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Adaptive Immune Response and Systemic Inflammation Predict Survival in High-Grade B-Cell Lymphoma with MYC/BCL2/BCL6 Rearrangements

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ABSTRACT

High-grade B-cell lymphomas with concurrent MYC and BCL2 and/or BCL6 rearrangements (HGBL-DH/TH) represent an aggressive subgroup of B-cell malignancies with limited therapeutic options and poor survival outcomes. Despite growing recognition of the tumor microenvironment (TME) as a determinant of prognosis in hematologic cancers, its role in HGBL-DH/TH has not been systematically evaluated. Here, we characterize the adaptive immune landscape in 47 HGBL-DH/TH cases compared with 27 triple-negative diffuse large B-cell lymphomas (tnDLBCL) using next-generation sequencing of T-cell receptor (TCR) β-chain repertoires. Systemic inflammation was assessed using the Glasgow Prognostic Score (GPS), and TME features were further profiled immunophenotypically.

Our findings reveal that HGBL-DH/TH harbors a distinctive TCR repertoire, including clonotypes absent in tnDLBCL, indicative of selective tumor-driven immune responses. Measures of TCR clonality and dominant clone frequency correlated with overall survival, whereas GPS was predictive of both overall and progression-free survival. Multivariate analysis identified GPS and the revised International Prognostic Index (R-IPI) as the strongest independent prognostic indicators. These results suggest that a combination of systemic inflammatory status and the composition of tumor-directed T-cell responses defines outcome in HGBL-DH/TH, highlighting potential avenues for immune-based risk stratification and therapeutic intervention.

Keywords: Immune profiling, T-cell receptor, High-grade B-cell lymphoma, Systemic inflammation

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Introduction

The most recent World Health Organization (WHO) classification of hematopoietic and lymphoid neoplasms recognizes high-grade B-cell lymphoma with concurrent MYC and BCL2 and/or BCL6 rearrangements (HGBL-DH/TH) as a provisional entity [1]. This aggressive subgroup is challenging to categorize, as it exhibits overlapping features of Burkitt lymphoma (BL) and diffuse large B-cell lymphoma (DLBCL), including cytogenetic alterations in MYC, BCL2, and BCL6 [2]. Clinically, HGBL-DH/TH remains a significant therapeutic challenge, frequently presenting with adverse prognostic factors such as advanced-stage disease, elevated revised International Prognostic Index (R-IPI) scores, and increased involvement of bone marrow and the central nervous system (CNS). Outcomes with conventional immunochemotherapy regimens, including R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone) ± high-dose consolidation followed by stem-cell transplantation, remain poor [3, 4].

Accumulating evidence highlights the critical role of the tumor microenvironment (TME) in shaping outcomes across multiple malignancies [5–8]. Among TME components, tumor-infiltrating lymphocytes (TILs) — representing adaptive immunity — have attracted considerable attention. Increased infiltration of T-cells has been linked to improved prognosis in diverse cancers, underscoring the importance of interactions between immune and tumor cells [7]. T-cell receptor (TCR) diversity, largely determined by the hypervariable complementarity-

determining region 3 (CDR3) of the TCR β-chain, is central to antigen recognition via MHC-peptide complexes [9]. As a molecular marker, the TCRβ CDR3 sequence reliably identifies individual T-cell clones, and the composition and diversity of these clones influence clinical outcomes. Prior studies, including our work in sporadic Burkitt lymphoma, have demonstrated that high T-cell infiltration correlates with favorable outcomes in both DLBCL and solid tumors [5–7, 10]. Conversely, restricted TCR repertoires have been associated with poor prognosis [5, 11]. However, the relevance of TCR repertoire diversity in HGBL-DH/TH, relative to DLBCL, has not been established.

Systemic inflammation also modulates the TME, influencing cytokine levels and the activity of tumor-infiltrating immune cells. Biomarkers such as C-reactive protein (CRP) and serum albumin reflect inflammatory and nutritional status and have been integrated into prognostic scoring systems. The Glasgow Prognostic Score (GPS), which incorporates CRP and albumin levels, categorizes patients into three risk groups (0–2 points) and has demonstrated prognostic utility in multiple cancers, including DLBCL [12].

In this study, we perform large-scale next-generation sequencing (NGS) of the TCR β-chain CDR3 region in a cohort of HGBL-DH/TH patients and compare these findings to a previously characterized cohort of triple-negative DLBCL (tnDLBCL) lacking MYC, BCL2, or BCL6 rearrangements [10]. We also investigate the relationship between TCR repertoire diversity and systemic inflammation, as measured by GPS, in the context of clinical outcomes in this rare lymphoma subtype.

The study cohort comprised 74 patients, including 47 with HGBL-DH/TH and 27 with tnDLBCL. The median age was 71.5 years (range: 18–89), with males representing 54.1% of the population. Age distribution was similar between HGBL-DH/TH and tnDLBCL patients (p = 0.1379). Median follow-up was 30.5 months (range: 1–115 months; interquartile range: 17.3–51.7 months). Regarding revised International Prognostic Index (R-IPI), two patients (2.7%) had a score of 0, 33 patients (44.6%) had scores of 1–2, and 39 patients (52.7%) had scores greater than 2. Extranodal involvement at diagnosis was observed in 51 patients (68.9%), and six patients (8.1%) presented with an ECOG performance status >2. Baseline staging showed that 43 patients (58.1%) had advanced disease (Ann Arbor stage III/IV), and B-symptoms were present in 38 patients (51.4%). No statistically significant differences were noted between HGBL-DH/TH and tnDLBCL regarding individual R-IPI components. A detailed summary of baseline clinical characteristics is provided in **Table 1**.

Table 1. Clinical characteristics of the study group.

Characteristics	DHL (n = 40)	THL (n = 7)	Triple-Negative DLBCL (n = 27)
Age (yrs.; median + range)	73.0 (35–89)	69.0 (42–79)	70 (18–87)
Sex			
Female	19 (47.5%)	2 (28.6%)	13 (48.1%)
Male	21 (52.5%)	5 (71.4%)	14 (51.9%)
R-IPI			
0	1 (2.5%)	-	1 (3.7%)
1–2	16 (40.0%)	2 (28.6%)	15 (55.6%)
>2	23 (57.5%)	5 (71.4%)	11 (40.7%)
Stage (Ann Arbor)			
I	7 (17.5%)	-	2 (7.4%)
II	8 (20.0%)	3 (42.8%)	11 (40.7%)
III	5 (12.5%)	2 (28.6%)	8 (29.6%)
IV	20 (50.0%)	2 (28.6%)	6 (22.2%)
B-Symptoms			
Yes	22 (55.0%)	4 (57.2%)	12 (44.4%)
No	18 (45.0%)	3 (42.8%)	15 (55.6%)
Extranodal sites			
0	10 (25.0%)	3 (42.8%)	10 (37.0%)
1–2	28 (70.0%)	4 (57.2%)	17 (63.0%)
>2	2 (5.0%)	-	-
ECOG PS			
0–2	37 (92.5%)	7 (100.0%)	24 (88.9%)
>2	3 (7.5%)	-	3 (11.1%)

