

ARTICLE



Molecular Diagnostics

Circulating tumor DNA from blood and cerebrospinal fluid in DLBCL: simultaneous evaluation of mutations, IG rearrangement, and IG clonality

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BACKGROUND: Circulating tumor DNA (ctDNA) is a promising biomarker for monitoring minimal residual disease (MRD), assessing disease status, and guiding treatment in diffuse large B-cell lymphoma (DLBCL). Current ctDNA assays rarely detect immunoglobulin (IG) fusions. This study evaluates a novel assay that simultaneously detects mutations, IG fusions, and IG V(D)J clonality in plasma and cerebrospinal fluid (CSF) to enhance molecular profiling and CNS monitoring.

METHODS: A prospective analysis was conducted in 57 DLBCL patients. Genomic alterations in plasma and CSF ctDNA were compared to those in tumor tissue using targeted next-generation sequencing (NGS).

RESULTS: Mutations, IG fusions, and IG V(D)J clonality were detected in 100%, 72.2%, and 78.6% of plasma ctDNA samples, respectively. Plasma ctDNA also revealed additional mutations absent in tumor tissue, reflecting clonal heterogeneity. The incorporation of IG fusion detection enabled molecular subtyping without requiring FISH. In CSF, ctDNA analysis identified genomic alterations in 8 cases, whereas conventional imaging and cytology confirmed CNS involvement in only 3, demonstrating the superior sensitivity of ctDNA for CNS surveillance.

CONCLUSION: This ctDNA assay offers a non-invasive, integrated approach for genomic profiling and disease monitoring in DLBCL, with improved sensitivity for CNS detection and potential to inform personalized treatment strategies.

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BACKGROUND

IG fusions are present in approximately half of mature B-cell tumours and are both diagnostically and therapeutically significant. For example, the *IGH::CCND1* rearrangement is a diagnostic hallmark of mantle cell lymphoma, while *IGH* can juxtapose with *PD-L1* in Hodgkin lymphomas, leading to *PD-L1* overexpression. This overexpression renders patients responsive to *PD-L1* inhibitors [1–4]. Traditionally, the detection of IG rearrangements has relied on fluorescence in situ hybridization (FISH) and karyotyping. However, recent studies have demonstrated the effectiveness of DNA next-generation sequencing (NGS) in identifying these rearrangements [5]. These assays require sufficient tumor cells or tissue, which are not always available in clinical practice. ctDNA can be readily obtained from peripheral blood or cerebrospinal fluid (CSF) [6]. While ctDNA has been widely used to detect mutations, copy number variations (CNVs), and IG V(D)J clonality, its potential to detect IG fusions from small ctDNA fragments remains uncertain [7–11]. In this study of 57 consecutive DLBCL

cases, we present evidence that plasma ctDNA can effectively capture IG fusions.

IG fusions are also crucial biomarkers for the molecular subtyping of DLBCL. DLBCL can be classified into 6 genome-based subtypes using the LymphGen algorithm: MCD (*MYD88/CD79B* mutations, chronic B-cell receptor signalling), BN2 (*IG::BCL6*, *NOTCH2* mutations), N1 (*NOTCH1* mutations), EZB (*EZH2* mutations, *IG::BCL2*), ST2 (*SGK1* and *TET2* mutations, immune-privileged microenvironment), and A53 (*TP53* mutations, genomic instability). These subtypes guide therapeutic strategies; for instance, the MCD subtype responds well to Bruton tyrosine kinase (BTK) inhibitors such as ibrutinib [12]. The simultaneous detection of all genomic aberrations required for molecular subtyping, including IG fusions, in a single assay reduces the need for multiple testing platforms. This streamlined approach is particularly valuable in DLBCL when tumour cells are limited.

Central nervous system (CNS) involvement in DLBCL, occurring in approximately 5% of cases, is associated with poor prognosis

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and poses significant clinical challenges due to the difficulty of most chemotherapy drugs crossing the blood-brain barrier [13]. Therefore, early diagnosis and preventive therapy are critical, with high-risk patients receiving intrathecal or systemic CNS-directed therapies. CNS involvement is typically evaluated using imaging and cytomorphologic and flow cytometric analyses of CSF; however, these traditional methods often lack sensitivity. To address these limitations, our study extends ctDNA analysis to CSF, offering a potentially more sensitive and comprehensive approach for detecting CNS involvement in DLBCL.

METHODS

Clinical information

This single-center observational study was performed on patients with suspected DLBCL or potential DLBCL recurrence/progression between March 2021 and March 2024 at the Second Affiliated Hospital of Soochow University. DLBCL was diagnosed according to the criteria of the WHO classification of Hematolymphoid Tumors (5th ed) [14]. Circulating cell-free DNA (cfDNA) was collected from plasma and CSF in Streck tubes prior to any treatment. A contrast-enhanced CT or 18F-PET-CT was performed at the time of diagnosis and during the follow-up. Treatment response was analyzed according to the Lugano classification criteria. The CNS prophylaxis strategy followed the NCCN guidelines. Patients with a high CNS-IPI score (4–6), double/triple-hit HGBL, or high-risk extranodal site involvement (such as testicular, breast, primary cutaneous, or renal/adrenal) received intrathecal methotrexate and cytarabine (4 doses) or high-dose methotrexate (3–3.5 g/m² for 2–4 cycles) during or after their treatment course. The date of the last follow-up was 16 August 2024. Clinical information was gathered from hospital records. Follow-up data on treatment responses and the timing of the first event (relapse, progression, or death from any cause) were also collected. This study was approved by the Medical Ethics Committee of the Second Affiliated Hospital of Soochow University. Informed consent was obtained from all participants before participation.

