

ORIGINAL RESEARCH

Clonal evolution in diffuse large B-cell lymphoma with central nervous system recurrence

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Background: The prognosis of patients with secondary central nervous system lymphoma (SCNSL) is poor and despite massive advances in understanding the mutational landscape of primary diffuse large B-cell lymphoma (DLBCL), the genetic comparison to SCNSL is still lacking. We therefore collected paired samples from six patients with DLBCL with available biopsies from a lymph node (LN) at primary diagnosis and the central nervous system (CNS) at recurrence.

Patients and methods: A targeted, massively parallel sequencing approach was used to analyze 216 genes recurrently mutated in DLBCL. Healthy tissue from each patient was also sequenced in order to exclude germline mutations. The results of the primary biopsies were compared with those of the CNS recurrences to depict the genetic background of SCNSL and evaluate clonal evolution.

Results: Sequencing was successful in five patients, all of whom had at least one discordant mutation that was not detected in one of their samples. Four patients had mutations that were found in the CNS but not in the primary LN. Discordant mutations were found in genes known to be important in lymphoma biology such as *MYC*, *CARD11*, *EP300* and *CCND3*. Two patients had a Jaccard similarity coefficient below 0.5 indicating substantial genetic differences between the primary LN and the CNS recurrence.

Conclusions: This analysis gives an insight into the genetic landscape of SCNSL and confirms the results of our previous study on patients with systemic recurrence of DLBCL with evidence of substantial clonal diversification at relapse in some patients, which might be one of the mechanisms of treatment resistance.

Key words: diffuse large B-cell lymphoma, secondary central nervous system lymphoma, clonal evolution, massively parallel sequencing

INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin's lymphoma and approximately 70% of patients are cured using the current standard of first-line combined chemotherapy and antibody therapy.^{1,2} However, the prognosis of patients with refractory or recurrent disease is limited, and it is especially dismal for those experiencing relapse to the central nervous system (CNS). During the course of the disease approximately

2%-5% of patients with DLBCL suffer from CNS involvement and their reported median overall survival (OS) is 2-4 months.^{3,4} The genetic drivers of systemic DLBCL at initial diagnosis and those of primary central nervous system lymphoma (PCNSL) were already identified by several groups. These showed that although several genes, for example *MYD88*, *PIM1*, *CD79B* or *TP53* are recurrently mutated in both systemic DLBCL as well as in PCNSL, others, such as *TOX* and *PRKCD*, were described in PCNSL but are found less commonly in systemic DLBCL, suggesting specific genomic signatures of the two diseases.⁵⁻¹⁴ Up to now, the mutational profile of secondary CNS lymphoma (SCNSL) was largely unknown, which might be caused by a lack of both adequate biopsies at the time of recurrence and centralized care in some parts of the world. However, the genetic background of this disease should be of diagnostic and

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therapeutic interest, as we and others have shown that there can be substantial clonal diversification from initial diagnosis to recurrence in several hematological diseases including systemic DLBCL with recurrence outside the CNS.¹⁵⁻¹⁷ Therefore, we collected paired samples from six patients with systemic DLBCL and recurrence to the CNS to investigate the genetic signature of SCNSL and clonal evolution by applying targeted resequencing of the exons of 216 selected genes known to be important in lymphoid malignancies.

PATIENTS AND METHODS

Patients

For earlier projects, we characterized 246 patients with systemic DLBCL who were diagnosed and treated with R-CHOP-like regimens at the Third Medical Department of the Paracelsus Medical University between 2004 and 2014.^{18,19} Out of 16 (6.5%) patients who experienced secondary relapse to the CNS we identified 6 patients that were biopsied in both a lymph node (LN) at primary diagnosis and the CNS at relapse. In one patient an additional sample from a lymphoma infiltration of the skin was taken during clinical routine at primary diagnosis and used in this analysis. The tumor cell content was assessed with hematoxylin and eosin staining, and the Hans algorithm was used to determine the cell of origin.²⁰ Fluorescence *in situ* hybridization (FISH) for the detection of *MYC* translocations was carried out for all patients using Split Signal Code Y5410 (Dako Denmark A/S, Glostrup). The patient characteristics were investigated by chart-based review and the OS was calculated from primary diagnosis to the last follow-up or death from any cause and progression free survival (PFS) was defined from diagnosis to progression of disease or death from any cause. In some cases patient follow-up was carried out via telephone interviews with the patients' general practitioners. All patients gave their written informed consent and the study was approved by the Ethics Committee of the provincial government of Salzburg, Austria (415-EP/73/127-2012).

