Mutational Landscape and Tumor Burden Assessed by Cell-free DNA in Diffuse Large B-Cell Lymphoma in a Population-Based Study

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ABSTRACT

Purpose: We analyzed the utility of cell-free DNA (cfDNA) in a prospective population-based cohort to determine the mutational profile, assess tumor burden, and estimate its impact in response rate and outcome in patients with diffuse large B-cell lymphoma (DLBCL).

Experimental Design: A total of 100 patients were diagnosed with DLBCL during the study period. Mutational status of 112 genes was studied in cfDNA by targeted next-generation sequencing. Paired formalin-fixed, paraffin-embedded samples and volumetric PET/CT were assessed when available.

Results: Appropriate cfDNA to perform the analyses was obtained in 79 of 100 cases. At least one mutation could be detected in 69 of 79 cases (87%). The sensitivity of cfDNA to detect the mutations was 68% (95% confidence interval, 56.2–78.7). The mutational landscape found in cfDNA samples was highly consistent with that shown in the tissue and allowed genetic classification in 43% of the cases. A higher amount of circulating tumor DNA (ctDNA) significantly correlated with clinical parameters related to tumor burden (elevated lactate dehydrogenase and β2-microglobulin serum levels, advanced stage, and high-risk International Prognostic Index) and total metabolic tumor volume assessed by PET/CT. In patients treated with curative intent, high ctDNA levels (>2.5 log hGE/mL) were associated with lower complete response (65% vs. 96%; $P < 0.004$), shorter progression-free survival (65% vs. 85%; $P = 0.038$), and overall survival (73% vs. 100%; $P = 0.007$) at 2 years, although it did not maintain prognostic value in multivariate analyses.

Conclusions: In a population-based prospective DLBCL series, cfDNA resulted as an alternative source to estimate tumor burden and to determine the tumor mutational profile and genetic classification, which have prognostic implications and may contribute to a future tailored treatment.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous entity from a biological and clinical standpoint. Two main molecular subtypes are recognized according to their cell-of-origin (COO): the germinal center and activated B-cell subtypes, with up to 15%–18% of cases being unclassifiable (1, 2). More recently, new genetic subtypes have been identified beyond COO, incorporating mutations, copy-number alterations (CNA), and structural variants, aimed at grouping patients based on common mechanisms of lymphomagenesis (3–5).

Genetic studies are usually performed in tumor tissue. However, in recent years, the interest for the detection and study of cell-free DNA (cfDNA) has provided a noninvasive tool for diagnosis, disease monitoring, clinical decision-making, and treatment selection in oncology (6). In the field of lymphoid neoplasms, some studies have highlighted the potential of cfDNA in determining the mutational profile of DLBCL, as well as the COO molecular subtype (7, 8), whereas others have focused on the prognostic utility of the amount of circulating tumor DNA (ctDNA), as a surrogate of tumor burden, compared with CT or PET imaging (9–11). Moreover, Kurtz and colleagues (12) showed that pretreatment ctDNA levels and molecular responses, either at diagnosis or relapse, were independent prognostic markers in DLBCL.

On the other hand, new biomarkers obtained at baseline PET/CT are under investigation, the total metabolic tumor volume (TMTV) and the total lesion glycolysis (TLG; ref. 13). High TMTV has been associated with a worse progression-free survival (PFS) and/or overall survival (OS) in DLBCL and other lymphoma subtypes (13–15).

The aim of this study was to prospectively study in a real-life setting the utility of plasma cfDNA to determine the mutational profile, assess

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Translational Relevance

Previous studies have highlighted the potential of cell-free DNA (cfDNA) to assess the mutational profile in diffuse large B-cell lymphoma (DLBCL). This population-based prospective study showed that cfDNA is a reliable source for DLBCL genotyping, allowing us to classify the cases into the recently described genetic subtypes, providing evidence to use cfDNA as a source for molecular classification. Also, baseline circulating tumor DNA (ctDNA) levels significantly correlated with clinical and volumetric PET/CT parameters of tumor burden. Moreover, high ctDNA levels (>2.5 log hGE/mL) were associated with lower complete response, shorter progression-free, and overall survival. To our knowledge, this is the first confirmation of this finding in a prospective single-center real-life series.

tumor burden, and estimate its impact on response and outcome of patients with DLBCL.

Materials and Methods

Patients

A total of 100 patients consecutively diagnosed with DLBCL according to the World Health Organization classification (1) were prospectively enrolled from September 2016 to March 2019. High-grade B-cell lymphoma not otherwise specified (NOS) and high-grade B-cell lymphoma with *MYC* and *BCL2* and/or *BCL6* rearrangements were also included, whereas primary mediastinal large B-cell lymphomas, posttransplant lymphoproliferative disorders or other immune-related lymphomas, and cases with a low-grade lymphoma component were excluded. Sufficient cfDNA to assess the mutational profile was obtained from 79 cases, which constituted the subjects of the study. Sample collection and processing are detailed in Supplementary Materials and Methods.