LDH			
Normal	11 (27.5%)	1 (14.3%)	14 (51.9%)
Elevated	29 (72.5%)	6 (85.7%)	13 (48.1%)
Glasgow prognostic score (GPS)			
GPS 0	12 (30.0%)	1 (14.3%)	17 (63.0%)
GPS 1	8 (20.0%)	1 (14.3%)	7 (25.9%)
GPS 2	20 (50.0%)	5 (71.4%)	3 (11.1%)
CNS involvement at diagnosis			
Yes	1 (2.5%)	-	-
No	45 (97.5%)	7 (100.0%)	27 (100.0%)

DLBCL, diffuse large B-Cell Lymphoma; Yrs., years; CNS, central nervous system; LDH, Lactate dehydrogenase; ECOG; Eastern cooperative oncology group; PS, performance status.

Laboratory assessment

In the study cohort, median serum lactate dehydrogenase (LDH) was 412 U/L, ranging from 113 to 14,638 U/L, with elevated LDH observed in 48 patients (64.9%) at diagnosis. Median serum albumin was 37.3 g/L (range, 21.7–54.3 g/L), while median C-reactive protein (CRP) was 11.1 mg/dL (range, 1.0–44.4 mg/dL). Assessment of systemic inflammation using the Glasgow Prognostic Score (GPS) revealed that 13 HGBL-DH/TH patients (27.7%) and 17 tnDLBCL patients (63.0%) had a score of 0. A GPS of 1 was observed in 16 patients overall (21.6%), including nine HGBL-DH/TH and seven tnDLBCL cases. A GPS of 2 was present in 28 patients (37.8%), predominantly in the HGBL-DH/TH subgroup (25 patients) compared with three tnDLBCL cases.

Treatment modalities

The majority of patients (55/74, 74.3%) received a R-CHOP-like regimen as first-line therapy. Alternative treatment strategies were administered to 14 HGBL-DH/TH patients and two tnDLBCL patients. Overall response rate (ORR) across the cohort was 82.4%. Complete remission (CR) was achieved in 16 HGBL-DH/TH patients (34.0%) and 14 tnDLBCL patients (51.9%), whereas partial remission (PR) was observed in 20 HGBL-DH/TH cases (42.6%) and 11 tnDLBCL cases (40.7%). Progressive disease (PD) occurred in nine patients (12.2%), including seven HGBL-DH/TH and two tnDLBCL cases.

Hematopoietic stem cell transplantation (HSCT) — autologous or allogeneic — was performed in six HGBL-DH/TH patients (12.8%; three autoHSCT, three alloHSCT), predominantly in the relapsed or refractory setting (five of six cases, 83.3%). Two patients (2.7%) declined further therapy. Relapse was observed in 27 HGBL-DH/TH patients (57.4%; 21 DH, six TH) and in four tnDLBCL patients (14.8%).

Treatment-related severe adverse events occurred in 51 patients (68.9%), primarily hematologic (n = 27) or infectious (n = 23). Notably, 43.5% (10/23) of infectious complications were observed in patients with a GPS of 2. A comprehensive overview of adverse events is presented in **Table 2**.

Table 2. Treatment modalities of the study group.

Characteristics	DHL (n = 40)	THL (n = 7)	Triple-Negative DLBCL (n = 27)
Frontline Therapy regimen			
R-CHOP-like	27 (67.5%)	5 (71.4%)	23 (85.2%)
DR	7 (25.9%)	3 (60.0%)	10 (43.5%)
Others	12 (30.0%)	2 (28.6%)	3 (11.1%)
Refusal of treatment	1 (2.5%)	-	1 (3.7%)
Response rates			
CR	15 (37.5%)	1 (14.3%)	14 (51.9%)
PR	17 (42.5%)	3 (42.9%)	11 (40.7%)
SD	3 (7.5%)	1 (14.3%)	-
PD	5 (12.5%)	2 (28.6%)	2 (7.4%)
Relapse rate	21 (52.5%)	6 (85.7%)	4 (14.8%)
2nd line treatment regimens (n = 35)			
R-DHAP	7 (33.3%)	2 (33.3%)	1 (25.0%)
R-Bendamustine	3 (14.3%)	2 (33.3%)	1 (25.0%)
Auto/Allo HSCT	4 (19.4%)	1 (16.7%)	-

Others	5 (23.8%)	-	2 (50.0%)
Refusal of treatment	2 (9.5%)	1 (16.7%)	-
Toxicity profile (1st line)			
Cytopenia grade III/IV	13 (32.5%)	3 (42.9%)	11 (40.7%)
Polyneuropathy	5 (12.5%)	1 (14.3%)	7 (25.9%)
GI toxicity	2 (5.0%)	-	-
Mucositis	2 (5.0%)	2 (28.6%)	-
Pneumonia	3 (7.5%)	-	1 (3.7%)
Acute kidney injury	5 (12.5%)	-	-
Sepsis	12 (30.0%)	-	7 (25.9%)

DLBCL, diffuse large B-Cell Lymphoma; DR, dose-reduction; R, rituximab; CHOP, cyclophosphamide, doxorubicin, vincristine, prednisolone; Others, other rituximab-based regimen (e.g., R-Bendamustine), GMALL protocols or palliative cytoreductive treatment.

Histopathology, immunophenotyping, and FISH analysis

Examination of the tumor microenvironment (TME) in HGBL-DH/TH samples, combining morphological inspection with a standardized immunohistochemical antibody panel, revealed considerable heterogeneity in T-cell infiltration. Patterns ranged from sparse, scattered lymphocytes to concentrated clusters within tumor regions. Quantitative histopathology indicated that tumor cells comprised a median of 85% of the tissue samples.

Immunophenotypic profiling, including assessment of BCL2, BCL6, and MYC expression, along with determination of cell-of-origin (COO) according to the Hans algorithm, showed that 42 cases (56.8%) exhibited a germinal center B-cell (GCB) phenotype, whereas 32 cases (43.2%) were classified as non-GCB. These findings are summarized in **Table 3** [13].

Table 3. Histopathological characteristics in the study group.