Targeted massively parallel sequencing

For this analysis we used a selected set of 216 genes known to be recurrently mutated in DLBCL (for gene list see [Supplementary Table S1](https://doi.org/10.1016/j.esmooop.2020.100012), available at <https://doi.org/10.1016/j.esmooop.2020.100012>). After assessment of the tumor cell content, genomic DNA was extracted from formalin-fixed paraffin embedded (FFPE) LN, CNS and skin samples. Healthy tissue from non-tumor FFPE samples or peripheral blood mononuclear cells were available for all six patients and compared with the lymphoma samples to exclude germline mutations. Target enrichment with SureSelect® (Agilent Technologies, Inc., Santa Clara, CA) and sequencing on Illumina HiSeq 3000® and HiSeq 2500® (Illumina, Inc., San Diego, CA) was carried out as previously described (see [Supplementary Materials and Methods](https://doi.org/10.1016/j.esmooop.2020.100012), available at <https://doi.org/10.1016/j.esmooop.2020.100012>, for details).^{21,22} We

validated this approach for earlier projects using other platforms and reached a confirmation rate >95%.^{15,22} Furthermore, we visually controlled all calls using the Integrative Genomics Viewer browser®.

Statistical analyses

The 24th version of the software IBM SPSS Statistics (Armonk, New York, NY) was used for statistical analyses. Survival was estimated by Kaplan–Meier curve analyses and the log-rank test was applied for statistical comparisons. The Mann–Whitney U test was used for comparison of continuous data between two groups and the Pearson's chi-squared was used for comparison of categorical data between two groups. Statistical significance was reached with a *P* value <0.05.

RESULTS

Patient characteristics

Between 2004 and 2014, 16 patients at our cancer center were diagnosed with a CNS relapse of DLBCL, and 6 of these patients were biopsied both at an LN at primary diagnosis as well as at the CNS at relapse. Patient 4 had an additional skin biopsy at primary diagnosis that was also sequenced. Five of the patients (83.3%) had non-germinal center B-cell-like (non-GCB) DLBCL according to the Hans classifier, and none of the patients had an *MYC* translocation. All patients were HIV negative. At recurrence, lymphoma cells were detected in the cerebrospinal fluid of patient 1 and patient 2 had a lymphoma infiltration of the eye (see [Table 1](#) for detailed patient characteristics).

In order to estimate a possible selection bias, we compared the clinical characteristics of the 6 patients with available biopsies used for this analysis with the 10 patients without biopsies of the CNS at relapse. All 16 patients received R-CHOP-like regimens as first-line therapy and there were no statistically significant differences in age, performance score, stage and clinical outcome between the two patient groups. The median time to CNS recurrence was 17.0 months in the cohort with available biopsies, and 6.0 months in the group of patients without a biopsy (*P* = 0.33, see [Supplementary Table S2](https://doi.org/10.1016/j.esmooop.2020.100012), available at <https://doi.org/10.1016/j.esmooop.2020.100012>). The median PFS and OS of patients with available biopsies were 17.0 and 22.0 months, respectively, compared with 6.0 (*P* = 0.36) and 14.0 (*P* = 0.50) months in patients without biopsies. After CNS relapse the median OS of patients in this analysis was 3.0 months. Two patients were still alive at the last point of follow-up.

