Detection of second primary lymphoma in late diffuse large B-cell lymphoma recurrences

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Article

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Abstract

Approximately one-third of diffuse large B-cell lymphoma (DLBCL) patients relapse, who often require salvage chemotherapy followed by autologous stem cell transplantation. In most cases, the clonal relationship between the first diagnosis and subsequent relapse is not assessed, thereby missing the identification of second primary lymphoma. Here, the clonal relationship of 59 paired DLBCL diagnosis and recurrences was established by next-generation sequencing (NGS)-based detection of immunoglobulin gene rearrangements. In 50 cases with interpretable results, 43 DLBCL patients (86%) developed clonally related lymphoma and relapsed disease. In total, 100% of early recurrences (< 2 year), 80% of the recurrences with an interval between 2 and 5 years, and 73% of late recurrences (≥ 5 year) were clonally related. In contrast, 7 out of 50 patients (14%) showed distinct dominant clonotypes in primary DLBCL and its recurrences, suggesting the occurrence of second primary DLBCL, which all occurred at least 4 years after primary diagnosis. Moreover, 43% of clonally unrelated cases were Epstein-Barr virus-positive, while this was 3% in relapsed DLBCL. In conclusion, NGS-based clonality testing in late recurrences should be considered in routine diagnostics to distinguish relapse from second primary lymphoma, as this latter group of DLBCL patients may benefit from less intensified treatment strategies.

Introduction

Diffuse large B-cell lymphoma (DLBCL) displays large heterogeneity with respect to the molecular alterations that drive lymphoma development and treatment response (1, 2). First-line R-CHOP (rituximab, cyclophosphamide, hydroxydaunorubicin, oncovin and prednisolone) treatment achieves complete remission in about 60% of patients diagnosed with DLBCL. In contrast ~ 10% suffer from primary refractory disease, with progression during or right after treatment, and 30–40% relapse after achieving complete remission, which is associated with poor outcome (3–5). DLBCL relapsed disease predominantly occurs within 2 years after first-line immunochemotherapy (5, 6), and most of these patients will receive salvage chemotherapy regimens in combination with autologous stem cell transplantation (ASCT), when eligible (7). Remarkably, in about 15% of DLBCL patients relapsed disease occurs after an interval of 5 years or more, and these late relapsed DLBCL patients display a lower international prognostic index (IPI) score and stage of disease, accompanied with an improved treatment outcome as compared to patients with a shorter time to relapse (6, 8, 9).

In case of DLBCL recurrence, B-cell clonality assessment of the primary and subsequent DLBCL is generally not performed, and the identification of clonally unrelated second primary DLBCL may be missed. These recurrences represent *de novo* DLBCL, and may therefore not require intensified treatment regimens. Clonally unrelated DLBCL has only been described in relatively small cohort studies (10–16), whereby the exact prevalence of second primary DLBCL has remained largely unknown. Although the underlying mechanisms for the occurrence of second primary lymphomas are still poorly understood, it has been reported in patients with primary immunodeficiencies and hereditary cancer predisposition syndromes (17–19). Distinguishing true relapsed DLBCL from second *de novo* DLBCL, will improve diagnostics of DLBCL patients and allow better treatment decisions.

B-cell lymphomas have an unique molecular footprint of immunoglobulin (IG) gene rearrangements, which facilitates assessment of clonal relationships between diagnosis and recurrent lymphomas (20). Recently, the Euroclonality-NGS Working Group has developed a novel next-generation sequencing (NGS)-based assay combined with ARResT/Interrogate immunoprofiler platform for detecting both complete and incomplete IG heavy (IGH) and kappa light (IGK) chain gene rearrangements (21, 22). This IG-NGS approach allows accurate clonality assessment due to availability of the exact clonotype nucleotide sequence, and is highly suitable for formalin-fixed paraffine-embedded (FFPE) samples because of amplification of smaller-sized amplicons, especially for the IGK locus (21). The IG-NGS assay has been validated for both B-cell non-Hodgkin lymphoma (NHL) and classical Hodgkin lymphoma, showing improved clonality detection as compared to conventional Euroclonality-BIOMED-2/Genescan analysis (23, 24). In addition, NGS-based analysis of IG gene rearrangements allows in depth clonotype analysis, which may facilitate the interpretation of clonality, and the immediate identification of stereotyped B-cell receptors (BCR) (25, 26).

Here, we investigated the clonal relationship of DLBCL recurrences by performing IG-NGS analysis in a large cohort of paired diagnosis and recurrent DLBCL samples, which involved different time intervals between diagnosis and recurrence(s). By determining the specific clonotypes in both the primary lymphoma and subsequent presentations, we demonstrate the occurrence of clonally unrelated lymphoma in 14% of cases, all with a time to recurrence of ≥ 4 years.

Materials And Methods

Patient selection

A cohort of 59 patients was selected, diagnosed between 1991 and 2021 with *de novo* DLBCL not otherwise specified (NOS; 2017 World Health Organization classification), followed with a subsequent DLBCL relapse for which tissue biopsies were available. Clinical data and informed consent was available for 32 DLBCL patients. Patients where clinical information was not available (n = 27), recurrences were considered as relapse/refractory with < 1 year interval to prior diagnosis (n = 8), and as relapse with ≥ 1 year interval. For uniformity, all cases are described as relapse in our study descriptions. Patient data and tissue samples were retrieved from the following centers: Radboud University Medical Center (Radboudumc, Nijmegen), Rijnstate Ziekenhuis (Arnhem), Jeroen Bosch Ziekenhuis ('s Hertogenbosch), Leiden University Medical Center (Leiden) and Canisius Wilhelmina Ziekenhuis (Nijmegen). Histology of all DLBCL tissue biopsies (n = 142), which involved 107 FFPE and 35 fresh frozen (FF) tissue samples, was reviewed by an experienced hematopathologist (M.v.d.B. and H.v.K.). This study was approved by the local institutional Medical Ethical Committee (2020–6390).

Clonality analysis by NGS-based detection of immunoglobulin gene rearrangements

Clonality analysis was performed by detecting complete and incomplete IG heavy chain (IGH) and IG kappa chain (IGK) gene rearrangements with NGS as previously described (21, 27). For each tissue sample, three multiplex PCR reactions were performed with standardized primers and 40 ng input DNA (with a minimum of 10 ng for limited material samples), to detect IG gene rearrangements involving variable (V), diversity (D), and joining (J) genes, as well as kappa

deleting element (KDE) and intronic recombination signal sequence (RSS) element. The following targets were analyzed: Framework-3 (FR3) IGHV-IGHD-IGHJ, IGHD-IGHJ, IGKV-IGKJ, IGKV-KDE, and IGK-Intron RSS-KDE.

IG-NGS clonality analysis was performed using either a protocol compatible with the adaptor ligation protocol with the Ion Torrent platform (21), or a two-step PCR protocol for sequencing on Illumina platform (27). In short, with the adaptor ligation protocol, library preparation was performed using the Plus Fragment Library Kit (Thermo Fisher Scientific, Waltham, MA, USA). Thereafter, for each sample equal DNA concentrations were loaded on Ion Torrent 318 chip (12 ng/ml) and sequenced (Ion PGM template OT2 200 kit, Ion Chef, Ion One Touch 2 system; Thermo Fisher Scientific, Waltham, MA, USA). For Illumina PCR two-step protocol, after the first-step multiplex PCR using M13-tailed IG target specific primers (28), library preparation was performed with adaptor-tailed M13 primers and the FastStart™ High Fidelity PCR System, dNTPack kit (Roche, Bazel, Switzerland). Subsequently, the samples were pooled at equal molarity (4 nM), loaded an Illumina mid-output chip and sequenced with MiniSeq500. NGS data were analyzed using the bioinformatics pipeline ARResT/interrogate (22), with a required minimum of 1000 reads coverage for IGHV-IGHD-IGHJ (FR3) and IGKV-IGKJ targets, and 500 reads for IGHD-IGHJ,IGKV-KDE and Intron RSS-KDE targets in case of Ion Torrent protocol, and a minimum coverage of > 1000 reads for all targets with the two-step PCR Illumina protocol. A clonotype (e.g. V4-34 -3/16/-1 J2) was defined by the 5' (V4-34) and 3' (J2) gene annotation, and the number of nucleotides deleted from the 5' (-3) and 3'(-1) gene, and added at the junction (/16/) (including D-segment in case of IGHV-IGHD-IGHJ) (29).