Targeted DNA NGS

Genomic DNA was isolated from tumour tissues (lymphoid tissue or bone marrow) and peripheral blood mononuclear cells using the QIAamp DNA Blood Mini Kit (Qiagen) or ReliaPrep™ FFPE gDNA Miniprep System (Promega). For cfDNA isolation, peripheral blood and CSF were centrifuged at 2000 rpm for 15 min within 24 h of collection. The supernatant was transferred to 1.5 ml sterile Eppendorf tubes and centrifuged at 15,000 rpm for 2 min at 4 °C, the supernatant was aliquoted into 2 mL sterile Eppendorf tubes and stored at –80 °C until DNA extraction. QIAamp Circulating Nucleic Acid Kit (Qiagen) was used for cfDNA isolation. For DNA sequencing library preparation, gDNA was fragmented to 300–500 bp with a Bioruptor Pico (Diagenode) (no fragmentation needed for cfDNA), DNA end repaired, and dA-tailed for 30 min at 30 °C followed by 30 min at 65 °C. A unique Molecular Identifier (UMI) containing adaptor was added by incubating with ligase for 15 min at 20 °C. After purification by AMPure XP beads, 7 cycles of PCR were performed. The PCR products were incubated with a pool of biotin-labelled bait oligos that targeted 106 genes commonly involved in lymphoma for 16 h. Targeted regions were captured by pull-down with streptavidin beads, amplified by PCR, and sequenced in an Illumina NovaSeq sequencer. Sequencing results for single-nucleotide variations, insertion/deletion, copy number variations (CNVs), IG-related fusions, and IG V(D)J clonality were analyzed with laboratory-developed pipelines (Sano Medical Laboratories). The list of targeted genes included: *ASXL1*, *ARID1A*, *ARID1B*, *ATM*, *B2M*, *BCL2*, *BCL6*, *BCOR*, *BCORL1*, *BIRC3*, *BLNK*, *BRAF*, *BTK*, *CARD11*, *CCND1*, *CCND3*, *CD274*, *CD28*, *CD58*, *CD79A*, *CD79B*, *CDKN1B*, *CDKN2A*, *CDKN2B*, *CKS1B*, *CRBN*, *CREBBP*, *CXCR4*, *DDX3X*, *DIS3*, *DLEU2*, *DNMT3A*, *DNMT3B*, *EGR1*, *EP300*, *EPHA7*, *ERG*, *EZH2*, *FAM46C*, *FAS*, *FBXW7*, *FOXO1*, *GNAI3*, *HRAS*, *ID3*, *IDH1*, *IDH2*, *IGH*, *IGK*, *IGL*, *INO80*, *INPP5D*, *IRF4*, *ITPKB*, *JAK1*, *KDM6A*, *KLF2*, *KMT2A*, *KMT2D*, *KRAS*, *MALT1*, *MAP2K1*, *MAX*, *MEF2B*, *MYC*, *MYD88*, *NFKBIE*, *NOTCH1*, *NOTCH2*, *NRAS*, *PAX5*, *PIK3CA*, *PIM1*, *PLCG1*, *PLCG2*, *POT1*, *PRDM1*, *PRKD2*, *PTEN*, *PTPN11*, *PTPRD*, *RBPJ*, *REL*, *RHOA*, *SETD2*, *SF3B1*, *SGK1*, *SOC1*, *STAT3*, *STAT5B*, *STAT6*, *TCF3*, *TET2*, *TMEM14B*, *TNFAIP3*, *TNFRSF14*, *TP53*, *TRAF2*, *TRAF3*, *TRA*, *TRB*, *TRD*, *TRG*, *XPO1*, *ZFAT*, *ZFX4*.

After sequencing, read pairs were aligned to Human Reference Genome version GRCh37/hg19. The SNVs and Indels were screened according to the following filtering criteria: (1) Exclude SNVs or Indels with minor allele

frequency (MAF) greater than 1% that were present in genomAD, ESP6500, the 1000 Genomes Project; (2) SNVs or Indels with a variant allele frequency (VAF) $\geq 0.3\%$ for cfDNA and $\geq 3\%$ for gDNA were retained. Additionally, variants in cfDNA with VAFs between 0.2% and 0.3%, as well as those identified in gDNA with VAFs between 0.5% and 3%, were classified as positive if two conditions were simultaneously met: the variant was detected in both tumour tissue and matched cfDNA; and manual review using the Integrative Genomics Viewer (IGV) excluding background artifacts; (3) Variant interpretation was performed in accordance with the guidelines established by the Association for Molecular Pathology (AMP), the American Society of Clinical Oncology (ASCO), and the College of American Pathologists (CAP). Pathogenicity was assessed based on multiple criteria, including evidence of a well-established role in leukemia and lymphomas, functional data, known clinical significance, and prognostic or therapeutic relevance. Variants were also cross-referenced against public databases including COSMIC, OncoKB, ClinVar, cBioPortal, and CIViC. For rare or novel mutations, in silico AI-based modelling of protein function (AlphaMissense) was additionally used to support pathogenicity classification. (4) The IG fusion was classified as positive when the number of non-repeating fusion sequences was ≥ 3 reads; (5) The IG clonality was interpreted as positive when both the count of IG monoclonal sequences was no less than 30 and the proportion of monoclonal sequences was no less than 10%.