Characteristics	**DHL (n = 40)**	**THL (n = 7)**	**Triple-Negative DLBCL (n = 27)**
Hans classifier and IHC			
GCB	34 (85.0%)	6 (85.7%)	2 (7.4%)
Non-GCB	6 (15.0%)	1 (14.3%)	25 (92.6%)
BCL2	28 (70.0%)	5 (71.4%)	1 (3.7%)
BCL6	33 (82.5%)	6 (85.7%)	3 (11.1%)
MYC	25 (62.5%)	4 (57.1%)	-
CD10	31 (77.5%)	5 (71.4%)	3 (11.1%)
MUM1/IRF4	9 (22.5%)	2 (28.6%)	3 (11.1%)
Ki-67 (median, range)	85% (40–100%)	75% (50–95%)	85% (55–95%)
T-cell infiltration			
Percentage (median, range)	15% (1–30%)	10% (3–30%)	20% (5–30%)

DHL, double hit lymphoma; DLBCL, diffuse large B-Cell Lymphoma; IHC, immunohistochemistry; THL, triple hit lymphoma.

Given that loss of human leukocyte antigen (HLA) class I and II expression frequently occurs in the pathogenesis of aggressive B-cell lymphomas, and recognizing that effective T-cell receptor (TCR)-mediated anti-tumor responses require an intact HLA system, we investigated whether TCR clonality metrics were associated with HLA expression. Immunohistochemical analysis of HLA class I (beta-2-microglobulin, B2M) and HLA class II (HLA-DR) was performed in cases with sufficient FFPE tissue remaining after molecular studies (n = 18 and n = 21, respectively). Although a notable proportion of samples exhibited loss of B2M and/or HLA-DR, no significant relationship with TCR clonality measures was detected **(Figure 1)**.

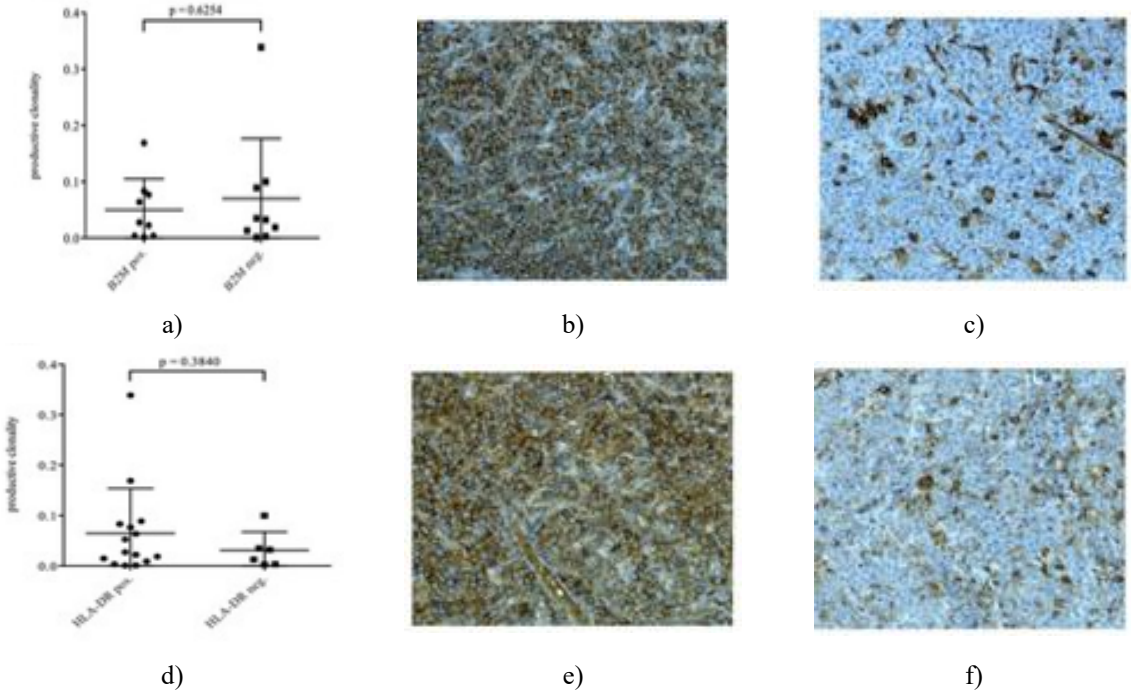

Figure 1. Immunohistochemical analysis revealed that HLA class I antigen (beta-2-microglobulin, B2M) was absent in a substantial subset of HGBL-DH/TH cases (b, c); however, this loss did not significantly affect TIL TCR clonality, as measured by productive clonality. Comparable results were observed for other clonality metrics, including the proportion of top 10 clones and maximum productive frequency (a; data not shown). In contrast, loss of HLA class II antigen (HLA-DR) expression was less common (e, f) and similarly showed no significant association with TCR clonality (d).

Fluorescence in situ hybridization (FISH) identified 47 HGBL-DH/TH cases, comprising 40 double-hit (DH) and seven triple-hit (TH) cases. For comparison, 27 triple-negative diffuse large B-cell lymphoma (tnDLBCL) cases were included. Epstein-Barr virus (EBV) status, assessed via EBER in-situ hybridization, was positive in 10 tnDLBCL cases, whereas no EBV-positive cases were detected among HGBL-DH/TH patients. To account for the higher frequency of EBV positivity in the tnDLBCL cohort, five EBV-positive cases were randomly excluded, and analyses were repeated in triplicate; these adjustments did not alter statistical significance, consistent with the lack of effect of EBV status on clonality in tnDLBCL. Furthermore, comparison between EBV-positive DLBCL, NOS, and tnDLBCL revealed no significant differences in any evaluated clonality measures.
The anatomical site of tumor origin did not appear to influence TIL TCR repertoire clonality, either in pooled analysis or when stratified by cytogenetic subtype (data not shown).

High-throughput TCR sequencing
Next-generation sequencing (NGS) of the TCRβ CDR3 region was performed on 65 samples, including all 47 HGBL-DH/TH patients with MYC and BCL2 and/or BCL6 rearrangements, as well as 27 tnDLBCL cases. Analysis was restricted to samples containing more than 100 productive TCRs per sample to prevent artifacts due to low T-cell counts.
Within the HGBL-DH/TH cohort, sequencing identified a median of 1,504.5 rearrangements per sample (range: 136–24,680). For patients with multiple analyzable samples (n = 27), all passing quality control, a strong correlation was observed between samples in terms of previously defined clonality metrics and sequence homology, confirming reproducibility and reliability of the TCR repertoire measurements **(Figures 2a–2d)**.