Identification of clonal rearrangements in DLBCL samples

To determine the lymphoma-associated clonotypes for each IG target, the abundance of the most dominant clonotype(s) was compared to the polyclonal background of non-malignant B-cell clonotypes. The technical and molecular scoring was based up on the principles previously described (23) (see supplementary information). For the technical scoring of each target, data output was classified as not evaluable (n.e.) in case of a lower number of reads than the minimum required reads (see above), or due to lack of reproducibility in technical replicates. In addition, data output was classified as polyclonal in case of sufficient reads but no dominant clonotype with a polyclonal background pattern, and as no clonal product (n.c.p) with sufficient reads but low heterogeneity in polyclonal background or an irregular polyconal pattern. Samples that showed interpretable results without any clonal rearrangements or only a single target, as well as all clonally unrelated cases were repeated as a technical replicate. In all clonally unrelated cases the recurrence-associated dominant clonotypes were not detected as subclone at first diagnosis in the top 15 of most abundant gene rearrangements, even with doubled amounts of input DNA, if sufficient material was available.

Additional information regarding Materials and Methods are provided in Supplementary Information.

Results

Clinicopathological characteristics of paired diagnosis-recurrence DLBCL patient cohort

In this study, paired diagnosis-recurrent DLBCL tumor samples of 59 patients were collected for clonality assessment. The majority of patients presented with one DLBCL recurrence (n = 44/59, 75%), while 20% had two recurrences and 5% had three DLBCL recurrences. (Table 1 and Supplementary Table 1). Approximately one-third of patients presented with stage IV disease (n = 11/31, 35%) at time of diagnosis, and most patients had low-risk IPI score (n = 9/24, 38%). The majority of patients were treated with (immuno)-chemotherapy (n = 30/32; 94%) after the initial diagnosis, and received (immuno)-chemotherapy with or without ASCT (n = 27/30, 90%) after the first recurrence (Supplementary Table 2).

Table 1
Clinicopathological data of relapsed DLBCL

Gender n = 59	
Female	26 (44%)
Male	33 (56%)
Age n = 59	
< 60	31 (53%)
≥ 60	28 (47%)
Stage n = 31	
I	5 (16%)
II	8 (26%)
III	7 (23%)
IV	11 (35%)
IPI n = 24	
Low	9 (38%)
Low-intermediate	7 (29%)
High-intermediate	6 (25%)
High	2 (8%)
First Line treatment n = 32	
Immuno-chemotherapy	12 (38%)
Chemotherapy	18 (56%)
Other	2 (6%)
Site of biopsy diagnosis n = 59	
Nodal	37 (63%)
Extranodal	22 (37%)
Immune-privileged	5 (9%)
Number of recurrences	
1	44 (75%)
2	12 (20%)
3	3 (5%)
Time to recurrence	
Interval	0.3 year-20.3yr
Cell-of-origin n = 46	
GCB	22 (48%)
Non-GCB	24 (52%)
Cell-of-origin was determined with Hans algorithm. IPI: International prognostic index. In case of nodal and extranodal biopsy, only nodal is indicated here. Immuno treatment indicates rituximab. (immuno-)chemotherapy was combined with radiotherapy in several patients.	

The time interval between primary diagnosis and first recurrence (time to relapse) varied between 5 months and 20.3 years (median 2.8 years), where 41% of DLBCL patients showed an early recurrence (< 2 years), and 31% presented with late recurrence (≥ 5 years). The remaining 17 patients (29%) displayed an intermediate interval between 2 and 5 years until the first relapse. In case of two or three recurrences (n = 12, two recurrences; n = 3, three recurrences), time from the first to the second recurrence involved a median interval of 2.7 years. Location of the tumor biopsies at time of the primary diagnosis predominantly involved nodal sites (63%), with the remainder of cases presenting at extranodal locations (37%), including the skin (27%), and immune-privileged (IP) sites (23%), such as the testis and central nervous system. Tumor biopsies at recurrence were either from nodal (n = 43/77, 56%) or extranodal sites (n = 34/77, 44%).

Cell-of-origin classification according to Hans algorithm of each case (n = 46) revealed a similar distribution of germinal center B-cell like (GCB) and non-GCB in this study cohort (Table 1 and Supplementary Table 1). In addition, MYC- and BCL2- positive double expressor lymphomas (DEL) were identified in 9 out of 46 cases (20%), which predominantly involved diagnosis samples, and 5 out of 45 cases (11%) showed EBV-positivity, which mostly corresponded to the non-GCB subtype (Supplementary Table 2). Detection of genomic rearrangements by FISH revealed the presence of *BCL2* translocations in 5 out of 34 cases (15%), *BCL6* translocations in 5 out of 33 cases (15%), and *MYC* translocations in 6 out of 37 cases (16%). Notably, only 2 out of 39 cases (5%) showed a *MYC* and *BCL2* translocation-positive double-hit lymphoma (DHL).

NGS- based clonality assessment in paired diagnosis-recurrence DLBCL samples

To determine the clonal relationship in our study cohort of 59 paired diagnosis-recurrence DLBCL samples, clonality analysis was performed in a total of 142 tissue specimens (n = 107 FFPE; n = 35 FF), with the EuroClonality IG-NGS assay (21) (Fig. 1A). This IG-NGS approach detects both productive and non-productive IGH (IGHV-IGHD-IGHJ (FR3) and IGHD-IGHJ) and IGK (IGKV-IGKJ, IGKV-KDE, and Intron RSS-KDE) gene rearrangements for each lymphoma sample (21). In each sample the dominant clonotype(s) for every target was determined according to defined guidelines (see suppl M&M; Supplementary Table 3). From 6 patients, lymphoma biopsies from two distinct anatomical locations were included, which yielded 63 diagnosis samples, and 73 recurrence samples. In 127 out of 142 samples (89%), a dominant clonotype was detected in at least one of the five targets. This involved in 103 out of 142 (73%) samples a clonal IGKV-IGKJ gene rearrangement, in 69 out of 142 (49%) samples a clonal IGHV-IGHD-IGHJ (FR3) gene rearrangement, in 64 out of 142 (45%) a clonal IGKV/Intron RSS-KDE rearrangement, and in 62 out of 142 (44%) a clonal IGHD-IGHJ gene rearrangement (Fig. 1B).

In 15 out of 142 samples (11%; n = 13 FFPE; n = 2 FF), IG-NGS failed to detect IG clonality, which included 7 diagnosis and 8 recurrence samples. In 9 samples this was related to inferior genomic DNA (gDNA) quality, and in the remaining 6 samples this was most likely related to the presence of somatic hypermutations (SHM) in IGHV and/or IGKV regions, in combination with low tumor load (Supplementary Table 3). Notably, four cases showed three or more clonal IGKV-IGKJ/IGKV-intron-KDE rearrangements, which was consistent with a single clone and related to the occurrence of IGK-inversions (30) (Supplementary Fig. 1).