Statistical analysis

All statistical analyses were performed using R software (version 4.3.1) and GraphPad Prism (version 9.0, RRID:SCR_002798). Descriptive statistics are expressed as means or medians for continuous variables and frequencies for categorical variables. Progression-free survival (PFS) was calculated from the date of diagnosis to the date of disease progression, relapse, or death from any cause. Overall survival (OS) was assessed from the date of diagnosis to the date of death from any cause. Both PFS and OS were plotted using the Kaplan-Meier (KM) method. A $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

Seventy-two patients with suspected DLBCL were enrolled in the study, with 57 confirmed cases eventually included based on histopathology. Of these, 49 patients had both tumour tissue and peripheral blood samples, while 8 patients had only blood samples due to the unavailability of tumour tissue. CSF samples were collected from 50 of the 57 patients; however, library construction failed in 13 cases due to insufficient cfDNA (<10 ng), and these cases were excluded from further analysis (Fig. 1a).

Among the 57 patients, the median age was 66 years. According to the Ann Arbor classification, 39 patients had stage III/IV diseases (68%), while 18 had stage I/II diseases (32%). Based on international prognostic index (IPI) scores, 29 patients had high scores ranging from 4 to 5 (51%). Additionally, 31 patients were classified as being at high risk of CNS relapse according to the CNS-IPI prognostic model (55%). Most patients received R-CHOP as first-line therapy. For those with MCD-like and BN2-like subtypes, combination therapy including BTK inhibitors was used when feasible. One patient died before the initiation of chemotherapy. Among the 54 evaluable patients at the end of first-line or salvage treatment, the overall response rate (ORR) was 83% (Supplementary Table 1).

Comparison of genomic alterations between tumor tissue, plasma ctDNA, and peripheral blood gDNA

Genomic profiling was performed on tumor tissue, plasma ctDNA, and peripheral blood gDNA from 49 patients with a targeted DNA NGS panel containing 106 lymphoma genes including *IGL*, *IGK*, and *IGH*. Gene mutations were identified in all tumor tissues (49/49, 100%), including 446 mutations in 66 genes, with a median of 7 somatic mutations per patient (range: 1–29). The most frequently mutated genes were *PIM1* (45%), *MYD88* (37%), *KMT2D* (31%), and *CD79B* (29%) (Fig. 1b). Eight types of immunoglobulin