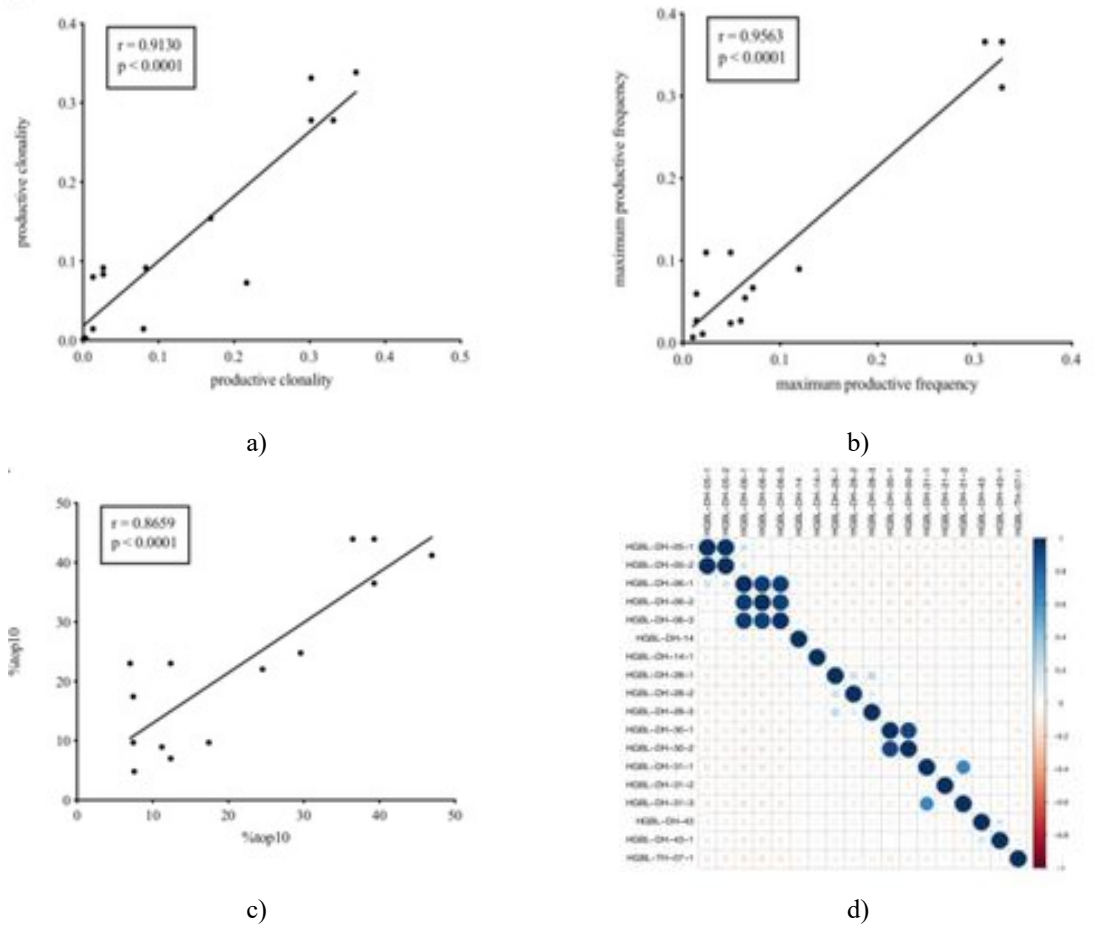

Figure 2. Comparative analysis of multiple samples from the same patient. Examination of two samples obtained from distinct anatomical sites at the same time point revealed a strong concordance in TCR repertoire metrics. Pearson correlation analysis demonstrated a high degree of agreement for productive clonality (a), maximum productive-clonotype frequency (b), and the proportion of the top 10 most expanded TCR clonotypes (c). TCR sequence overlap was also confirmed, illustrated by a linear heat map of sequence homology (d).

Across all analyzed samples, a total of 155,658 unique clonotypes were detected, with the majority occurring at low frequencies and in only a small subset of patients. **Figure 3** depicts the usage patterns of TCR Vβ and Jβ gene segments across individual samples. Notably, the distribution of Vβ- and Jβ-gene segment usage displayed borderline significant differences, with variability observed in the recombination of several Vβ segments and a single Jβ segment.

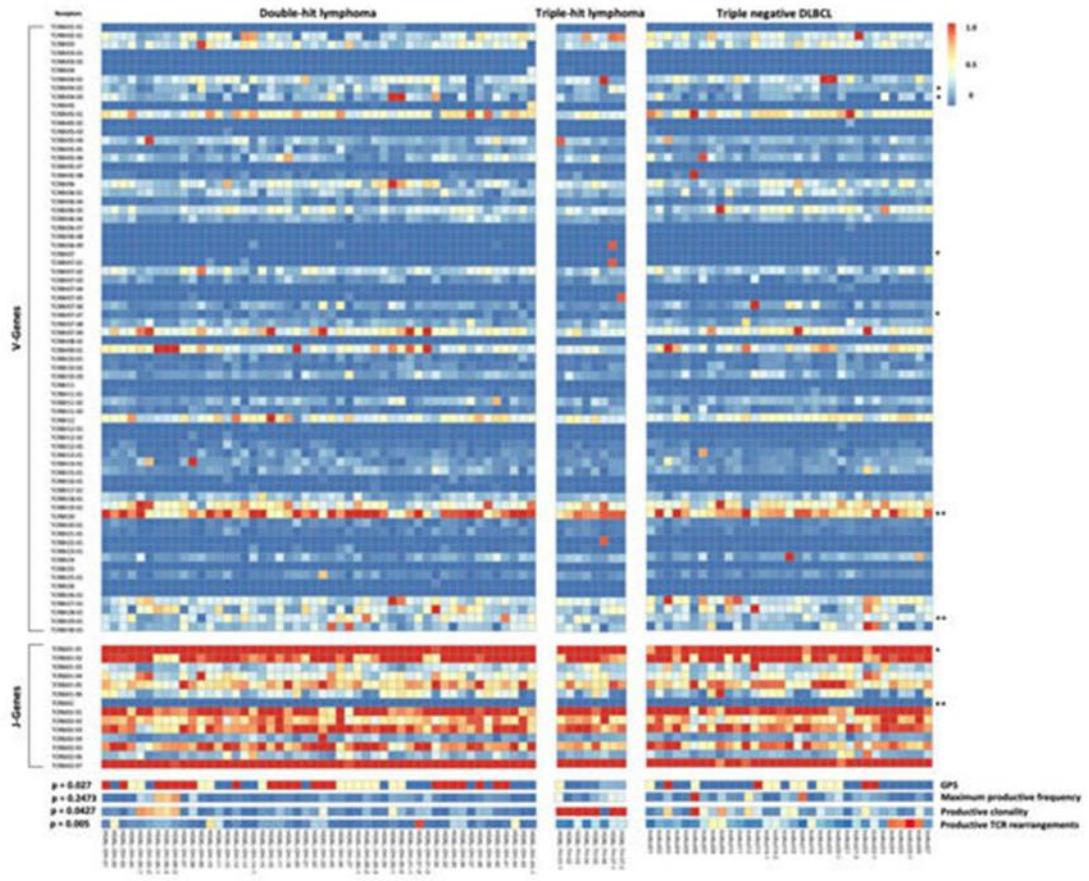

Figure 3. Vβ- and Jβ-gene segment usage in tumor samples from HGBL-DH/TH at diagnosis and relapse compared to tnDLBCL. A heatmap illustrates the frequency of Vβ- and Jβ-gene usage across samples. Several Vβ and Jβ segments exhibited differential usage between pooled HGBL-DH/TH samples at diagnosis and tnDLBCL, with borderline significance indicated by *p < 0.07 and statistical significance by **p < 0.05, as determined by two-tailed t-tests.

