviations: WT, wild type; BCR, B-cell receptor; HR, hazard ratio; CI, confidence interval. Bold values indicate a statistically significant result.

*Results are less reliable because of <5 events in 1 of the groups.

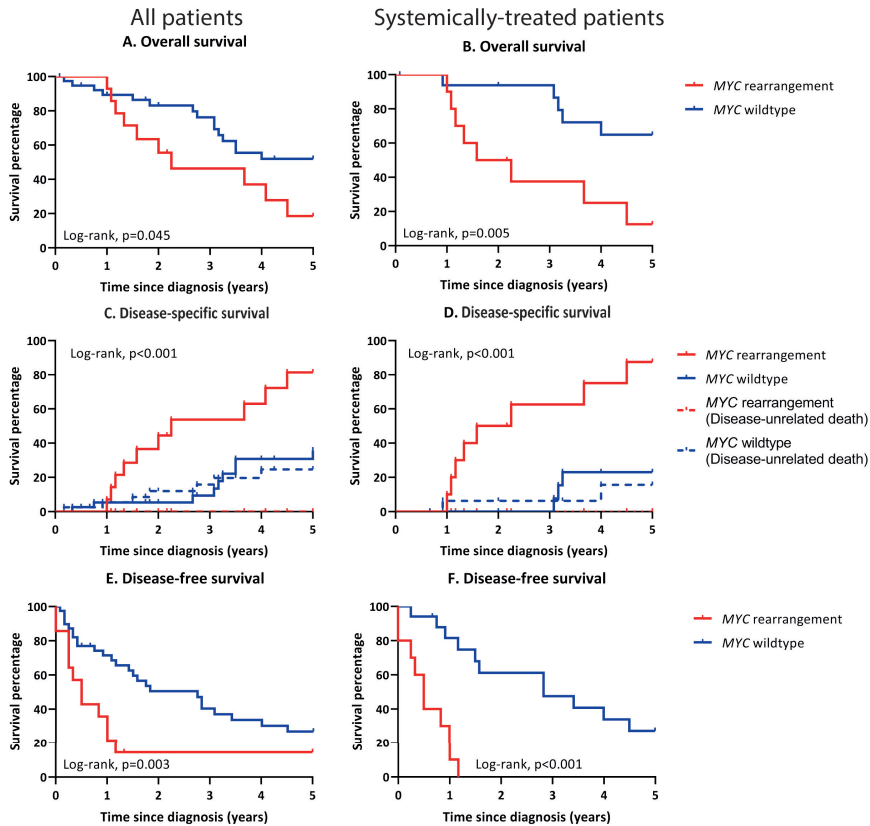


Figure 5. Survival curves for *MYC* status in all patients and the subgroup of systemically-treated patients with primary cutaneous diffuse large B-cell lymphoma, leg type. Patients with a *MYC* rearrangement demonstrate an inferior overall survival in (A) all patients ($p=0.045$; HR:2.23; 95%CI:1.00-4.98) and (B) systemically-treated patients ($p=0.005$; HR:4.47; 95%CI:1.43-13.99), an inferior disease-specific survival in (C) all patients ($p<0.001$; HR:4.16; 95%CI:1.63-10.60) and (D) systemically-treated patients ($p<0.001$; HR:7.51; 95%CI:1.95-28.87), and a shorter disease-free survival in (E) all patients ($p=0.003$; HR:2.85; 95%CI:1.39-5.86) and (F) systemically-treated patients ($p<0.001$; HR:13.77; 95%CI:3.59-52.85) compared with patients without a *MYC* rearrangement.

Survival analysis

To identify risk classifiers for relapsed/refractory disease and survival, we correlated the molecular profiles with the patient outcomes. In all 55 patients, survival analysis (Table 2 and Figure 5) demonstrated that only *MYC* rearrangements were associated with an inferior OS ($p=0.045$; HR:2.23; 95%CI:1.00-4.98), DSS ($p<0.001$; HR:4.16; 95%CI:1.63-10.60), and shorter DFS ($p=0.003$; HR:2.85; 95%CI:1.39-5.86). To assess the influence of treatment heterogeneity, the subgroup of systemically-treated patients only was also evaluated. This analysis revealed similar results, with only *MYC* rearrangements to be associated with an inferior OS ($p=0.005$; HR:4.47; 95%CI:1.43-13.99), DSS ($p<0.001$; HR:7.51; 95%CI:1.95-28.87), DFS ($p<0.001$; HR:13.77; 95%CI:3.59-52.85). Interestingly, none of the adverse molecular factors described in literature (*MYD88* L265P, loss of *CDKN2A*, and mutations in the BCR pathway) were associated with an inferior survival. In addition to the molecular alterations, age at diagnosis >70 years and male gender were associated with a significantly inferior survival (Supplemental Table 2).