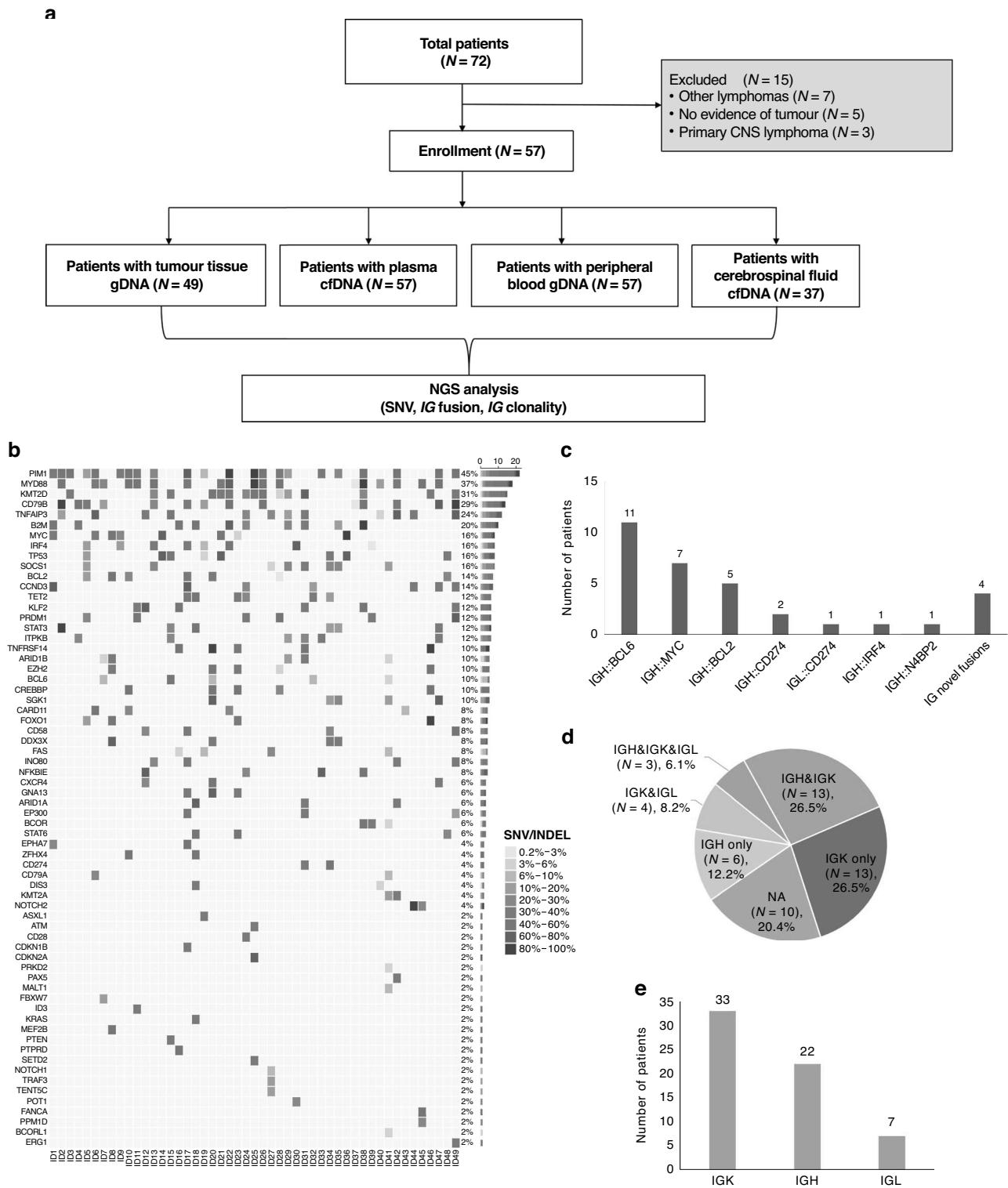


Fig. 1 Study design and analysis of primary tumor gDNA. a Flow diagram illustrating patient enrolment and specimen collection. **b** A total of 66 genes were mutated in tumor tissue, with the most commonly mutated genes listed at the top. Patient IDs are displayed at the bottom, and mutation prevalence for each gene is shown on the right. Mutation frequencies are represented using different colour shades. **c** Eight types of *IG* fusions were identified. **d** Summary of the types and proportions of *IG* clonality observed. **e** The number of patients with clonal *IGK*, *IGH*, and *IGL*, respectively.

(*IG*) fusions were detected, including *IGH::BCL6* (11 cases), *IGH::MYC* (7 cases), *IGH::BCL2* (5 cases), *IGH::CD274(PD-L1)* (2 cases), *IGL::CD274(PD-L1)* (1 case), *IGH::IRF4* (1 case), *IGH::N4BP2* (1 case), and *IGH::novel fusion partners* including *t(4;14)(q13;q32)* (1 case),

t(3;14)(q13;q32) (1 case), *t(8;14)(q12;q32)* (1 case), *t(1;14)(p35;q32)* (1 case) (Fig. 1c). Concurrent FISH assays were performed in 5 of 24 (20.8%) NGS-positive *IG* fusion cases, demonstrating concordant results between FISH and NGS for *IGH* rearrangement