Comparative analysis of the TCR repertoire in HGBL-DH/TH and tnDLBCL

TCR repertoire comparisons between HGBL-DH/TH and tnDLBCL are summarized in **Table 4**. Analysis revealed a significant difference in the number of productive TCR rearrangements, reflecting the total functional T-cell infiltrate within the tumor (HGBL-DH/TH: median 1504.5, range 136–24,680; tnDLBCL: median 3447.0, range 1642–17,745; p = 0.005). Further, tnDLBCL samples displayed a more restricted TCR repertoire as indicated by productive clonality (HGBL-DH/TH: median 0.036, range 0.0009–0.3614; tnDLBCL: median 0.08785, range 0.0013–0.4437; p = 0.0427) **(Figure 4d)**. No statistically significant difference was observed for the "% maximum frequency clone" metric between HGBL-DH/TH and tnDLBCL (p = 0.2473), which quantitatively reflects the most expanded T-cell clones within each tumor.

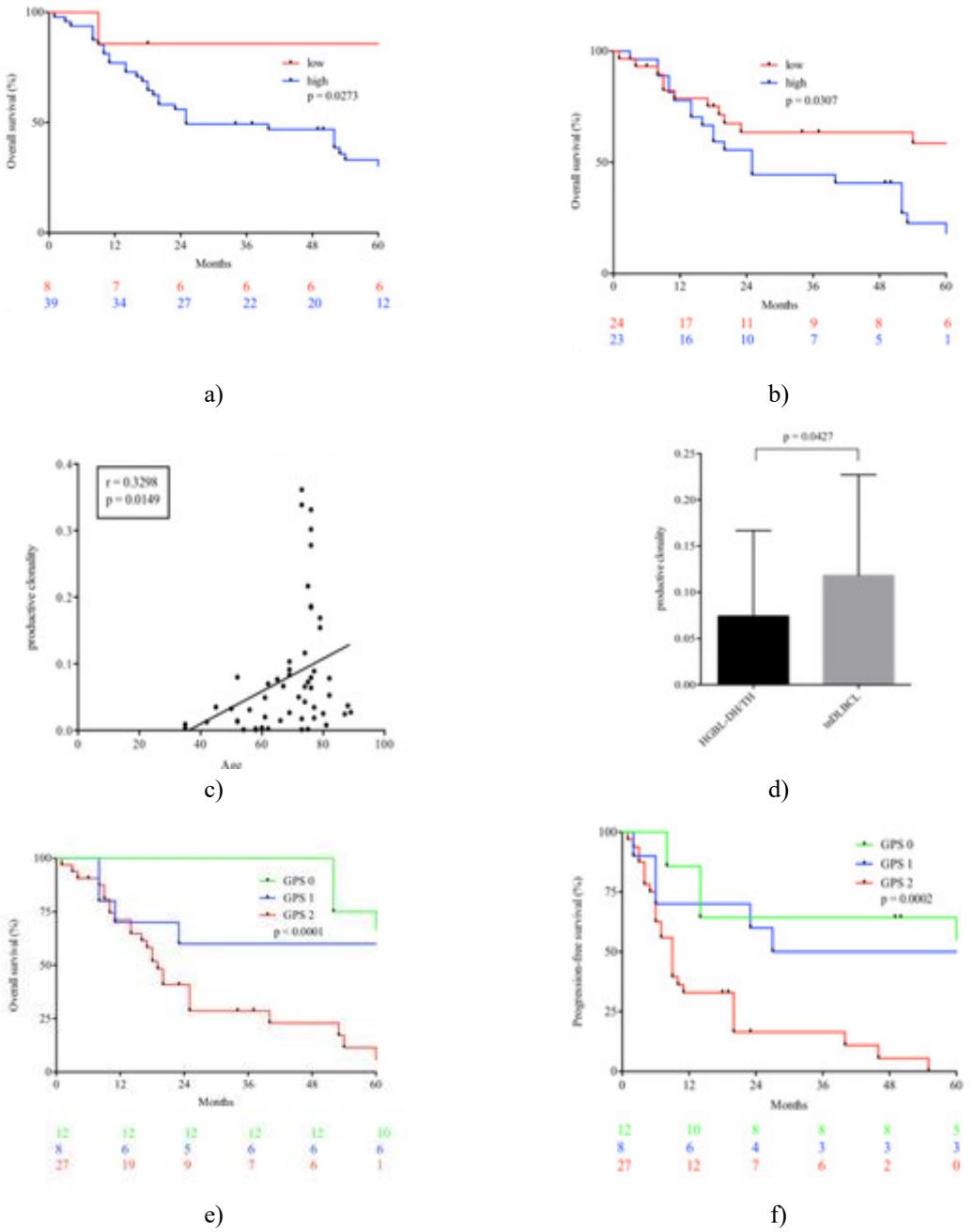

Figure 4. Prognostic significance of TCR repertoire clonality in HGBL-DH/TH. Overall survival (OS) in HGBL-DH/TH patients stratified by productive TCR clonality (A) and maximum productive frequency (B) demonstrates that the clonal structure of tumor-infiltrating T-cells holds predictive value for OS, whereas no significant impact was observed for progression-free survival (PFS; data not shown) (productive clonality: p = 0.0273; HR: 2.839; 95% CI: 1.124–7.169; maximum productive frequency: p = 0.0307; HR: 2.167; 95% CI: 1.074–4.370).

In HGBL-DH/TH patients, productive clonality correlated significantly with age (p = 0.0149; r = 0.3298) (C), whereas maximum productive frequency showed a trend toward correlation of borderline significance (p = 0.0599; r = 0.2577). Comparative analysis of clonality metrics between HGBL-DH/TH and tnDLBCL revealed a significant difference in productive clonality (p = 0.0427) (D).

Furthermore, systemic inflammation, as quantified by the Glasgow Prognostic Score (GPS), demonstrated an opposing prognostic effect to that of heightened adaptive intra-tumoral immune responses, showing significant associations with both OS (p < 0.0001) (E) and PFS (p = 0.0002) (F).

Table 4. Comparative TCR β sequencing of HGBL-DH/TH and tnDLBCL.