DISCUSSION

To provide insight into the genetic background and disease evolution of patients with PCDLBCL-LT, we comprehensively analysed the molecular profile of a relatively large cohort of patients ($n=55$), performed paired analysis of diagnostic and relapsed/refractory disease in 14 of these patients, and studied the association of recurrent molecular alterations with disease outcome.

As PCDLBCL-LT is a rare disease, previously, only smaller cohorts of patients were analysed for molecular alterations, including whole-exome sequencing in 31 patients and targeted sequencing in an additional 20 patients.^{21,22} Consistent with these studies, we found a high frequency of mutations in *MYD88* (72%), *CD79B* (51%), *PIM1* (36%), *TBL1XR1* (25%), and *KMT2D* (24%), and loss of *CDKN2A* (65%). This profile predominantly contributes to constitutive activation of the NF- κ B-signalling pathway and overlaps with the activated B-cell (ABC) subtype of DLBCL and, in particular, extranodal DLBCL, such as intravascular large B-cell lymphoma, primary central nervous system lymphoma (PCNSL), and primary testicular lymphoma

(PTL).²³⁻²⁵ This profile was identified as the C5 signature by Chapuy et al.²⁶, with the major alterations being mutations in *MYD88* and *CD79B*, additional mutations in *ETV6*, *PIM1*, *TBL1XR1*, and *BTG1*, and ongoing SHM. At the same time, Schmitz et al.¹⁷ described the MCD group in DLBCL, with co-occurrence of mutations in *MYD88* and *CD79B*, further characterized by inactivating mutations in *PRDM1*, as well as alterations in the tumor suppressor genes *CDKN2A*, *ETV6*, *BTG1*, *BTG2*, *TBL1XR1*, and *KLHL14*. In our cohort of PCDLBCL-LT patients, we detected molecular features of both the C5 signature and the MCD genotype.

Additionally to the molecular similarities with ABC-DLBCL, we also identified *MYC* rearrangements in 26% of the PCDLBCL-LT patients, which are more frequently found in GCB-DLBCL.²⁷⁻²⁹ However, the association of *MYC* rearrangements with the GCB subtype accounts especially for double hits with *BCL2* and triple hits whereas single *MYC* rearrangements and double hits with *BCL6* show a rather equal distribution between the GCB and ABC subtypes.³⁰ In previous studies, which included small cohorts up to 25 PCDLBCL-LT patients, the frequency of *MYC* rearrangements was reported between 0% and 43%.³¹⁻³⁴ In addition to our previous study⁵, we currently describe 14 new patients of which 3 (21%) patients had a *MYC* rearrangement. In our cohort, the majority of the *MYC*-rearranged patients (10/14; 71%) also harbored *MYD88* and/or *CD79B* mutations.

In addition, Mareschal et al.²¹ report *MYC* mutations in 26% of patients with PCDLBCL-LT compared with 16% in our cohort.²¹ Interestingly, in our cohort, *MYC* mutations often co-occurred with *MYC* rearrangements (89%) and only 14% of the *MYC* rearrangements were present in patients without a *MYC* mutation. This corresponds to DLBCL, as described by Reddy et al.³⁵, with *MYC* rearrangements in >80% of the cases with a *MYC* mutation, while only 10% of the cases without a *MYC* mutation were *MYC*-rearranged.

Our results further indicate that PCDLBCL-LT patients with a *MYC* rearrangement may experience a more aggressive disease course, despite absence of double hits in *BCL2* and *BCL6*. In survival analysis, *MYC* rearrangements were the only molecular feature associated with an inferior OS, DSS, and DFS, in both all patients and the

subgroup of systemically-treated patients. These results support our previously published data on an association of *MYC* rearrangements with an inferior survival in PCDLBCL-LT.⁵ Recently, we demonstrated a prognostic importance of *MYD88* mutations in systemic DLBCL³⁶, as was previously also reported in PCDLBCL-LT by Pham-Ledard et al.⁴. However, in our current study, *MYD88* mutations, including the subgroup of *MYD88* L265P, were not associated with an adverse outcome. Accordingly, other previously described adverse molecular factors, i.e. loss of *CDKN2A* and mutations in the BCR pathway, were not associated with an inferior survival in our cohort.^{3,4,6} Results of survival analysis in relatively small patient cohorts, including the results of the current study, should always be interpreted with caution and should be confirmed by other studies. In PCDLBCL-LT, so far, none of the reported adverse molecular factors have been independently confirmed.