Massively parallel sequencing

The median tumor cell content of all 13 lymphoma biopsies was 90% (range 70%-90%). In all but one sample, 80% of the targets were sequenced to at least 30× mean target coverage (MTC), which was set as an internal quality criterion before this analysis. The average MTC was 175.9×. Patient 6 was excluded from further analyses because in the

Table 1. Patient characteristics								
Patient	COO	Diagnosis (year)	Sex	NCCN-IPI	PFS (months)	OS (months)	Status ^a	Sequencing successful
1	GCB	2012	Female	High	7	10	Dead	Yes
2	Non-GCB	2009	Female	High-int.	19	33	Dead	Yes
3	Non-GCB	2008	Female	Low-int.	19	120	Alive	Yes
4	Non-GCB	2010	Male	High-int.	17	22	Dead	Yes
5	Non-GCB	2007	Male	High-int.	6	8	Dead	Yes
6	Non-GCB	2008	Male	Low-int.	67	132	Alive	No

COO, cell of origin; int., intermediate; GCB, germinal center B-cell-like; NCCN-IPI, National Comprehensive Cancer Network-International Prognostic Index; OS, overall survival; PFS, progression free survival.

^a Last point of follow-up October 2019.

LN sample from this individual only 68.3% of the targets were sequenced 30×. A complete list of exonic mutations in all five patients is shown in [Supplementary Tables S3-S7](#), available at <https://doi.org/10.1016/j.esmooop.2020.100012>. Overall, we detected somatic, exonic mutations in 76 (35.2%) of the 216 selected genes and in 58 (26.9%) genes non-synonymous mutations were seen. The median number of exonic mutations detected per patient was 32 (range 12-50), and the median number of non-synonymous mutations detected per patient was 23 (range 10-36). In order to assess the genomic differences between the LN biopsies and the skin sample of patient 4 at primary diagnosis and the recurrent CNS manifestations, we calculated the Jaccard similarity coefficient for all patients. This coefficient describes the similarity of a sample set and was calculated as the number of concordant mutations found in both the peripheral lymphoma infiltrates as well as the CNS sample of one patient divided by the number of all mutations (concordant and discordant) per patient. The closer the coefficient is to 1 the more homogenous the samples of a patient are. A discordant mutation was defined as a mutation found with an allelic frequency (AF) of at least 10% in one of the biopsies (LN, skin or CNS) but not found in another lymphoma sample of the same patient. The 10% cut-off was chosen to investigate only mutations present at a meaningful clonal fraction. All patients had at least one discordant mutation (median 5, range 1-20), and four patients had mutations that were only present in the CNS but not the primary LN. Patient 4 had one mutation that was found in the LN and the CNS but not the skin sample. There was a wide range in the number of discordant mutations detected only at CNS recurrence per patient (0-10) with a median count of 5. [Figure 1](#) shows a list of all genes where we found exonic, non-synonymous mutations with an AF of at least 10% in one of the samples per patient. Several concordant mutations were found in genes known to be important for lymphoma biology, such as *BCL2*, *BTG2*, *CARD11*, *CD79B*, *MYC* and *MYD88*. There was no clear pattern of genetic change seen at CNS recurrence, meaning that the same gene mutations could only be detected in the LN or the CNS in different patients. New mutations in the CNS samples were seen in *HLA* genes and epigenetic regulators but also in *MYC*, *EP300* and *CCND3* in two patients. Mutations that were found in the primary LN but not in the recurrent CNS lesion were found, for example, in *CCND3*, *DUSP27*, *MYC* and *CARD11*. The median Jaccard similarity

coefficient for non-synonymous exonic mutations in this cohort was 0.76 (range 0.26-0.93, see [Figure 2](#)). Patient 2 and patient 3 had a Jaccard similarity coefficient closer to 0 (0.26 and 0.34) indicating more differences between the primary LN and the CNS relapse compared with patients 1, 4 and 5 where the Jaccard similarity coefficient was closer to 1 (0.90, 0.76 and 0.93, respectively). In this small cohort there were no statistically significant differences in survival comparing these two groups (median PFS 19.0 versus 7.0 months and median OS 22.0 versus 10.0 months in patients with more heterogeneous compared to more homogeneous samples).