Analyzed Parameter	HGBL-DH/TH (n = 47)	tnDLBCL (n = 27)	p-Value
Productive TCR rearrangements	1504.5 (136–24,680)	3447 (164–21,745)	0.0050
Productive Clonality	0.036 (0.0009–0.3614)	0.08785 (0.0013–0.4437)	0.0427
% Maximum frequency clone	0.407 (0.205–1.822)	4.1884579 (0.4035117–44.8000014)	0.2473

In addition, we identified fourteen public TCRβ CDR3 clonotypes (mean frequency ≥ 0.2%) that were recurrently observed in HGBL-DH/TH patients (n ≥ 3) but absent in tnDLBCL **(Table 5)**. Many of these clonotypes appeared at relatively high frequencies (median 0.407%; range 0.205–1.822%), suggesting potential biological relevance and selective expansion driven by tumor neoantigens. Cross-referencing these sequences against the largest publicly available dataset of healthy individuals and patients with defined clinical conditions revealed that all but one clonotype had not been previously reported. The single exception targets EBNA3A, a nuclear protein of Epstein-Barr virus, and was detected in three HGBL-DH/TH cases, none of which were EBV-positive.

Table 5. Major public T-Cell Receptor β CDR3 clonotypes found in HGBL-DH/TH but not tnDLBCL suggestive of a process of shared tumor-neoantigen selection within the tumor-infiltrating TCR repertoire, cross referenced with a publically available dataset.

Amino Acid	Present in HGBL-DH/TH	Mean Frequency (%)	Epitope Gene	Epitope SPECIES	V-Gene	J-Gene
CASSPLGETQYF	4	0.419	-	-	TCRBV18-01*01	TCRBJ02-05*01
CASRDTEAFF	4	1.822	-	-	TCRBV06	TCRBJ01-01*01
CASSPTRTGDYEQYF	3	1.593	-	-	TCRBV07-09	TCRBJ02-07*01
CASSDSSLTEAFF	3	0.201	EBNA3A	EBV	TCRBV06-04	TCRBJ01-01*01
CASSQGGSGEQFF	3	0.705	-	-	TCRBV04-03*01	TCRBJ02-01*01
CASSLGGSPEAFF	3	0.524	-	-	TCRBV07-02*01	TCRBJ01-01*01
CASSQGGSGELFF	3	0.468	-	-	TCRBV04-03*01	TCRBJ02-02*01
CASSLESGANVLTF	3	0.428	-	-	TCRBV07-06*01	TCRBJ02-06*01
CASSLGGAGANVLTF	3	0.395	-	-	TCRBV05-06*01	TCRBJ02-06*01
CASSLAGGGTEAFF	3	0.388	-	-	TCRBV28-01*01	TCRBJ01-01*01
CSARDRVSYNEQFF	3	0.302	-	-	TCRBV20	TCRBJ02-01*01
CSARDPGSSYEQYF	3	0.279	-	-	TCRBV20	TCRBJ02-07*01
CASSGTEAFF	3	0.216	-	-	TCRBV28-01*01	TCRBJ01-01*01
CSARSGNTEAFF	3	0.205	-	-	TCRBV20	TCRBJ01-01*01

Influence of TCR diversity and systemic inflammation on survival

Kaplan–Meier analysis and log-rank testing revealed that higher productive clonality and elevated percentages of the maximum frequency clone—reflecting TCR repertoire diversity and adaptive immune activity—were associated with significantly worse overall survival (OS) in HGBL-DH/TH patients (productive clonality: p = 0.0273; HR: 2.839; CI: 1.124–7.169; maximum productive frequency: p = 0.0307; HR: 2.167; CI: 1.074–4.370), whereas no significant effects were observed for progression-free survival (PFS) (productive clonality: p = 0.4459; maximum productive frequency: p = 0.5567) when dichotomized as previously described [14] **(Figures 4a and 4b)**. Consistent with prior observations by Simnica *et al*., both clonality metrics were influenced by patient age **(Figure 4c)**. Notably, these measures of intra-tumoral TCR architecture differed from tnDLBCL independent of age **(Figure 4d)** [15]. In contrast, an increased Glasgow Prognostic Score (GPS), reflecting systemic inflammation and innate immune activation, significantly predicted both OS and PFS **(Figures 4e and 4f)**. Multivariate analysis confirmed GPS (p = 0.029) and the revised International Prognostic Index (R-IPI, p = 0.006) as the only

independent prognostic factors for OS, while TCR clonality measures approached but did not reach statistical significance (maximum productive frequency: $p = 0.082$; productive clonality: $p = 0.294$). Furthermore, patients with a GPS of 0 exhibited a significantly higher number of productive TCR rearrangements compared with those with a GPS of 1 ($p = 0.0415$) or GPS of 2 ($p = 0.0227$).

This study presents a large-scale NGS analysis of the TCR β-chain repertoire in HGBL-DH/TH, aiming to elucidate the prognostic relevance of tumor-infiltrating T lymphocyte diversity. To our knowledge, this is the first study linking both systemic inflammation and the composition of the local adaptive immune response, specifically TCR diversity, to clinical outcomes in HGBL-DH/TH.

The robustness of immunosequencing from FFPE-derived genomic DNA provides a major advantage over single-cell sorting or RNA-based approaches, as it is less susceptible to transcriptional variability [11, 16, 17]. Future studies incorporating RNAseq from fresh-frozen tissue could further refine analysis by focusing on actively expressed TCR subsets.

Our findings regarding the predominance of the germinal center B-cell (GCB) phenotype in HGBL-DH/TH are in line with prior large-scale studies. Rosenwald *et al*. observed that cases with BCL2 rearrangements exclusively exhibited a GCB profile, while BCL6-rearranged cases could display either GCB or non-GCB phenotypes [18]. Similarly, Sha *et al*. reported a 75% GCB frequency in HGBL-DH/TH, contrasting with the higher proportion of post-germinal center phenotypes in double-expressor lymphomas [19]. In the current study, tnDLBCL cases had a higher prevalence of non-GCB subtypes, which are generally linked to poorer prognosis [18, 20], allowing for a comparative analysis of two clinically relevant and distinct aggressive B-cell lymphoma subgroups.

Previous studies across multiple cancer types have highlighted the prognostic significance of a dynamic and complex TME [21, 22]. Consistent with this, Keane *et al*. demonstrated that the TCR repertoire plays a critical role in defining the TME in B-cell lymphomas [11]. In our high-throughput immunosequencing data, HGBL-DH/TH exhibited a more diverse TCR repertoire than tnDLBCL, while tnDLBCL showed a higher number of functional tumor-infiltrating T-cells (productive rearrangements). However, no significant differences were observed regarding total T-cell infiltration or maximum productive clone frequency between the two groups.

In line with the age-associated decline in TCR repertoire diversity reported by Simnica *et al*., measures of productive clonality in our cohort were significantly influenced by age [15]. Importantly, age distributions were comparable between HGBL-DH/TH and tnDLBCL, yet age-adjusted clonality metrics differed between these subgroups. Findings for tnDLBCL were consistent with previous observations by Keane *et al*. [11].