The clonal relationship of paired diagnosis-recurrence DLBCL

Next, the clonal relationship of the paired diagnosis-recurrence DLBCL samples was established in 50 cases for which at least one dominant clonotype was detected in both primary diagnosis and subsequent recurrence. In 43 of 50 cases (86%), identical clonotypes could be detected for at least one of the five targets, indicating that diagnosis and recurrence samples were clonally related (Fig. 2; see suppl M&M for details). For a few of the clonally related cases (n = 6), some differences in the junctional clonotype sequence were observed between diagnosis and relapse, most likely due to ongoing somatic hypermutation (SHM) (Supplementary Tables 3 and 4). In contrast,7 out of 50 cases (14%) showed distinct dominant clonotypes for one or more IG gene rearrangements, and were identified as clonally unrelated recurrences.

In 8 cases, a possible clonal relationship could not be assessed due to the absence of a clonal gene rearrangement in the diagnosis, recurrence or both. In addition, one patient (DLBCL-154), who developed two subsequent DLBCL recurrences (lymph node biopsies), showed clonal rearrangements with different dominant clonotypes compared to the primary DLBCL (skin biopsy) for the IGKV targets (Supplementary Table 3). These gene rearrangements were, however, already subclonally present in the primary DLBCL, which was also the case for the second IGHV-IGHD-IGHJ (FR3) gene rearrangement. Therefore, this case may represent a biclonal DLBCL at primary diagnosis, where one of the clones eventually relapsed. Both primary diagnosis and first recurrence were EBV-positive, but displayed a different histomorphology, with more background inflammatory infiltrate and pleomorphic tumor cells that showed a Hodgkin and Reed/Sternberg (HRS) cell-like morphology in the recurrence.

Clonally related DLBCL recurrences are identified in both early and late relapsed disease

In the 43 clonally related cases, identical clonotypes in both diagnosis and recurrence were detected for either a single IG gene rearrangement (n = 14), or two or more IG targets (n = 29) (Supplementary Fig. 2). Here, 100% of early recurrences (< 2 year), 80% of the recurrences with an interval between 2 and 5 years, and 73% of late recurrences (≥ 5 year) were clonally related (Fig. 3). Furthermore, several cases (n = 12) involved DLBCL patients with multiple recurrences in which identical clonotypes were identified. Notably, case (DLBCL-107) showed three consecutive relapses with clonally related lymphoma and time intervals of 2.1, 4.8 and 5.5 years inbetween (Fig. 4). EBV-positivity was observed in only 1 out of 34 (3%) clonally related relapsed DLBCL cases. Clonally related DLBCL recurrences included DLBCL biopsies taken at nodal, extranodal and a combination of both sites for both diagnosis and relapse (Supplementary Fig. 3). Tissue biopsies taken at different anatomical locations within the same patient at time of diagnosis (n = 6) (interval of < 2 months between biopsies), were all found to be clonally related (Supplementary Tables 1 and 3).

Detection of clonally unrelated DLBCL in patients with a late recurrence

Clonally unrelated cases displayed distinct clonotypes for either one (n = 1), two (n = 4) or three IG targets (n = 2) (Supplementary Figs. 2 and 4). Notably, all second primary DLCBL that were identified by IG-NGS occurred after a longer interval period (median 6 years, range between 4.3 and 10.3 years) (Supplementary Table 5). Within the group of late DLBCL recurrences with a time to relapse of ≥ 4 years, 7 of 19 were clonally unrelated (37%), while for DLBCL recurrences with a time to relapse of ≥ 5 years, 4 out of 15 were clonally unrelated DLBCL (27%). Patients who presented with clonally unrelated DLBCL had a median age of 59 years at first diagnosis (range 43–72 years), and both the primary DLBCL and the clonally unrelated recurrence were the same subtype in most cases (4 cases GCB; 2 cases non-GCB). However, one patient harbored a DLBCL with a GCB phenotype at first diagnosis, but a non-GCB at second presentation (Supplementary Table 6). In 1 out 7 cases (DLBCL- 106), the histomorphology differed between the primary and subsequent lymphoma, with an EBV- positive Hodgkin-like morphology at primary diagnosis, and an EBV-positive DLBCL morphology at second primary diagnosis (Fig. 5, Supplementary Fig. 5). EBV positivity was observed in 3 out of 7 clonally unrelated DLBCL at diagnosis (43%) (n = 2 both diagnosis and recurrence; n = 1 only at diagnosis), which is significantly higher than the EBV-positivity rate of 3% in the clonally related DLBCL cases (n = 1; relapse).

IGHV gene usage and stereotyped B-cell receptor in relapsed DLBCL

Antigenic stimulation of the B-cell receptor (BCR) is an important survival mechanism of both normal and malignant B cells, and particular antigens have been linked to lymphomagenesis (31). This has been supported by the identification of stereotyped groups of BCR with highly similar CDR3 sequences in different subtypes of B-NHL (32). To determine potential skewing in IGHV gene usage and the presence of stereotyped CDR3 sequences in the relapsed DLBCL cohort, the IGHV gene for each IGHV-IGHD-IGHJ gene rearrangement of the clonally related cases was analyzed (n = 35), and compared to the IGHV gene distribution in reactive lymph nodes (RLN; n = 20) (Supplementary Table 7). IGHV4-34 represented the most abundant IGHV gene, and was detected in 31% of cases as compared to 5% in RLN (Fig. 6). Likewise, although less prevalent, IGHV3-43 was also enriched in relapsed DLBCL compared to RLN (6% versus 1%). Other IGHV genes were not different between DLBCL and RLN, including IGHV3-23 (9% versus 8%) and IGHV4-39 (6% versus 5%). Due to short sequence avialibility of the IGHV gene within the current FR3 IG-NGS assay and high similarity among multiple V3 genes, the exact IGHV3 gene usage could not be determined in 6 DLBCL cases. Moreover, DLBCL cases in our relapse cohort that presented with IGHV4-34 were mostly combined with IGHD2-21 or IGHD3-22 (each 20%) and IGHJ4 (64%) (Table 2). Notably, 50% of IGHV4-34 cases for which we retrieved the CDR3 sequence of both diagnosis and relapse (n = 8) showed alterations in the relapsed DLBCL junction, resulting from ongoing SHM, although no common pattern could be identified (Table 2).