DISCUSSION

For this analysis we used a targeted massively parallel sequencing approach of the exons of 216 genes known to be recurrently mutated in DLBCL in order to investigate the genetic landscape of SCNSL and to elaborate on clonal evolution. We successfully sequenced 10 paired LN and CNS samples of five patients who experienced CNS relapse of DLBCL during the course of their disease. Additionally, we analyzed a lymphoma infiltrate of the skin of one patient. Due to the high tumor cell content of all samples, we were able to detect several mutations that were present at similar AFs in the paired primary LN as well as the CNS sample of each patient. These shared mutations combined with the typical clinical course, with the CNS lymphoma occurring within 24 months of the primary diagnosis, show that our patients suffered from SCNSL and makes a second lymphoid malignancy in these individuals very unlikely.

However, all patients had at least one discordant mutation that was not present in one of their samples, and four out of five patients had at least one mutation that was only detected in the CNS. The Jaccard similarity coefficients of the five patients in our study were in a similar range as the scores of 0.5 and 0.61 reported in two patients with follicular lymphoma, where samples were also taken at two timepoints.²³ However, patient 2 and patient 3 in our cohort had Jaccard similarity coefficients closer to 0 suggesting substantial clonal diversification between initial diagnosis of systemic DLBCL and CNS relapse in these individuals. These findings are in line with the data reported by Isaev et al., who also described the presence of new mutations in the CNS relapse of five patients with systemic DLBCL.²⁴ The two patients with many newly acquired

Function	Genes	P1		P2		P3		P4			P5	
		LN	CNS	LN	CNS	LN	CNS	skin	LN	CNS	LN	CNS
cell adhesion	<i>NRXN3</i>											
cell adhesion	<i>POSTN</i>											
cell cycle	<i>BCL2</i>	4	4									
cell cycle	<i>BTG2</i>											
cell cycle	<i>CCND3</i>											
cell cycle	<i>DNAH5</i>			1		1						
cell cycle	<i>MYC</i>	2	1	2	1							
cell cycle	<i>PIM1</i>			2	2	2	2	1	1	1	1	
cell cycle	<i>STAT3</i>											
cell cycle	<i>UBE2A</i>											
cell-cell interaction	<i>CDH9</i>											
cell-cell interaction	<i>PCDHB11</i>											
cellular stress response	<i>SGK1</i>	2	2									
cytoskeleton	<i>ACTB</i>											
cytoskeleton	<i>DST</i>											
cytoskeleton	<i>PCLO</i>											
cytoskeleton	<i>TMSB4X</i>											
epigenetic regulators	<i>EP300</i>											
epigenetic regulators	<i>HIST1H1C</i>											
epigenetic regulators	<i>HIST1H2AG</i>											
epigenetic regulators	<i>KMT2D</i>	2	2									
G protein signalling	<i>OR10A2</i>											
G protein signalling	<i>P2RY8</i>		2									
G protein signalling	<i>PKD1</i>											
immune response	<i>CD58</i>											
immune response	<i>CD83</i>											
immune response	<i>HLA-A</i>				2							
immune response	<i>HLA-B</i>											
immune response	<i>HLA-C</i>											
immune response	<i>IGLL5</i>			4	4	1	3	1	1			
immune response	<i>MPEG1</i>											
immune response	<i>MYD88</i>											
iron metabolism	<i>HEPH</i>											
lipid receptor	<i>OSBPL10</i>											
neuronal development	<i>TENM4</i>							2	2	2		
NFKB/BCR signalling	<i>BCL10</i>											
NFKB/BCR signalling	<i>CARD11</i>			1	1	1						
NFKB/BCR signalling	<i>CD79B</i>											
NFKB/BCR signalling	<i>KLHL14</i>											
NFKB/BCR signalling	<i>LYN</i>											
Notch signalling	<i>NOTCH2</i>											
phosphatase activity	<i>DUSP27</i>											
regulation of endosomes	<i>DIAPH2</i>											
sarcomeric signaling	<i>OBSCN</i>											
sarcomeric signaling	<i>TTN</i>											
transcription factor	<i>FOXO1</i>											
transcription factor	<i>IKZF3</i>											
transcription factor	<i>MEF2B</i>											
transcription factor	<i>PRDM1</i>											
transcription factor	<i>TBL1XR1</i>			1		1						
transcription factor	<i>TOX</i>					1		2				
transcription factor	<i>TSC22D1</i>											
transcription factor	<i>ZNF608</i>											
uncharacterized	<i>KIAA1614</i>											
uncharacterized	<i>LRRN3</i>											
Wnt signalling	<i>APC2</i>											