In a previous study, we observed no clear association between restricted HLA-DR expression and the behavior of tumor-infiltrating T-cells, and its impact on patient survival appears limited to the pre-rituximab era [23]. This aligns with findings by Tada *et al*., who reported that HLA restriction does not significantly influence clinical outcomes in aggressive B-cell lymphomas treated in the rituximab era [24]. Given that loss of both HLA class I and II expression is a recurring feature in the pathogenesis of aggressive B-cell lymphomas, and that TCR-mediated anti-tumor responses require an intact HLA system, it was noteworthy to identify a substantial subset of patients exhibiting immunohistochemical loss of B2M and/or HLA-DR; however, this did not affect TCR clonality measures **(Figures 1a–1e)** [25, 26]. Future studies should aim for more comprehensive HLA typing and immunohistochemical assessment of the class I and II machinery to better understand how potential HLA restrictions influence specific TCR sequences and clonality.

The observation of a more diverse TCR repertoire may reflect an insufficient adaptive immune response, potentially suppressed by malignant B-cells. Reports from solid tumors suggest that higher T-cell clonality correlates with a more focused and effective anti-tumor immune response. For instance, CTLA-4 blockade can lead to TCR repertoire diversification and worse outcomes, whereas PD-1 blockade tends to increase clonality and is associated with improved prognosis [27, 28]. Therefore, a higher percentage of the maximal clone frequency may indicate increased responsiveness to anti-PD-1 therapies [29]. Since no patients in the current cohort received checkpoint inhibitors, this hypothesis requires validation in prospective clinical trials. Regardless, our data indicate that highly expanded TCR clonotypes may be linked to adverse outcomes in HGBL-DH/TH.

Univariate analysis suggested that restricted TCR repertoires, as measured by clonality, were associated with worse overall survival (OS), though not progression-free survival (PFS). However, these measures did not retain independent prognostic significance in multivariate Cox regression analyses, indicating that additional features of the tumor microenvironment (TME) must be considered when evaluating the functional capacity of tumor-infiltrating T-cells.

When comparing TCR diversity and productive TCR rearrangements between HGBL-DH/TH and tnDLBCL, our findings suggest that tnDLBCL may be more amenable to immunotherapeutic strategies [11]. Productive clonality, which influences response to immunotherapy, differed significantly between these groups ($p = 0.0427$), and higher TCR diversity may reduce the likelihood of tumor antigen escape [30].

Interestingly, fourteen public clonotypes were recurrently identified in HGBL-DH/TH, implying potential tumor neoantigen selection. These subdominant clones could be capable of targeting specific tumor antigens [31]. It is plausible that these clonotypes reflect immune responses directed against cytogenetic alterations characteristic of HGBL-DH/TH, consistent with similar findings recently reported in Burkitt lymphoma [10]. Targeted sequencing has also revealed higher tumor mutational and neoantigen burdens in HGBL-DH/TH compared to tnDLBCL [32]. The presence of distinct public clonotypes in HGBL-DH/TH aligns with Linnemann *et al*., who demonstrated a correlation between neoantigen-specific T-cells and tumor mutational load [33]. While T-cell diversity in tumors often differs from peripheral blood [34], recent work by Wu *et al*. detected expanded TCR clonotypes across tumor tissue, adjacent normal tissue, and blood [35]. Therefore, future studies should employ whole-exome sequencing (WES), transcriptomic profiling, and proteomics in larger cohorts to characterize tumor neoantigens, and include peripheral blood assays to clarify the uniqueness of HGBL-DH/TH clonotypes relative to tnDLBCL. To functionally validate our findings and link dominant HGBL-DH/TH clonotypes to their target antigens, we plan to complement DNA-based TCR sequencing with single-cell RNA sequencing, as recently described [36]. Additionally, employing cytokine-capturing antigen-presenting cells or lentiviral delivery of epitope libraries for MHC presentation (T-scan) may help identify the specific antigens recognized by these T-cells [37, 38].

Although we identified a TCR β CDR3 clonotype recognizing the EBV nuclear protein EBNA3A, none of the HGBL-DH/TH cases were EBV-positive by EBER in-situ hybridization, suggesting non-tumor-specific T-cell infiltrates. Lack of EBV serology data limits further analysis. These results should also be interpreted in light of findings by Mundo *et al*., who reported a substantial fraction of B-cell lymphomas falsely negative for EBV using routine detection methods [39].

Increasing evidence highlights the substantial influence of the Glasgow Prognostic Score (GPS) on outcomes across multiple malignancies, including lymphomas such as DLBCL, classical Hodgkin lymphoma, multiple myeloma, and various solid tumors [40, 41]. To our knowledge, this is the first investigation to demonstrate the combined prognostic relevance of GPS and TCR clonality in HGBL-DH/TH.

Despite being one of the largest cohorts studied in this rare entity, the current analysis has limitations, most notably its sample size and retrospective design. Validation of these findings in larger, prospectively collected cohorts is essential before clinical recommendations for personalized risk stratification and potential treatment intensification can be made. Given the rarity of HGBL-DH/TH, the retrospective approach was unavoidable. Ideally, prospective collection of peripheral blood samples for comparison with tumor tissue would have strengthened the study, but this was not feasible in the current exploratory framework. Furthermore, clinical data were derived from medical records, which may be incomplete in certain cases.

Although the CDR3 region of the TCR β-chain provides a robust measure of T-cell clonality, it represents only a partial view of the TCR repertoire. As a result, clonal diversity may have been underestimated relative to analyses incorporating both TCR α- and β-chains, which could also have led to overestimation of shared TCRαβ clonotypes between patients, as potential α-β pairings remain undetected. Additionally, MHC polymorphisms were not considered. A more comprehensive assessment, integrating gene expression profiling and broader immunohistochemical characterization of HLA components, would have been informative but was beyond the scope of this study and should be addressed in future investigations.

Despite these limitations, our findings have potential clinical implications. As efforts continue to identify patients requiring upfront therapeutic intensification, improved baseline risk stratification and a deeper understanding of adaptive immunity in this challenging lymphoma subtype remain urgently needed.

Materials and Methods

Patient samples

Patients with complete cytogenetic data for BCL2, BCL6, and MYC rearrangements were identified from our institutional cytogenetics database. All consecutive HGBL-DH/TH samples submitted to the Reference Centre for Hematopathology Lübeck (Prof. Feller) between 1 January 2008 and 31 July 2017, fulfilling the 2016 WHO

diagnostic criteria for hematopoietic and lymphoid tumors, were screened [1]. Diagnoses were confirmed independently by two experienced hematopathologists.

Application of internal quality control measures led to the exclusion of 14 out of 79 HGBL-DH/TH samples due to insufficient numbers of productive templates (<100). The final analysis included 65 formalin-fixed, paraffin-embedded (FFPE) diagnostic samples from 47 patients. Both nodal and extranodal primary manifestations were represented. Although extranodal involvement was common, many biopsy samples were obtained from nodal sites, leading to classification of some extranodal cases as "nodal" for analytic purposes.