Besides their prognostic effect, the predominant drivers of lymphomagenesis in PCDLBCL-LT patients, i.e. *MYD88/CD79B* mutations, *MYC* rearrangements, and, to a lesser extent, *CDKN2A* loss were persistently present in diagnostic and relapse/refractory disease. These findings suggest that these are early events in disease evolution of PCDLBCL-LT, and that, despite large reductions in lymphoma volume by initial therapy in the vast majority of the patients, minimally residual disease persisted that was not detected by our currently standard methods of clinical examination and PET-CT scanning. Such minimal residual disease may be detected with targeted analysis of liquid biopsies for circulating tumor DNA harbouring hotspot mutations like *MYD88* L265P in the future.³⁷ In addition, subclonal variations between diagnosis and relapse were present and commonly caused by ongoing SHM. This corresponds with the recent study by Ducharme et al.⁶, demonstrating mutations in SHM motifs in 33% to 100% of several affected genes, including *PIM1*, *IRF4*, *MYC*, *BCL2*, and *CARD11*, and expression of activation-induced cytidine-deaminase, the enzyme that is responsible for SHM, as was reported by Dijkman et al.³⁸

The increasing knowledge of the molecular landscape of PCDLBCL-LT, including 55 patients in the current study, provides a rationale for novel, more targeted treatment strategies in the near future. So far, several case reports and a phase

II clinical trial demonstrate promising results of treatment with lenalidomide in relapsed/refractory PCDLBCL-LT patients.³⁹⁻⁴² Additionally, 1 patient in our cohort with progressive disease under immune-polychemotherapy and radiotherapy achieved an enduring complete remission for over a year on lenalidomide monotherapy. Also, several relapsed/refractory PCDLBCL-LT cases have been reported with response to ibrutinib⁴³⁻⁴⁵, an inhibitor of the BCR pathway. Given the importance of *MYD88/CD79B* as drivers of lymphomagenesis in the majority of PCDLBCL-LT patients, the NF- κ B pathway and its affiliated signaling pathways might be targeted in several alternative ways by future systemic therapies, as reviewed by De Groen et al.¹⁸ In addition, *MYC* and the cyclin-dependent kinases downstream of *CDKN2A* could be considered as targets for novel therapeutic approaches, either as monotherapy or in combination with inhibition of the NF- κ B pathway.^{46,47} Despite a large spread in reported frequency of *MYC* rearrangements, *MYC* protein expression is reported in the majority of patients with PCDLBCL-LT, with 67% in our cohort and 83% in the cohort reported by Menguy et al.³² It seems plausible that not only the *MYC*-rearranged patients, but also the *MYC* expressors, may benefit from *MYC*-directed treatment strategies.⁴⁶ Finally, as mutations in epigenetic modifiers were present in a significant subgroup of patients (64%), these genes could also provide attractive targets for novel therapeutic approaches.⁴⁸ Currently, we stand at the beginning of precision medicine in PCDLBCL-LT, and it will be highly interesting to see the efficacy of these and other targeted treatment strategies in clinical trials for relapse/refractory disease but also as first-line therapy. In case of novel targeted treatments, ongoing SHM that results in subclonal variation may become clinically relevant, as for example *CARD11* mutations that may cause resistance to ibrutinib.¹⁸

In conclusion, our data corroborate the importance of *MYD88/CD79B* mutations, *CDKN2A* loss, and *MYC* rearrangements as early events and persistent oncogenic drivers of disease evolution of PCDLBCL-LT. In addition, disease evolution is characterized by ongoing SHM. From these predominant drivers, only *MYC* rearrangements are associated with an inferior survival. These results support the idea that activation of the NF- κ B pathway, *MYC* signaling, and loss of *CDKN2A* play a critical role in pathogenesis of PCDLBCL-LT and that these molecular alterations could provide attractive targets for novel therapeutic approaches.