concordant mutations found in LN/skin and CNS

discordant mutations not found in one of the samples per patient

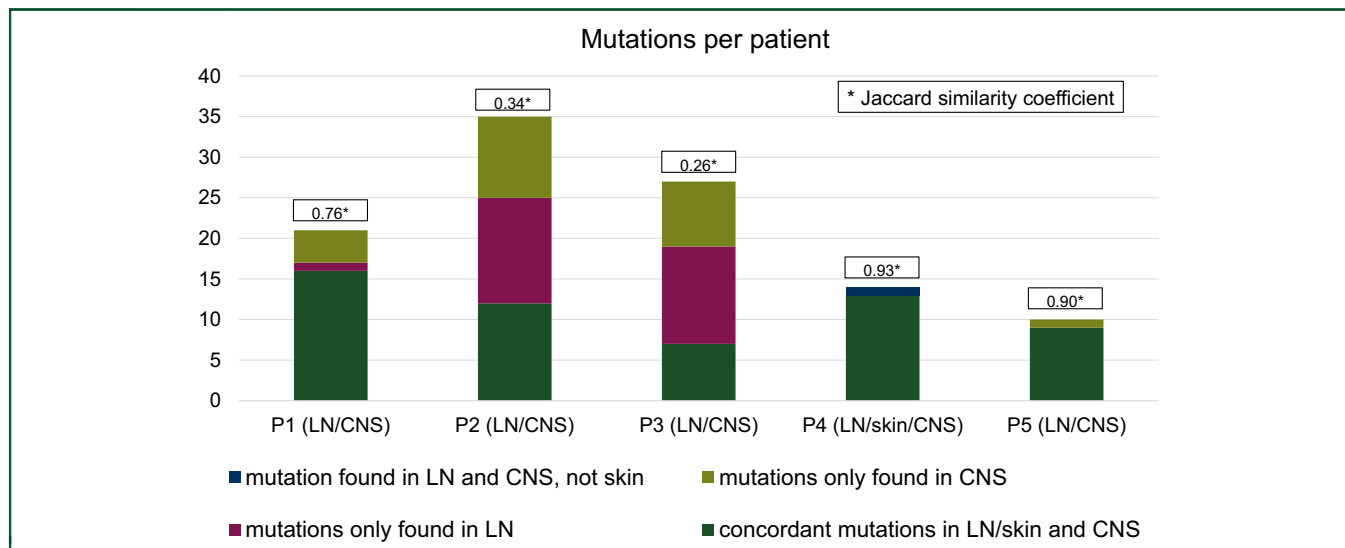


Figure 2. Number of mutations per patient.

The number of non-synonymous mutations per patient with an allelic frequency of at least 10% in one of the samples. Concordant mutations found in lymph node (LN)/skin and central nervous system (CNS) of one patient and discordant mutations are shown in indicated colors. The Jaccard similarity coefficient including synonymous and non-synonymous mutations is shown in [Supplementary Table S8](https://doi.org/10.1016/j.esmooop.2020.100012), available at <https://doi.org/10.1016/j.esmooop.2020.100012>.

mutations at CNS relapse show a non-statistically significant trend towards longer PFS and OS compared with the other three patients in our sample set. Interestingly, the majority of the mutations that were only detected in the CNS in these two patients had an AF above 30% and therefore represented the major clone in these biopsies. Similar patterns were seen in patients with gliomas where treatment associated mutations at relapse were mostly clonal but not associated with a worse prognosis.^{25,26} As all patients in our cohort were treated with R-CHOP-like regimens at primary diagnosis we are unable to tell if the newly acquired mutations seen in patients 2 and 3 might also be caused by chemotherapy.