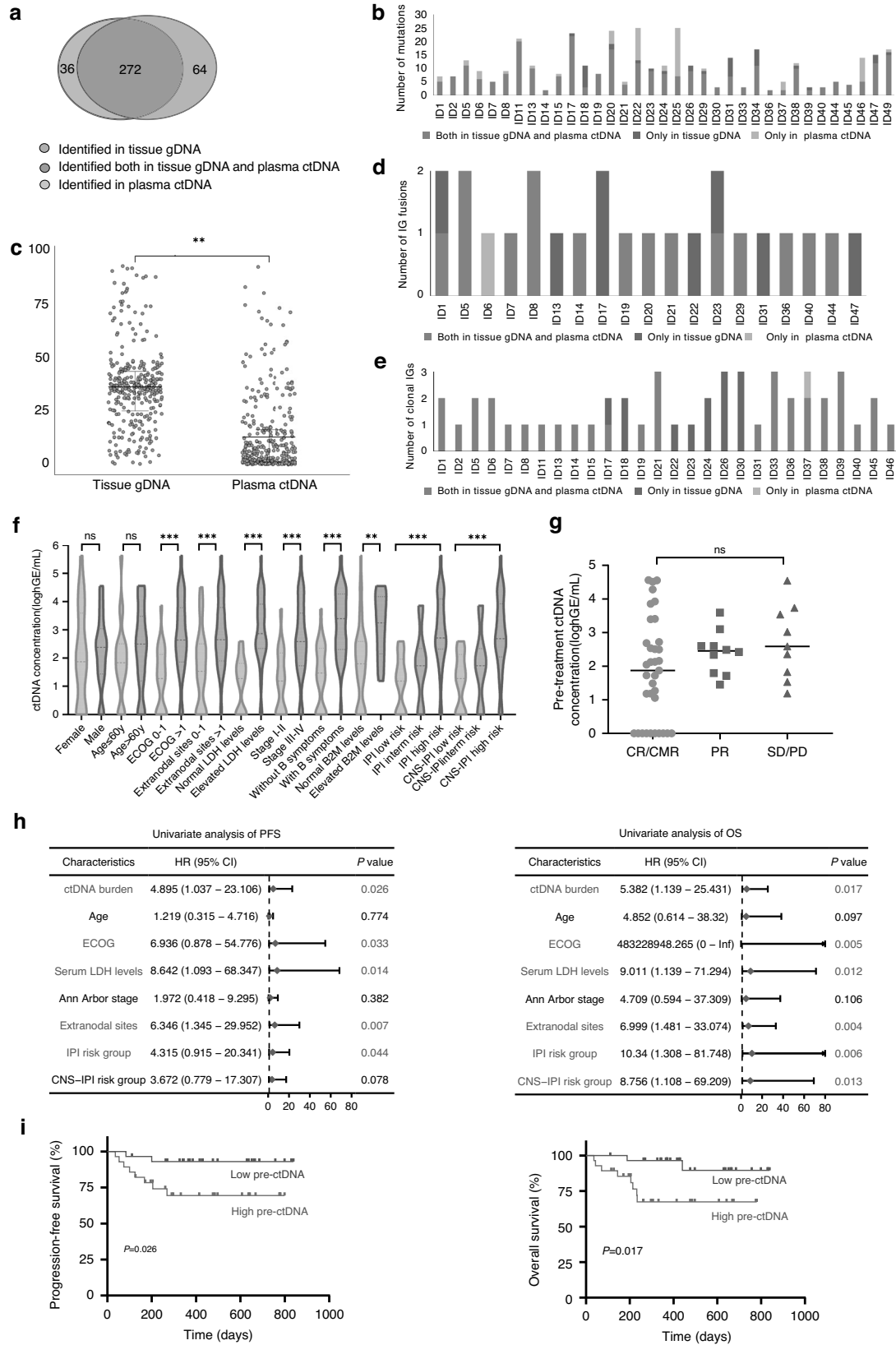


Fig. 2 Comparison of mutational profiles in primary tumor gDNA and Plasma ctDNA. **a** Venn diagram showing the number of mutations detected in plasma ctDNA, tumor tissue, or both (N = 35). **b** Mutation concordance between plasma ctDNA and matched tumor tissue gDNA for each patient. Red: mutations present in both tissue gDNA and plasma ctDNA; blue: mutations present only in tissue gDNA; green: mutations present only in plasma ctDNA. **c** Mutation frequency is significantly higher in tumor tissue compared to plasma ctDNA. **d** IG fusion concordance between plasma ctDNA and matched tissue gDNA for each patient. **e** Clonal IG concordance between plasma ctDNA and matched tissue gDNA for each patient. **f** Plasma ctDNA concentration was correlated with various clinical parameters, with significant associations observed with ECOG status, elevated serum LDH levels, multiple extranodal sites of involvement, increased serum B2M levels, advanced Ann Arbor stage, and the presence of B symptoms. **g** Analysis of pre-treatment plasma ctDNA concentration in relation to treatment response, including CR/CMR, PR, or SD/PD. While patients with elevated ctDNA concentrations tended to have poorer therapeutic responses, this trend was not statistically significant. **h** Univariate Cox analysis of PFS and OS with various clinical parameters. **i** Kaplan-Meier estimates of PFS and OS based on pre-treatment ctDNA status. The group with higher plasma ctDNA levels is significantly associated with poorer PFS and OS. ns: not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; CR complete response; CMR complete metabolic response; PR partial response; SD stable disease; PD progressive disease; LDH lactate dehydrogenase; B2M beta-2 macroglobulin; PFS progression-free survival; OS overall survival.

(Supplementary Table 2). Notably, 3 DLBCL cases had the *IG::PD-L1* fusion, suggesting that these patients are likely to respond to PD-L1 inhibitors. Clonality analysis revealed that clonal *IG* was detected in 39 of 49 patients (79.6%). This included clonal *IGK* in 13 cases, clonal *IGH* in 6 cases, clonal *IGK* and *IGH* in 13 cases, clonal *IGK* and *IGL* in 4 cases, and clonal *IGK*, *IGL*, and *IGH* in 3 cases (Fig. 1d, e).

Among the above 49 cases, genomic alterations were detected in plasma ctDNA from 35 patients (71.4%), including 336 mutations in 60 genes. Compared to the 308 mutations identified in tumor tissues, 272 mutations were shared between tumor tissue and plasma ctDNA, while 64 mutations were unique to plasma ctDNA and 36 were unique to tissue specimens (Fig. 2a). Specifically, 10 cases (28.6%) showed identical mutations in both plasma ctDNA and tissue, 17 cases (48.6%) had additional mutations in plasma ctDNA, 14 cases (40%) had additional mutations in tissue specimens, and 6 cases (17.1%) displayed unique mutations in both plasma ctDNA and tissue specimens (Fig. 2b). The average variant allele frequency (VAF) in plasma ctDNA was 13% (range: 0.2%–92.3%), significantly lower than that in tissue specimens, which averaged 36.5% (range: 0.5%–92.7%) ($p < 0.001$) (Fig. 2c). *IG* fusions were detected in 14 of 35 cases (40%) using plasma ctDNA. In the same group of 35 cases, *IG* fusions were identified in 18 cases using tumor tissue (51.4%), indicating that plasma ctDNA can capture most *IG* fusions. Notably, an *IGH* fusion with a novel partner, *t(1;14)(p21;q32)*, was detected exclusively in plasma ctDNA (Fig. 2d). Clonal *IG* was identified in 22 of 35 cases (62.9%) from plasma ctDNA. In the same group of 35 cases, clonal *IG* were identified in 28 cases using tumor tissue (80%) (Fig. 2e). Taking together, plasma ctDNA captured 13 of the 18 total *IG* fusions (72.2%) and 22 of the 28 clonal *IGs* (78.6%) (Supplementary Table 3).

In 8 cases, only plasma ctDNA was available, as no tumor tissue was obtained (Supplementary Table 4). Somatic mutations were detected in 7 of the 8 cases (87.5%), with the most frequently mutated genes being *BCL2* (37.5%) and *MYD88* (37.5%). *IGL::BCL2* fusion was identified in 1 patient (ID56). Clonal *IG* was identified in 4 of the 8 cases (50%), with clonal *IGK* being the most common type.

Genomic alterations were identified in 8 of 49 peripheral blood gDNA samples (16.3%) after excluding age-related mutations. These findings indicate that genomic profiling of plasma ctDNA provides significantly more comprehensive information compared to peripheral blood gDNA.

High plasma ctDNA concentration is associated with poor prognosis

The concentration of plasma ctDNA was measured in haploid genome equivalents per milliliter (hGE/mL). The median ctDNA value was 2.13 log hGE/mL (range: 0–5.62 log hGE/mL) (Supplementary Table 5). We analyzed ctDNA concentrations in relation to various clinical parameters, including gender, age, ECOG

performance status, serum lactate dehydrogenase (LDH) levels, extranodal involvement, disease stage, B symptoms, beta-2 macroglobulin (B2M) levels, IPI, and CNS-IPI scores (Fig. 2f). High ctDNA concentrations were significantly associated with poor ECOG status ($p < 0.001$), elevated serum LDH levels ($p < 0.001$), multiple extranodal sites of involvement ($p < 0.001$), increased serum B2M levels ($p = 0.001$), advanced Ann Arbor stage ($p < 0.001$), and the presence of B symptoms ($p < 0.001$). No significant differences in ctDNA concentrations were observed on gender or age. Furthermore, ctDNA concentrations strongly correlated with IPI and CNS-IPI risk scores. For IPI risk groups, the mean ctDNA concentrations were 1.12 ± 0.96 log hGE/mL for low risk, 1.71 ± 1.35 log hGE/mL for intermediate risk, and 2.96 ± 1.32 log hGE/mL for high risk ($p < 0.001$). Similarly, for CNS-IPI risk groups, the mean ctDNA concentrations were 1.19 ± 0.98 log hGE/mL for low risk, 1.71 ± 1.35 log hGE/mL for intermediate risk, and 2.81 ± 1.41 log hGE/mL for high risk ($p < 0.001$).