Table 2
IGHV4-34 CDR3 sequences in relapsed DLBCL

		VH gene	DH gene	JH gene	Length CDR3 (AA)	Junction (AA)	Nucleotide sequence
DLBCL.118	R1	IGHV4-34	IGHD2-21	IGHJ4	20	CARGGPYDYCGGGSCYFDFW	TGTGCGAGAGGGGGCCCCTACGATTATTGTGGTGGTGGCA(
DLBCL.144	R1	IGHV4-34	IGHD3-22	IGHJ4	16	CARVFYYDSSGYPDYW	TGTGCGAGAGTTTTCTATTATGATAGTAGTGGTTATCCCGA(
DLBCL.159	R1_2	IGHV4-34	IGHD6-19	IGHJ4	11	CARGPAVASIW	TGTGCAAGAGGCCCCGCAGTGGCTTCGATCTGG
DLBCL.101	D	IGHV4-34	IGHD2-15	IGHJ4	19	CAGGLPSCSGGRCY#SGYW	TGTGCGGGAGGACTACCATCTTGTAGTGGTGGTAGGTGTTA
	R1	IGHV4-34	IGHD2-15	IGHJ4	19	CAGGLPSCSGGRCY#SGYW	TGTGCGGGAGGACTACCATCTTGTAGTGGTGGTAGGTGTTA
DLBCL.102	D	IGHV4-34	IGHD3-9	IGHJ4	20	CARRQFRPFDWFFRGHFDFW	TGTGCGAGACGTCAATTCCGACCTTTTGACTGGTTTTTTAG
	R1	IGHV4-34	IGHD3-9	IGHJ4	20	CARRQFRPFDWFFRGHFDFW	TGTGCGAGACGTCAATTCCGACCTTTTGACTGGTTTTTTAG
	R2	IGHV4-34	IGHD3-9	IGHJ4	20	CARRQIRPFDWLFRGHFDFW	TGTGCGAGACGTCAAATCCGACCTTTTGACTGGTTATTTAG
DLBCL.107	D	IGHV4-34	IGHD2-21	IGHJ3	14	CARGPVVTHSFDIW	TGTGCGAGAGGCCCGGTGGTGACGCATAGTTTTGATATCTG
	R1	IGHV4-34	IGHD2-21	IGHJ3	14	CARGPVVTHSFDIW	TGTGCGAGAGGCCCGGTGGTGACGCATAGTTTTGATATCTG
	R2	IGHV4-34	IGHD2-21	IGHJ3	14	CARGPVVTHSFDIW	TGTGCGAGAGGCCCGGTGGTGACGCATAGTTTTGATATCTG
	R3	IGHV4-34	IGHD2-21	IGHJ3	14	CARGPVVTHSFDIW	TGTGCGAGAGGCCCGGTGGTGACGCATAGTTTTGATATCTG
DLBCL.108	D	IGHV4-34	IGHD3-22	IGHJ1	18	CANYYYDSTGYYKTFQHW	TGTGCGAATTATTACTATGATAGTACTGGTTATTATAAGACC
	R1	IGHV4-34	IGHD3-22	IGHJ1	18	CASYYYDSSGYYKTFQYW	TGTGCGAGTTATTACTATGATAGTAGTGGTTATTATAAGACC
	R2	IGHV4-34	IGHD3-22	IGHJ1	18	CASYYYDSSGYYKTFQYW	TGTGCGAGTTATTACTATGATAGTAGTGGTTATTATAAGACC
DLBCL.111	D	IGHV4-34	IGHD4-4	IGHJ5	19	CARHRGDSNDVITTWFDPW	TGTGCGAGACATCGCGGCGACAGTAACGACGTCATTACCAC
	R1	IGHV4-34	IGHD4-4	IGHJ5	19	CARHRGDSNDVITTWFDPW	TGTGCGAGACATCGCGGCGACAGTAACGACGTCATTACCAC
DLBCL.114	D_2	IGHV4-34	-	IGHJ4	10	CARRGGIDYW	TGTGCGAGACGAGGTGGCATCGACTACTGG
	D_3	IGHV4-34	-	IGHJ4	10	CARRGGIDYW	TGTGCGAGACGAGGTGGCATCGACTACTGG
	R1	IGHV4-34	-	IGHJ4	10	CARRGGIDYW	TGTGCGAGACGAGGTGGCATCGACTACTGG
DLBCL.119	D	IGHV4-34	IGHD5-24	IGHJ5	14	CVRQQEMSQFFDPW	TGTGTGAGACAACAAGAGATGTCCCAATTCTTCGACCCCTG
	R1	IGHV4-34	-	IGHJ5	14	CVRQQDMSQFFDPW	TGTGTGAGACAACAAGACATGTCCCAATTCTTCGACCCCTG
DLBCL.163	D	IGHV4-34	IGHD3-10	IGHJ4	13	CARGNTIGYISDW	TGTGCGCGGGGAAATACAATTGGCTACATTTCTGACTGG
	R2	IGHV4-34	-	IGHJ4	13	CAVGAPLGYISHW	TGTGCGGTAGGGGCCCCACTTGGCTACATTTCTCATTGG

For each clonally related case with a dominant IGHV4-34 clonotype, CDR3 amino acid (AA) and nucleotide sequence were retrieved from Arrest/Interrogate. Ir
detected in both diagnosis and relapse, clonal evolution was evaluated and sequence differences between the two sequences in CDR3 are underscored. Diag

Discussion

Approximately 40% of DLBCL patients suffer from refractory or relapsed disease after standard R-CHOP treatment (3, 5). Disease recurrence mostly occurs within 2 years after initial diagnosis (early relapse), but in some cases, time to relapse may even take more than 5 years (late relapse) (5, 6, 8, 9). It still remains to be established if in both scenarios, the recurrences indeed represent true relapsed disease, or whether these involve a second primary DLBCL, in which case intensified treatment regimens may not be required. In fact, the exact prevalence of clonally unrelated DLBCL in both early and late relapse has remained largely unknown.

In this study, the recently established and validated EuroClonality IG-NGS assay was employed to assess the clonal relationship of 59 paired DLBCL diagnosis and recurrence samples, including patients with multiple DLBCL recurrences. In contrary to conventional BIOMED-2/Genescan analysis, where solely fragment lengths of IG gene rearrangements can be compared (if required combined with Sanger sequencing) (16), the Euroclonality IG-NGS retrieves the exact clonotype sequence immediately, thereby overcoming potential false interpretations. In this study, clonality could be assessed in 127 out of 142 DLBCL tissue samples (89%). In the remaining samples no clonal rearrangements were identified due to either poor gDNA quality, low tumor load, or potential mismatches between target and primers sequences related to SHM. Consequently, in 50 of the 59 cases (85%), the clonal relationship between the primary diagnosis and recurrences could be unequivocally established. DLBCL recurrences with a time to relapse of < 2 years were all clonally related to the initial DLBCL, while clonally unrelated cases were observed in 20% of patients with an intermediate time interval between 2 and 5 years, and 27% in patients with a late relapse (≥ 5 years). In total, 14% of DLBCL recurrences were identified as clonally unrelated lymphomas.

Interestingly, in one case (DLBCL-154), the dominant clonotypes between diagnosis and recurrence were different, but the clonal relapse-associated clonotypes could be detected subclonally at diagnosis, indicating the presence of clonal diversity at time of diagnosis. Except for this one case, our data provide no substantial evidence for biclonality in relapsed DLBCL, confirming the observation that occurrence of biclonality in lymphomas is rare and mostly concerns the co-occurrence of two distinct diseases (33–35). Cases with multiple IGK rearrangements, possibly caused by inversion, were identified in ~ 8% (n = 4) of cases, which is in accordance of previous findings (30).

For clonally related DLBCL recurrences, analysis of IGHV gene usage in these relapsed DLBCL cases showed a predominance for IGHV4-34 compared to reactive lymphoid tissue. Presence of IGHV4-34 has been shown to correlate with chronic activated BCR signaling (36), and is required for cell survival in non-GCB DLBCL (37), due to autoreactivity caused by binding to glycoproteins on the cell surface (38). The prevalence of IGHV4-34 in our cohort of relapsed DLBCL (31%), which mostly involved non-GCB DLBCL, is similar to previous findings in primary DLBCL (~ 24%) (ref. 36). Other frequently observed IGHV genes in relapsed DLBCL involved IGHV3-23 and IGHV4-39, but prevalence was not different in RLN, and these are known to be positively biased in regular pro-B cell IG rearrangements compared to other IGHV genes (39), and have also previously been identified as overrepresentated in unbiased DLBCL cohort (40). When comparing the junctional amino acid sequence of IGHV-34 between primary diagnosis and relapse sample, this sequence differed in 50% of cases, but no specific motifs could be identified. Analysis of longer IGHV gene sequences is required to provide more detailed information on clonal evolution, particularly since binding of IGHV4-34 to self-proteins mostly relies on the FR1 region (38). This is in line with a previous study of paired diagnosis-relapse DLBCL samples, which did not detect significant evolution of the antigen selection pressure related to changes in IG sequences, suggesting that lymphoma-associated genetic alterations, but not antigen selective pressure, drive relapse development (41).