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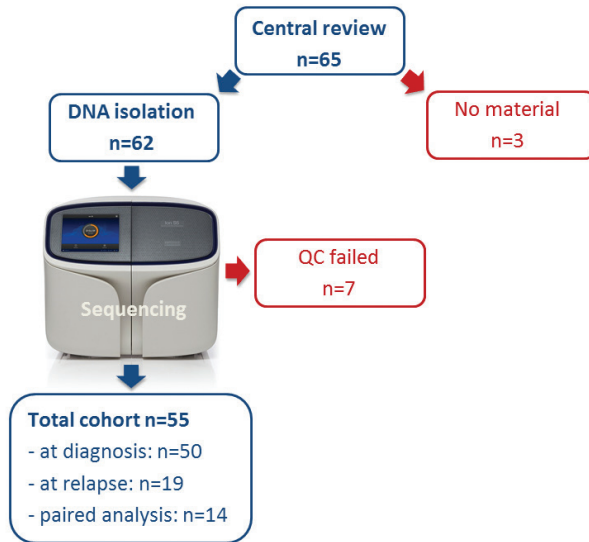
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SUPPLEMENTAL DATA



Supplemental Figure 1. Flow-chart of study inclusion.
Abbreviations: QC, quality control.

Supplemental Table 1. Average read count per patient with primary cutaneous diffuse large B-cell lymphoma, leg type

Sample	Average read count	Sample	Average read count	Sample	Average read count
1	2804.5	18R	2265.0	37	1029.9
2	2984.7	19	3164.4	38	1955.7
3	1739.6	20P	1642.5	39	1811.4
4	2817.4	20R	4973.4	40P	1132.5
5	2726.6	21	4219.1	40R	2911.3
6P	3515.8	22	1865.0	41	3036.5
6R	1568.5	23	3467.5	42	2014.0
7	1172.2	24P	7595.6	43	2794.2
8	1635.5	24R	1941.8	44P	2094.9
9	2077.3	25	3223.2	44R	1859.3
10	1200.5	26	5526.0	45	2349.3
11	1197.8	27	3172.8	46P	1974.7
12P	2109.1	28	1946.1	46R	1657.8
12R	2273.8	29	4411.7	47	1149.1
13	3127.6	30P	3376.9	48	3628.5
14P	1059.0	30R	3638.6	49	2462.9
14R	1611.8	31	3803.5	50	3748.4
15P	1332.1	32	4046.2	51P	2839.7
15R	2690.5	33	1248.0	51R	3266.1
16P	2590.4	34	3097.6	52	2102.8
16R	3501.4	35	3330.8	53	1157.3
17	3202.3	36P	2207.4	54	1857.4
18P	2801.7	36R	2478.2	55	1191.4

Abbreviations: P, primary sample; R, relapse sample.

Supplemental Table 2. Survival analysis of clinical factors in patients with primary cutaneous diffuse large B-cell lymphoma, leg type

	Overall survival		Disease-specific survival		Disease-free survival	
	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)
A. All patients (n=55)						
Age at diagnosis ² (≤70 vs >70 years)	0.004*	10.49 (1.42-77.77)	0.021*	1.09 (1.08-1.16)	0.041	2.56 (0.99-6.62)
Sex (male vs female)	0.005	3.14 (1.35-7.28)	0.003*	5.23 (1.71-16.03)	0.178	-
Disease extension ² (single vs multiple lesions)	0.730	-	NR	-	0.848	-
B. Systemically-treated patients (n=27)						
Age at diagnosis ² (≤70 vs >70 years)	0.023*	7.60 (0.98-59.01)	0.047*	1.07 (0.99-1.15)	0.059*	-
Sex (male vs female)	0.005*	5.30 (1.44-19.52)	0.004*	6.97 (1.49-32.53)	0.026	2.93 (1.07-8.01)
Disease extension ² (single vs multiple lesions)	NR	-	NR	-	NR	-

Abbreviations: HR, hazard ratio; CI, confidence interval; NR, not reliable.

*Results are less reliable because of <5 events in 1 of the groups.

Remark: disease extension was excluded from analysis in disease-specific survival for all patients and in overall-survival, disease-specific survival, and disease-free survival for the systemically-treated patients because of ≤1 event in the single lesion arm.