Based on therapeutic response, patients were grouped as complete response (CR)/complete metabolic response (CMR), partial response (PR), or stable/progressive disease (SD/PD), according to the Lugano response criteria. While patients with elevated ctDNA concentrations tended to have poorer therapeutic responses, this trend was not statistically significant ($p = 0.2006$) (Fig. 2g). However, high ctDNA concentrations were significantly associated with poor prognosis. Patients with a median ctDNA value of 2.13 log hGE/mL or above had significantly worse PFS and OS compared to those with a median ctDNA value below 2.13 log hGE/mL. The differences were statistically significant, with $p = 0.026$ for PFS and $p = 0.017$ for OS (Fig. 2h, i). Although these associations were significant in univariate analysis, they did not remain significant in the multivariate Cox regression model (PFS: $p = 0.900$; OS: $p = 0.904$), which included ctDNA, age, ECOG performance status, LDH, extranodal involvement, disease stage, IPI, and CNS-IPI scores (Fig. S1).

CSF ctDNA and CNS involvement of DLBCL

Among 57 DLBCL patients enrolled, CSF samples were collected from 50 individuals. Of these, 37 cases underwent successful sequencing, while 13 cases failed library construction due to insufficient cfDNA (< 10 ng). Genomic aberrations in CSF cfDNA were identified in 8 of 37 cases (21.6%). A total of 20 mutated genes were detected, with the most common mutations being *MYD88* (25%), *CD79B* (25%), *KMT2D* (25%), and *ASXL1* (12%). Additionally, 4 *IG* fusions were detected in 2 cases: one case (ID7) harbored the *IGH::BCL6* fusion, while the other (ID9) had 4 rearrangements, including *IGH::BCL6*, *IGH::IRF4*, *IGL::REL*, and *IGH::novel fusion partner/t(2;14)(p23;q32)*. Clonal *IG* V(D)J, all *IGK*, was identified in 4 of the 8 cases (Fig. 3a).

Of the 8 CSF ctDNA-positive patients, 6 also had genomic results from tumor tissues. All 6 cases had overlapping genomic abnormalities between CSF ctDNA and tumor tissue, including 5