Clonally unrelated DLBCL recurrences were observed in 14% of cases, which all involved lymphoma patients with a time to recurrence of ≥ 4 years. However, the frequency of late recurrences (≥ 5 years) in our DLBCL cohort (n = 15/50; 30%) is higher than what has been reported for unbiased cohorts of DLBCL patients (~ 15%) (ref. 6, 8, 9). Nonetheless, the frequency of second primary lymphoma within the cohort of late recurrences should not be affected by this overrepresentation. In the clonally unrelated DLBCL samples, the clonotype of the second DLBCL could not be identified in the primary DLBCL as a detectable subclone, which excludes expansion a minor B-cell clone already present at time of diagnosis. Previous studies in DLBCL have also demonstrated that clonally unrelated lymphomas mostly involve longer time to relapse intervals, where 70% of the patients (n = 10) developed an unrelated lymphoma after ≥ 4 years (10–16). Thus, a long time (≥ 4 years) to recurrence is correlated with a second primary DLBCL.

In our study, the cell-of-origin profile according to Hans algorithm was identical between the primary and second primary DLBCL for 6 out 7 cases (86%). This may suggest that the susceptibility for a second *de novo* lymphoma within one patient might be linked to a specific DLBCL genetic subtype (42, 43). In addition, most of the cases showed a similar DLBCL histomorphology between primary and subsequent lymphoma, with exception of one case. As could have been expected, EBV-positivity was more frequent in the clonally unrelated DLBCL cases (n = 3/7; 43%) as compared to the relapse cohort (n = 1/34; 3%), suggesting a role for a diminished immune response in these patients. Interestingly, case 154 with biclonal DLBCL was EBV-positive, confirming that EBV infection may be linked to the occurrence of multiple DLBCL clones within one patient. Therefore, patients with second primary DLBCL may have an underlying immunodeficiency, which can lead to EBV reactivation and subsequent lymphoma development (44). Similarly, the occurrence of clonally unrelated B-cell lymphomas have been linked to EBV- (45, 46), and immunodeficiency disorders (47, 48). Second primary lymphoma may also relate to germline mutation in cancer predisposition genes (18, 49), but accurate numbers have not yet been reported, which requires further investigations.

In conclusion, second primary DLBCL are present in 37% of DLBCL patients with a time to recurrence of ≥ 4 years. As such, clonality testing in cases with a longer time interval (> 4 years) between the primary DLBCL and subsequent recurrence should be considered in routine diagnostics, as for patients presenting with a second unrelated lymphoma other treatment options than the current intensified treatment modalities may be considered.

Declarations

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Author contributions

HvK and BS designed the research and conceived the project. MB, JR and JL performed the experimental research, MB, DvB, LK, KH, MvdB, PG and BS analyzed the data. MB, EH, FdG, BH, AH, PN, EvdS, JV, CH, WS and MvdB collected the histological material and/or clinical data. MB and BS wrote the manuscript. MvdB and HvK examined the histopathology.

Data availability

The raw datasets generated in this study are available from the corresponding author on reasonable request.

Conflict of interest disclosure

The authors declare no competing financial interests.

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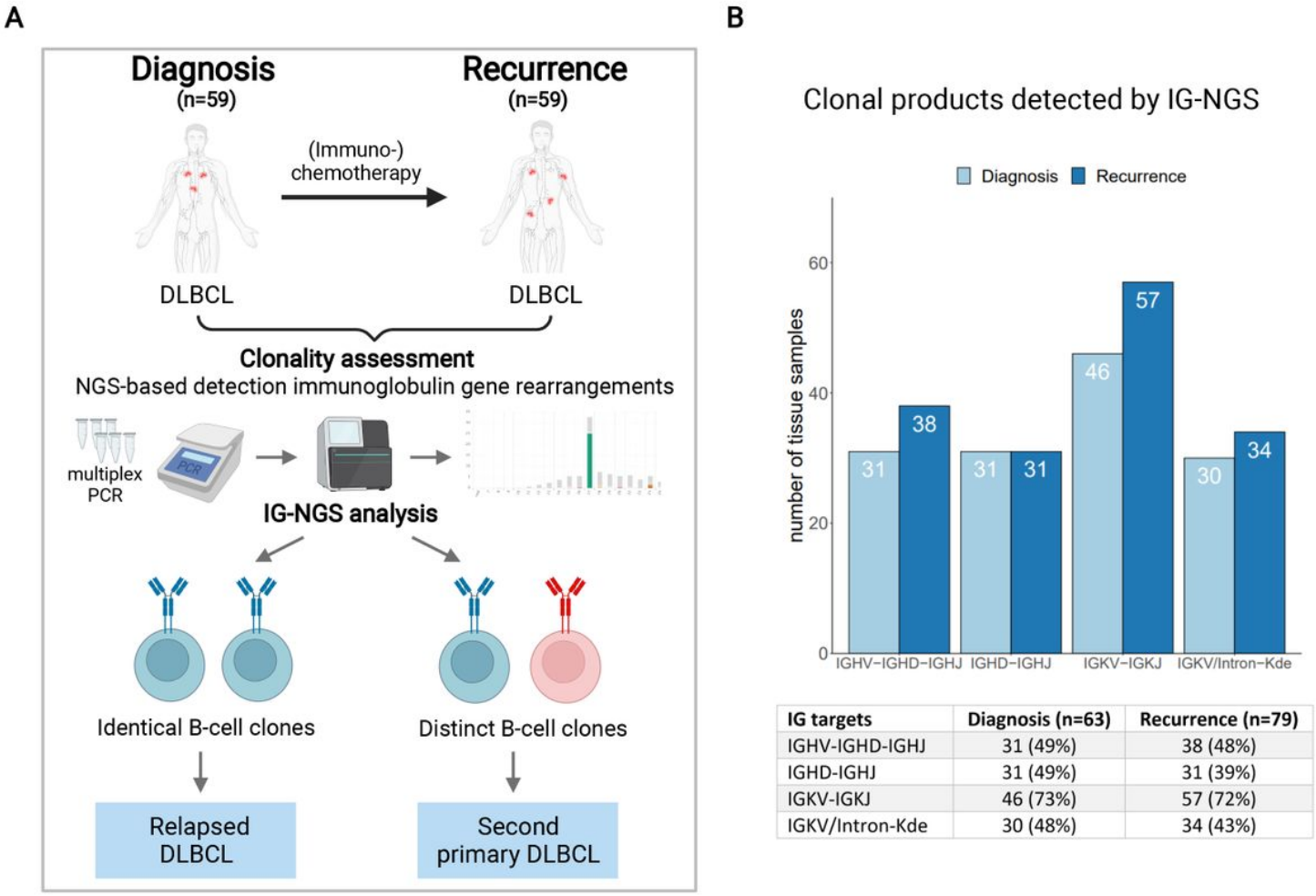
Figures



IG targets	Diagnosis (n=63)	Recurrence (n=79)
IGHV-IGHD-IGHJ	31 (49%)	38 (48%)
IGHD-IGHJ	31 (49%)	31 (39%)
IGKV-IGKJ	46 (73%)	57 (72%)
IGKV/Intron-Kde	30 (48%)	34 (43%)

Figure 1

NGS-based clonality assessment in paired diagnosis-recurrence DLBCL cohort.

Next-generation sequencing (NGS)-based clonality analysis in paired diagnosis-recurrence cohort of 59 diffuse large B-cell lymphoma (DLBCL) patients. (A) Outline of the study in which IG-NGS clonality data of 142 tissue specimens (FFPE: n=107, FF: n=35) was obtained, which included 63 diagnosis (n=4 tissue samples from two independent locations) and 79 recurrence samples (n=2 tissue samples at the same time at two independent locations). Recurrences with identical DLBCL-associated IG gene rearrangements between diagnosis and recurrence were indicated as relapsed DLBCL, and with distinct dominant gene rearrangements as second primary lymphoma (B) The number of tissue samples in which a dominant clonotype was identified for IG targets IGHV-IGHD-IGHJ, IGHD-IGHJ, IGKV-IGKJ and IGKV/Intron-Kde. The results of IGHV-IGHD-IGHJ indicate FR3 target analysis.