To investigate the influence of immunochemotherapy and the TME at relapse, seven longitudinal samples from seven patients were included (median interval: 22 months). For comparative purposes, 34 diagnostic samples from 28 triple-negative DLBCL patients (negative for MYC, BCL2, and BCL6 by FISH; 10 EBV-positive, 24 EBV-negative) were analyzed. Case selection for the control group was based solely on availability of consecutive biopsy specimens with appropriate cytogenetics. While this cytogenetically defined reference group included 10 EBV-positive DLBCL cases, potential confounding was minimized by excluding patients with suspected immunosuppressive disorders. All cases meeting the predefined criteria underwent subsequent clinical evaluation following confirmation by the hematopathology panel.

Clinical evaluation

The study included 47 patients with HGBL-DH/TH whose diagnoses were confirmed both histologically and cytogenetically. For comparison, 27 patients with de novo DLBCL were also analyzed, as described previously [10]. Patients were excluded if follow-up was incomplete, or if they had primary CNS lymphoma, post-transplant lymphoproliferative disorder, HIV-associated lymphoma, or transformed indolent lymphoma. Key clinical and pathological characteristics are summarized in **Table 1**.

Clinical data were obtained from medical records and encompassed performance status (ECOG), disease stage, administered treatments, therapeutic responses, relapse patterns, baseline lactate dehydrogenase (LDH) levels, revised International Prognostic Index (R-IPI) [42], and survival outcomes. All information was anonymized and linked to histopathological findings and TCR sequencing results. Disease extent was staged according to the Cotswold-modified Ann Arbor system [43]. Additionally, the Glasgow Prognostic Score (GPS) was calculated to provide an inflammation-based risk assessment. GPS assigns one point for CRP ≥10 mg/dL and one point for serum albumin ≤35 g/L, yielding three categories (0, 1, or 2 points) [44]. Following initial evaluation, treatment was administered according to the treating physician's discretion, with institutional guidelines guided by contemporary DSHNHL/GLA recommendations.

Immunophenotyping, tumor-infiltrating T-Cell assessment, and FISH

Immunophenotypic characterization was performed using a panel of antibodies on FFPE tissue sections. Slides were reviewed by VO, ACF, HM, and NG to assess both qualitative and quantitative features of tumor-infiltrating lymphocytes (TILs), showing high reproducibility (data not shown). Importantly, the absolute number of productive TCR rearrangements—but not clonality—correlated significantly with the quantity of T-cells within a sample.

Cytogenetic evaluation of MYC, BCL2, and BCL6 was performed using dual-color break-apart FISH probes (Abbott Vysis, Des Plaines, IL, USA) according to manufacturer instructions. Samples were included only if t(8;14)(q24;q34) was present and no additional BCL2 or BCL6 rearrangements were detected.

High-throughput TCRβ CDR3 sequencing

Genomic DNA was extracted (see Supplementary Methods), and the hypervariable CDR3 region of the TCRβ chain was sequenced using the ImmunoSEQ assay (Adaptive Biotechnologies, Seattle, WA, USA) with a target depth of 30,000 reads per sample. A minimum of 500 ng DNA per sample was required. Each sample was amplified in two aliquots via two-step multiplex PCR, incorporating sample-specific barcodes and adapter sequences, followed by correction for primer bias as described [45].

In the first PCR, forward and reverse primers specific to each V and J gene segment amplified the CDR3 region. Barcode and Illumina® adapter sequences were added in the second step. Amplicons (87 bp covering CDR3 and flanking V and J segments) were sequenced on an Illumina® MiSeq (v3 chemistry), with up to 23 samples per lane.

Raw sequencing data were demultiplexed using barcodes, and adapter, primer, germline, and contaminant sequences were removed. Sequences were clustered using a modified nearest-neighbor approach to account for technical errors, and annotated according to the IMGT database (accessed 27 November 2020). Data were processed using Adaptive Biotechnologies' pipeline and further analyzed in Analyzer 3.0.

TCR data analysis

Normalized CDR3 sequences were quantified against synthetic TCRβ templates to correct amplification bias [46]. Shannon entropy was used to assess repertoire diversity, with higher values reflecting greater TCR heterogeneity [46]. Clonality, defined as the reciprocal of normalized entropy, reflects the dominance of specific clones, independent of sequencing depth.

To evaluate clonal expansion, the relative frequency of the most abundant clone (% maximal frequency) and the combined frequency of the ten most expanded clones (% top10) were calculated. Frequencies were normalized for sequencing depth to allow comparison across samples [11].

Comparative clonotype analysis with DLBCL

Clonotypes that met predefined expansion thresholds were considered indicative of potential tumor neoantigen-driven selection. These expanded sequences were then cross-referenced with a large, publicly available database encompassing healthy donors and patients with diverse conditions (VDJdb) to annotate potential biological relevance (Supplementary Methods) [47]. Comparative analyses were performed on 65 HGBL-DH/TH samples and 34 tnDLBCL samples.

Statistical methods

Differences between patient subgroups were evaluated using chi-square tests and paired two-tailed Student's t-tests as appropriate. Associations between continuous variables were assessed using Pearson correlation. Progression-free survival (PFS) and overall survival (OS) were measured from the date of initial diagnosis. Survival estimates were generated using the Kaplan–Meier method and compared using univariate log-rank tests. Variables demonstrating significant impact on OS or PFS were included in subsequent multivariate Cox proportional hazards models. Statistical analyses were conducted using Microsoft Excel, GraphPad PRISM 6, R-Studio, and SPSS 25. Optimal cut-off points for survival analysis were determined using an AUC-based approach as described by Budczies *et al*. (productive clonality: 0.0033; maximum productive frequency: 0.0212) [14].

Ethical considerations

All samples were collected as part of routine clinical care. The study protocol was approved by the Ethics Committee of the University of Lübeck, in accordance with the Declaration of Helsinki (reference 18-356). Patients provided written informed consent for both diagnostic and research use of their biopsy specimens, as well as for inclusion of anonymized clinical data.

Data availability

Processed sequencing datasets will be made publicly accessible through the immuneAccess database (Adaptive Biotechnologies, Seattle, WA, USA) under a dedicated project titled "TCR repertoire in HGBL with MYC, BCL2 and/or BCL6 rearrangements" upon publication of the manuscript.

Conclusion

This study demonstrates that highly expanded intra-tumor TCR clonotypes are associated with adverse prognosis in HGBL-DH/TH. The clonal architecture of tumor-infiltrating T-cells in HGBL-DH/TH is distinct from tnDLBCL, independent of age-related effects. Several clonotypes exclusive to HGBL-DH/TH, potentially driven by tumor neoantigens, were identified. Additionally, elevated baseline GPS is associated with increased relapse rates, refractory disease, and reduced overall survival. These findings highlight the complementary roles of adaptive and innate immune parameters as prognostic indicators, providing a potential framework for refined risk stratification and treatment guidance, which warrants validation in future prospective studies.

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