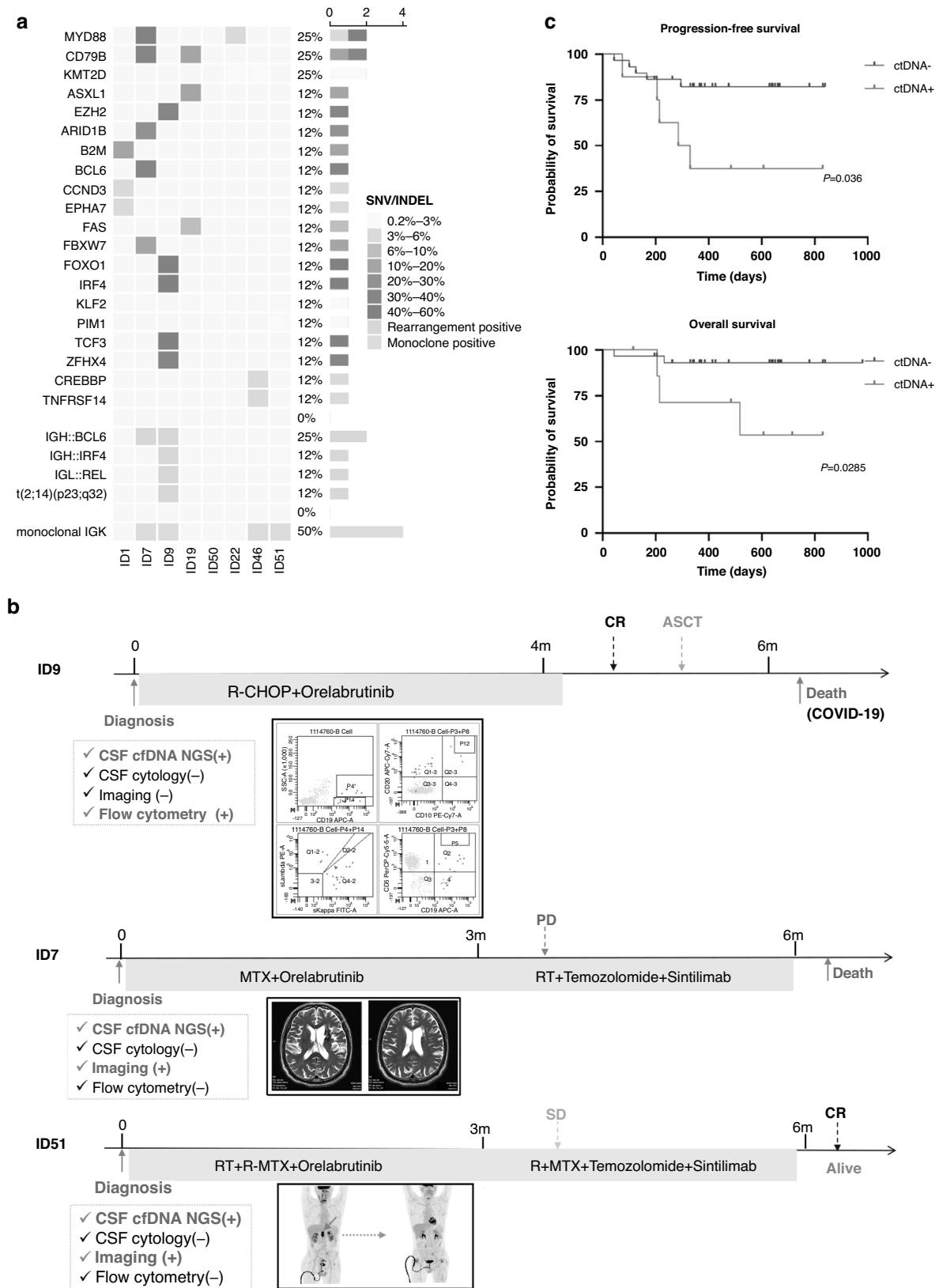


Fig. 3 Analysis of CSF ctDNA. **a** A total of 20 genes were mutated in 8 cases of CSF ctDNA, with the most frequently mutated genes listed at the top. Patient IDs are shown at the bottom, and mutation prevalence for each gene is indicated on the right. Mutation frequencies are shown in varying colour shades. *IG* fusions and clonal *IG* are also included. **b** Three patients demonstrated clinical evidence of CSF involvement: ID9 had positive flow cytometry, while ID7 and ID51 showed positive imaging findings. All three of these patients tested positive for CSF ctDNA. **c** Kaplan-Meier estimates of PFS and OS in patients with CSF ctDNA-positive versus CSF ctDNA-negative status. The CSF ctDNA-positive group showed significantly shorter PFS and OS. CSF cerebrospinal fluid; ASCT Autologous stem cell transplantation; R Rituximab; RT Radiation therapy; MTX methotrexate.

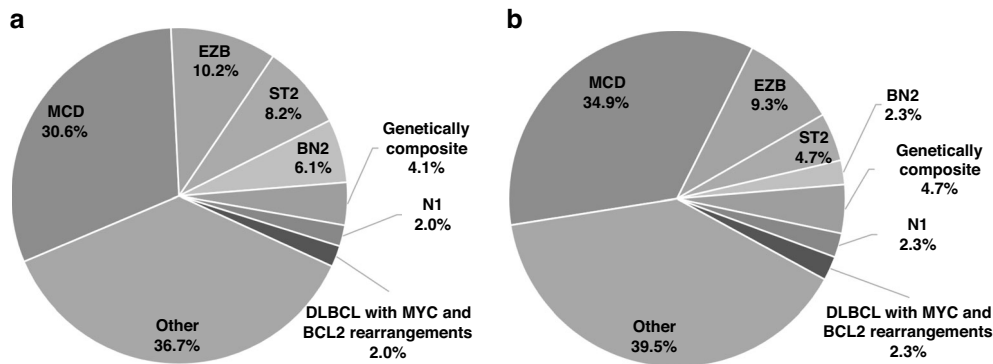


Fig. 4 Molecular subtypes of DLBCL identified from different types of specimens. **a** tumor tissue gDNA (N = 49); **b** plasma ctDNA (N = 43).

had at least one common mutation, and the remaining case shared an *IG* fusion and monoclonal *IG* sequence. Furthermore, 2 of the 5 cases shared *IG* fusions, and 3 of the 6 cases shared monoclonal *IG* V(D)J sequences (Supplementary Table 6).

Clinical evidence of CNS involvement was observed in 3 cases in this series, including positive imaging findings in 2 patients (ID7 and ID51) and positive flow cytometry in 1 patient (ID9) (Fig. 3b). All 3 cases had positive CSF ctDNA. The remaining 5 CSF ctDNA-positive patients showed no evidence of CNS involvement based on standard laboratory assessments. Follow-up showed that two patients died shortly after diagnosis due to COVID-19 and discontinued therapy. The other three patients showed no signs of CNS involvement at 4, 20, and 27 months of follow-up, respectively. All three received CNS prophylactic treatment, including intrathecal chemotherapy or the BTK inhibitor orelabrutinib, which is known to cross the blood-brain barrier and may provide protective effects against CNS disease (Fig. S2). Finally, A survival analysis was performed by comparing CSF ctDNA-positive and CSF ctDNA-negative patients, which showed significantly shorter PFS ($p = 0.036$) and OS ($p = 0.0285$) in the CSF ctDNA-positive group (Fig. 3c).

Molecular subtyping of DLBCL

Our current assay, which simultaneously evaluates mutations and *IG* fusions, was evaluated for its performance on molecular subtyping using the LymphGen algorithm. Using genomic information from tumor tissue DNA, 31 of 49 patients were successfully subtyped (63.3%), including MCD (30.6%), EZB (10.2%), ST2 (8.2%), BN2 (6.1%), genetically composite (4.1%), DLBCL with *MYC* and *BCL2* rearrangements (2.0%), and N1 (2.0%) (Fig. 4a). These results align with previous reports using both molecular and FISH assays, which demonstrated that 63.1% of DLBCL cases could be molecularly subtyped [12].