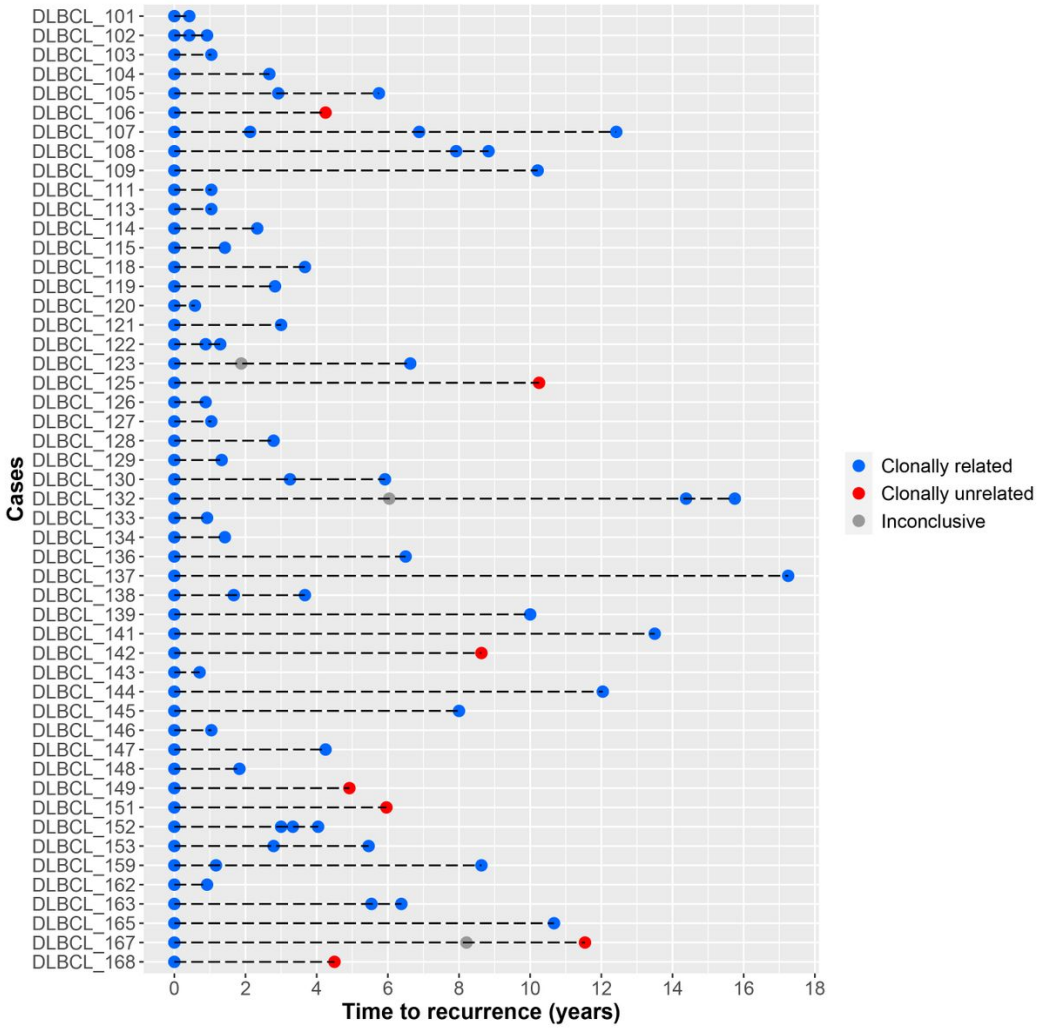


Figure 2

Overview of clonality assessment of paired diagnosis-recurrence DLBCL cohort. Clonal relationship could be established for 50 paired diagnosis-recurrence samples, where NGS-based clonality assessment identified DLBCL-specific clonotype(s) in both diagnosis and subsequent follow-up samples for each patient. These include clonally related DLBCL samples (n=43; indicated in blue), clonally unrelated DLBCL samples (n=7; indicated in red), and a few intermediate samples without a detectable DLBCL-specific clonotype (n=3), which resulted in inconclusive data (indicated in grey). Per case (indicated on the y-axis), the time interval between biopsies is indicated in years on the x-axis.

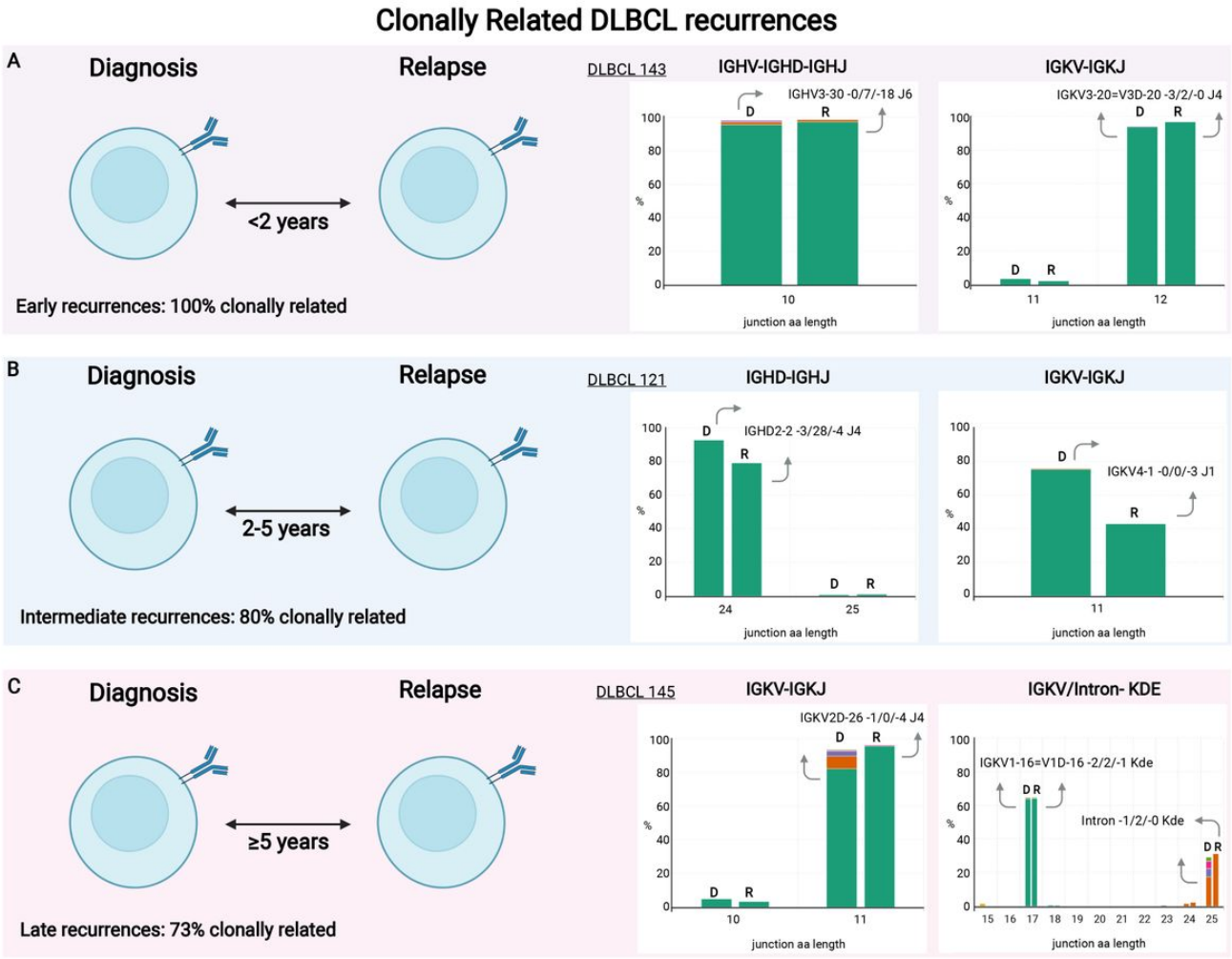


Figure 3

Clonally related DLBCL recurrences as identified by NGS-based clonality assessment