Despite its known association with a poor prognosis in both systemic DLBCL and PCNSL, none of the five patients in this cohort with CNS recurrence harbored a *TP53* mutation. The genes that were most frequently mutated in our analysis were *IGLL5*, *HLA-C*, *KMT2D*, *PRDM1* and *TMSB4X*, some of which were reported to be recurrently mutated in double- and triple-hit lymphomas.²⁷ However, none of the patients in our cohort had an *MYC* translocation in FISH analyses. In three of our patients we saw multiple mutations in *PIM1* and *BCL2*. These genes are known targets of somatic hypermutation in systemic DLBCL as well as in PCNSL and were also found to be recurrently mutated in 25 patients with systemic relapse of DLBCL. While the role of these hypermutated genes for the biology of the disease is unclear, they may enable us to track clonal evolution in our patients.^{9,15,28,29} *MYD88*, *CD79B* and *TBL1XR1* mutations are known drivers for PCNSL and were also found in five of our patients: two *MYD88*, two *CD79B* and one *TBL1XR1*.⁶⁻¹²

A review of the literature showed that overall, 46 (60.5%) of the mutations seen in our patients were also described in patients with PCNSL. However, in our cohort we also found mutations in several genes such as *FOXO1*, *HLA-A*, *HLA-B*, *LYN* or *STAT3* that have not been described in the major reports on PCNSL, suggesting a different genetic background compared with SCNSL.⁶⁻¹⁴ Genes with mutations that were only detected in the CNS but not in the primary LN are involved in the cell cycle, such as *MYC*, *CCND3* and *STAT3*, but are also transcription factors such as *TOX* and *TBL1XR1*, epigenetic regulators *HIST1H1C* and *HIST1H2AG* or involved in immune response, *HLA-A* and *HLA-C*. Mutations of *CARD11* are important driver mutations of lymphomagenesis.⁵ Therefore, it is interesting that a COSMIC annotated *CARD11* mutation was not detected in the CNS relapse of one patient, despite its presence in the primary LN and high coverage in this region. This is comparable with what we have seen in two patients with systemic recurrence of DLBCL and might be explained by the previous report on the varying biological impact of different *CARD11* mutations on the evolution and resistance mechanisms of the disease.^{15,30} One patient also had discordant *MYC* mutations in the LN and the CNS. While it could be that different clones acquire different mutations in genes that are important drivers for lymphomagenesis, it is also known that *MYC* mutations have a varying effect on gene function.³¹

The clinical data of our cohort as well as previous literature show that patients with SCNSL have a poor prognosis.^{2,3} One of the reasons is that the CNS is protected by the blood-brain barrier which limits the concentration

Figure 1. List of genes with concordant and discordant mutations.

All genes with somatic, exonic, non-synonymous mutations and an allelic frequency of at least 10% in one of the samples per patient. In some genes several mutations were found in one patient. The number within the cells marks the mutation count for these genes. CNS, central nervous system; LN, lymph node.

of conventional chemotherapies reaching intracerebral tumors.³² However, recent therapies with small molecules, such as ibrutinib and lenalidomide, have shown promising activity in both PCNSL and SCNSL.³³⁻³⁵ Notably, for these types of targeted approaches it will be important to find predictive markers to select patients for the optimal therapy. Mutations in *CARD11* and *CD79B* that were found in three of our patients are known resistance mechanisms in patients with PCNSL treated with ibrutinib.³⁶ Furthermore, the presence of oncogenic *CARD11* and *MYD88* mutations, detected in two patients in our cohort, were reported to play a role in lenalidomide sensitivity of non-GCB DLBCL.³⁷ This shows that the greater insight into the genomic profile of SCNSL might also improve current and future treatment strategies for this disease.

The approach of targeted resequencing of a selected group of genes has a limitation in finding new mutations. However, this technique was feasible and made it possible to further our insight into the genetic landscape of SCNSL and differentiate it from PCNSL. Furthermore, we demonstrated that as in systemic recurrence of DLBCL, there can be substantial clonal diversification between initial diagnosis and CNS recurrence in some patients, which might be one of the mechanisms of resistance in this disease. Another limitation of our study is the small sample size which is caused by the fact that biopsies of SCNSL are often not safely possible due to the location of the tumor, the urgency for treatment or the performance score of the patients. In the future, it will therefore be interesting to see if the heterogeneity seen in our patients can also be depicted by the analyses of circulating tumor DNA as this technique will be easier to implement in clinical routine.

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DISCLOSURE

The authors declare no conflict of interest.

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