We further explored the feasibility of molecularly subtyping DLBCL using plasma ctDNA. Of 43 plasma ctDNA-positive DLBCL cases, 26 were successfully subtyped (60.5%), including MCD (34.9%), EZB (9.3%), ST2 (4.7%), BN2 (2.3%), genetically composite (4.7%), DLBCL with *MYC* and *BCL2* rearrangements (2.3%), and N1 (2.3%) (Fig. 4b). Additionally, we compared molecular subtyping results from 35 patients who were positive for both tumor tissue and plasma ctDNA. The consistency between tissue- and plasma-derived subtyping was 94.3% (33/35), highlighting the reliability of plasma ctDNA for molecular characterization.

DISCUSSION

We evaluated a streamlined targeted DNA NGS assay capable of detecting mutations, *IG* fusions, and *IG* V(D)J clonality in a single workflow. This assay was used for genomic profiling in 49 DLBCL, achieving molecular subtyping in 63.3% of the cohort. Notably,

this molecular subtyping incorporates *IG* fusions traditionally identified via FISH. However, FISH assays are limited in detecting cryptic *IG* fusions caused by small insertions and “non-genic breakpoints” outside the typical FISH coverage regions. In contrast, DNA-based NGS assays readily identify these FISH-negative *IG* rearrangements [15, 16]. Additionally, this assay facilitates the discovery of non-traditional *IG* novel fusion partners. For instance, in this study, 3 DLBCL cases were identified with *PD-L1* rearrangements, including 2 cases of *IGH::PD-L1* fusions and 1 case of *IGL::PD-L1* fusion. These patients responded favourably to immunotherapy targeting *PD-L1*, underscoring the potential clinical utility of such findings [17].

Plasma ctDNA is a convenient and minimally invasive source of tumor material that can be repeatedly sampled. It has been used in lymphomas for mutation profiling at diagnosis and for detecting clonal *IG* V(D)J for MRD monitoring [18–20]. Our study demonstrates, for the first time, that ctDNA can also be used for the detection of *IG* fusions, albeit with reduced efficiency compared to genomic DNA from tumor tissue. ctDNA detected 72.2% of *IG* fusions in our cohort. This decreased efficiency likely reflects the fragmented nature of ctDNA (~160 base pairs), which can limit the ability to detect fusions that require sufficient DNA sequence from both sides of the breakpoint. Consistent with previous studies [21], ctDNA identified additional mutations not detected in tumor tissue. This discrepancy reflects the genetic heterogeneity of DLBCL, which comprises various subclones with distinct mutations. DNA extracted from a localised tumor biopsy may fail to capture mutations present in other tumor regions. In contrast, ctDNA is shed from the entire tumor, offering a more comprehensive representation of the tumor’s genomic landscape. These additional mutations can offer insights into clonal evolution, tumor progression, and potentially inform targeted therapy.

High ctDNA concentrations in our study were significantly associated with poor prognostic markers, including poor ECOG performance status, elevated serum LDH, multiple extranodal sites, increased serum B2M, advanced Ann Arbor stage, and the presence of B symptoms. Moreover, high ctDNA levels correlated with shorter PFS and OS. These findings align with previous studies in DLBCL and other cancers, indicating that high ctDNA levels likely reflect a greater tumor burden, increased tumor aggressiveness, and resistance to therapy [22]. This highlights ctDNA’s potential as a valuable tool for risk stratification and disease monitoring.

In this cohort of 49 confirmed DLBCL cases, plasma ctDNA revealed abnormal genomic alterations in 71.4% of patients, a sensitivity comparable to previous studies. Several strategies could further enhance the ctDNA detection sensitivity. These include optimising sample handling, increasing input DNA quantity, and employing deeper sequencing. Additionally, recent advances targeting circulating nucleases or liver-resident macrophages

involved in cfDNA degradation have achieved a greater than tenfold increase in cfDNA levels [23]. This scale of improvement in ctDNA yield could significantly enhance detection sensitivity.

Our study also demonstrated the utility of CSF ctDNA in assessing CNS involvement in DLBCL. Among 37 cases analyzed for CSF ctDNA, 8 showed genomic aberrations similar to those from tumor tissue from the same patients, confirming CNS involvement. Notably, of these 8 CSF ctDNA-positive cases, only 3 were diagnosed as having CNS involvement by conventional imaging or flow cytometry. The early detection of CNS involvement using CSF ctDNA allowed for timely therapeutic intervention.

In summary, we evaluated a comprehensive targeted DNA NGS assay capable of detecting mutations, *IG* fusions, and *IG* V(D)J clonality simultaneously, enabling molecular subtyping of DLBCL in a single assay. This assay is also applicable to ctDNA from plasma and CSF. Notably, CSF ctDNA outperformed standard imaging, CSF cytology, and flow cytometry in determining CNS involvement in DLBCL. Furthermore, we demonstrated, for the first time, that ctDNA, despite its fragmented nature, can be utilised for detecting *IG* fusions. These findings underscore the potential of NGS-based ctDNA analysis as a robust tool for molecular characterization, risk assessment, and disease monitoring in DLBCL.

DATA AVAILABILITY

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS

BL and SX contributed to the study conception and design. Material preparation, data collection, and analysis were performed by HZ, LL, XZ, PC, XL, JF, YW, YX and SW. The first draft of the manuscript was written by HZ and LL, and all authors reviewed the manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Medical Ethics Committee of the Second Affiliated Hospital of Soochow University. Informed consent was obtained from all participants before participation.

ETHICAL STATEMENT

All methods were performed in accordance with the relevant guidelines and regulations.

CONSENT FOR PUBLICATION

Informed consent was obtained from all participants.

ADDITIONAL INFORMATION

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