Clonally related DLBCL recurrences were identified by NGS-based clonality analysis and occured at different frequencies in early and late relapse: (**A**) early relapse (interval time to relapse <2 years), (**B**) intermediate time to relapse of 2 to 5 years , and (**C**) late relapse with time to relapse ≥ 5 years. For each time interval group, the percentage (%) clonally related cases is indicated. A representative example for each time interval category as obtained by IG-NGS analysis and ARResT/Interrogate bioinformatics are shown. The abundance of each clonotype per indicated target (IGHV-IGHD-IGHJ, IGHD-IGHJ, IGKV-IGKJ, IGKV/Intron-KDE) is depicted on the y-axis and the junction amino acid (aa) length on the x-axis. A clonotype is defined by the 5' and 3' gene annotation and the junctional nucleotide sequence of the rearrangement. For each case, the dominant clonotype for diagnosis (D) and relapse (R) is indicated.

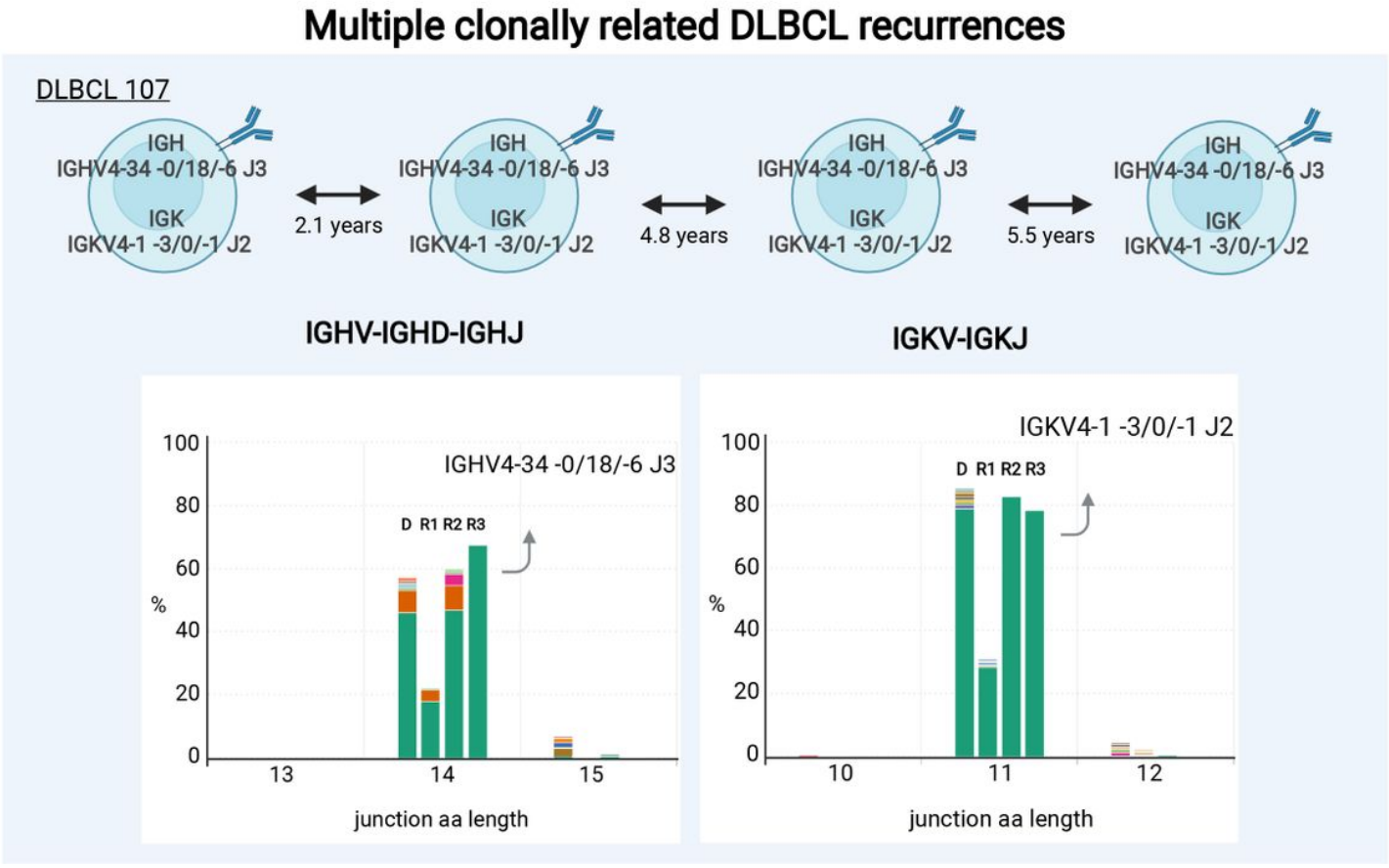


Figure 4

NGS-based clonality assessment of multiple consecutive recurrences within one patient

IG-NGS clonality analysis of a representative DLBCL patient with multiple consecutive recurrences reveals clonally related lymphomas as determined by the presence of identical clonotypes for two immunoglobulin gene rearrangement targets. Indicated are the time intervals in years between each relapse. Clonal gene rearrangements (indicated in green bars) of diagnosis (D), first relapse (R1), second relapse (R2) and third relapse (R3) are depicted for both IGHV-IGHD-IGHJ and IGKV-IGKJ targets. The abundance of each clonotype (each represented by a different color) is visualized on the y-axis and the junction amino acid (aa) length on the x-axis, in which a clonotype is defined by the 5' and 3' gene annotation and the junctional nucleotide sequence of the rearrangement.

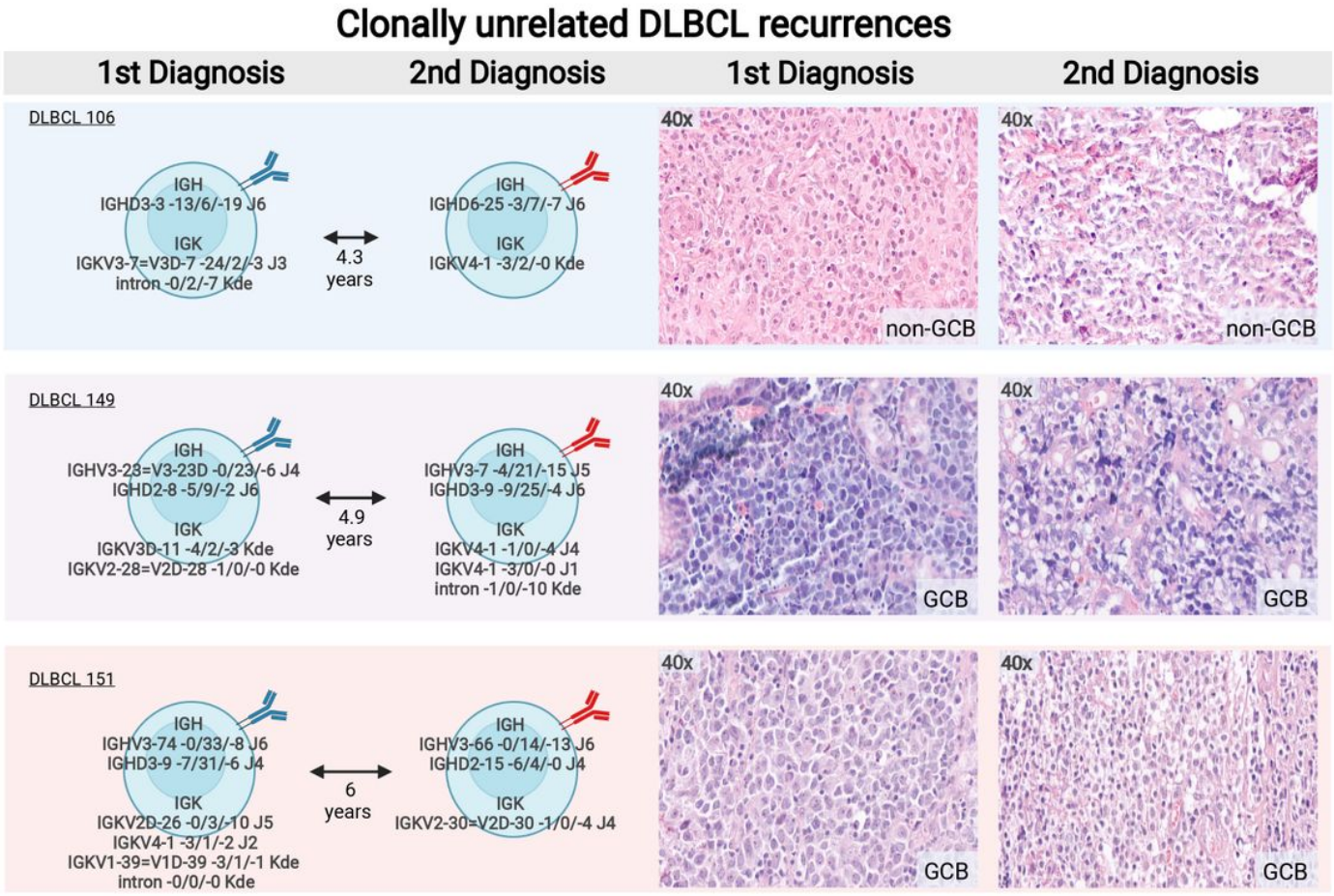


Figure 5

NGS-based clonality assessment and histopathology overview of clonally unrelated DLBCL recurrences representing second primary lymphoma.

Distinct clonotypes were identified in clonally unrelated DLBCL recurrences (left panel), and the data of three representative cases are shown. For each clonally unrelated case, time interval in years and the dominant gene rearrangements for the specific IG-targets are depicted for both first and second primary diagnosis, in which the dominant clonotype is defined by the 5' and 3' gene annotation and the junctional nucleotide sequence of the rearrangement. Cell-of-origin classification (GCB and non-GCB) was determined according to the Hans algorithm. Hematoxylin eosin (HE) stainings (40X) for both primary and second unrelated DLBCL are shown in the right panels.

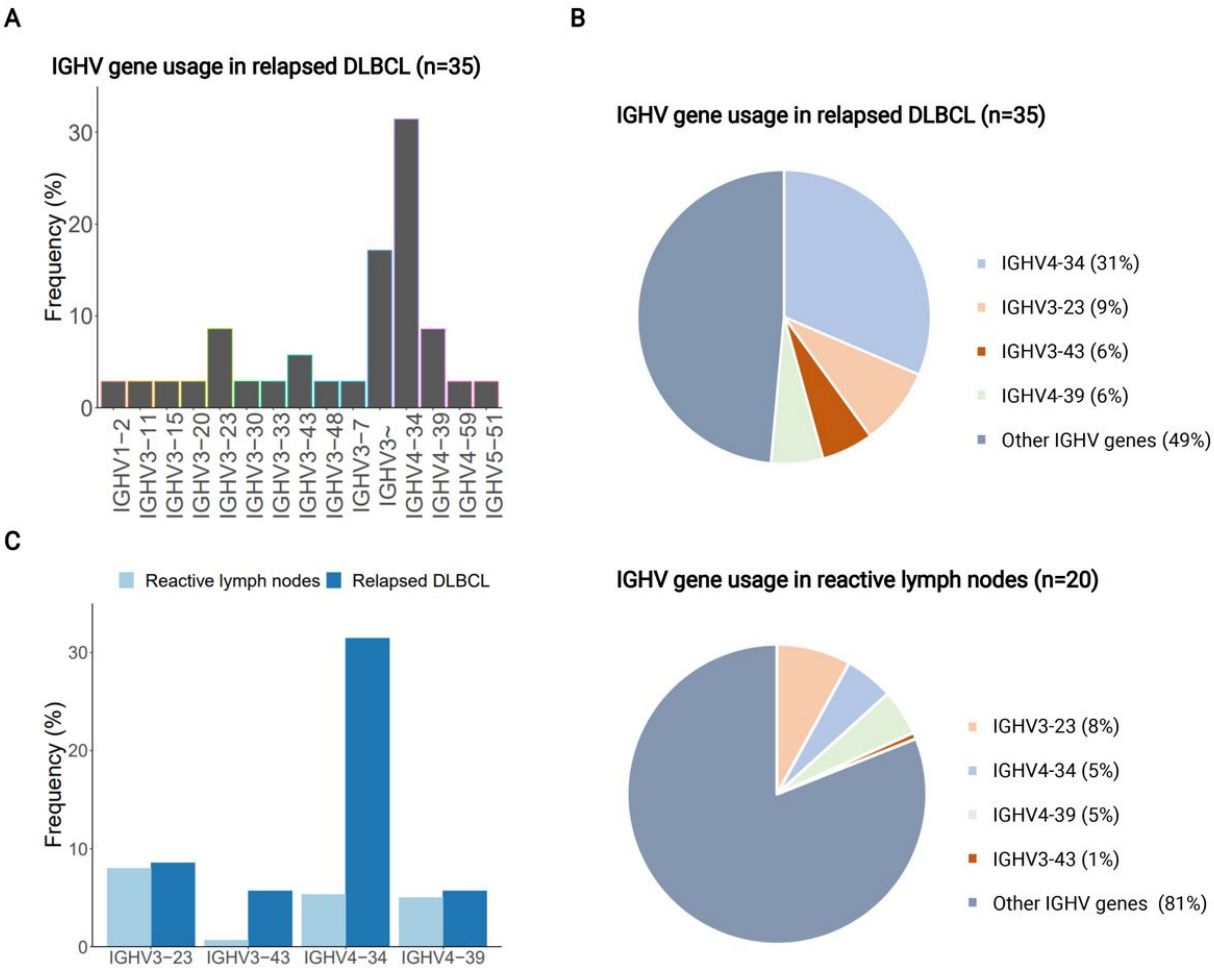


Figure 6

IGHV gene repertoire in relapsed DLBCL cohort

Immunogenetic analysis of paired diagnosis-relapse cohort revealed IGHV gene usage in relapsed DLBCL, which was compared to IGHV gene frequencies in reactive lymph nodes. (**A**) Relative frequency (y-axis) of all IGHV genes (x-axis) in relapsed DLBCL cohort (n=35). (**B**) Frequency of the four most dominant IGHV genes (present in ≥2 cases) in relapsed DLBCL (n=35), while the other IGHV genes are grouped together, and the relative frequency of IGHV3-23, IGHV4-34, IGHV4-39 and IGHV3-43 gene usage in reactive lymph nodes (n=20). (**C**) Relative frequencies of IGHV3-23, IGHV4-34, IGHV4-39 and IGHV3-43 gene usage in relapsed DLBCL and reactive lymph nodes.

Supplementary Files

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