The main clinicobiological and evolutionary characteristics were recorded and analyzed as described previously (16). These variables included: (i) clinical data: age, gender, performance status (PS) according to the Eastern Cooperative Oncology Group (ECOG) scale, presence of B symptoms, and bulky disease (defined as a tumor diameter >7 cm); (ii) hematologic and biochemical parameters: white blood cell and lymphocyte counts, hemoglobin, serum lactate dehydrogenase (LDH), and β2-microglobulin levels; (iii) tumor burden data: nodal and extranodal involvement, number of extranodal involved sites, palpable splenomegaly, bone marrow infiltration, and Ann Arbor Stage; and (iv) the International Prognostic Index (IPI).

Staging was performed according to standard procedures, including PET/CT and unilateral bone marrow biopsy (17). All, but two patients, who died before starting therapy, were treated with chemoimmunotherapy, mostly ($N = 62$, 78%) R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone; **Table 1**). Responses were assessed by end-of-therapy PET/CT according to standard guidelines (18). The median follow-up for surviving patients was 23.5 months (range, 8.6–42 months). Patients provided written informed consent in accordance with the Declaration of Helsinki, and the study was approved by the institutional review board.

Histologic review

Histologic diagnosis, including morphology and IHC, was reviewed by O. Balagué, N. Castrejón de Anta, and E. Campo. The percentage of tumor infiltration and the expression of MYC, BCL2, BCL6, and p53 were semiquantitatively estimated. *MYC*, *BCL2*, and *BCL6* rearrangements were routinely assessed by FISH using both fusion and break-apart probes. COO assessment was performed by means of Lymph2Cx Assay (NanoString Technologies; Supplementary Materials and Methods).

Table 1. Main baseline features, treatment, and response of the 79 patients with DLBCL.

Characteristics	*N* (%)
Median age (range)	63 (20–94)
Male/female	42/37 (53/49)
Histology	
DLBCL, NOS	63 (80)
High-grade B-cell lymphoma, NOS	8 (10)
High-grade B-cell lymphoma with *MYC* and *BCL2* and/or *BCL6* rearrangements	8 (10)
COO classification[a]	
Germinal center B-cell	39 (59)
Activated B-cell	19 (29)
Unclassified	8 (12)
ECOG PS ≥ 2	29 (37)
B symptoms	31 (39)
Stage	
I/II	12/17 (15/22)
III/IV	7/43 (9/54)
Bone marrow infiltration[a]	20 (27)
Bulky mass (>7 cm)	21 (27)
LDH > normal	45 (57)
IPI	
Low risk	24 (30)
Low–intermediate risk	17 (21.5)
High–intermediate risk	17 (21.5)
High risk	21 (27)
PET/CT baseline parameters[b]	
Median TMTV (range)	207 (0–4,171)
Median TLG (range)	1,525 (0–26,295)
Treatment	
R-CHOP	62 (78)
Intensive chemoimmunotherapy	7 (9)
R-CVP/R-GEMOX	8 (10)
Died before starting treatment	2 (3)
Response to treatment	
Complete response	59 (75)
Partial response	4 (5)
Progressive disease	16 (20)

Abbreviations: R-CVP, rituximab, cyclophosphamide, vincristine, and prednisone; R-GEMOX, rituximab, gemcitabine, and oxaliplatin.
[a]The number of patients in whom the variable was available: bone marrow infiltration, 73; COO, 66.
[b]PET/CT was available in 63 cases.

Mutational profile

The mutational status of 112 recurrently mutated genes in B-cell lymphoma was examined by targeted next-generation sequencing (NGS; Supplementary Table S1). Libraries were performed with 15–30 ng of cfDNA and 150 ng of genomic DNA (gDNA) obtained from plasma and formalin-fixed, paraffin-embedded (FFPE) biopsy, respectively, using molecular-barcoded library adapters (ThruPLEX Tag-seq Kit, Takara) coupled with a custom hybridization capture–based method (SureSelect XT Target Enrichment System Capture strategy, Agilent Technologies Inc.) and sequenced in an MiSeq Instrument

(Illumina, 2 × 150 bp). Sequencing data have been deposited at the European Genome-Phenome Archive (http://www.ebi.ac.uk/ega/), which is hosted at the European Bioinformatics Institute, under accession number EGAS00001004733.

The bioinformatic analysis was performed using an updated version of our previously validated pipeline (19). Synonymous and intronic variants were removed. Because of the lack of matched germ line DNA, potential polymorphisms were filtered out on the basis of public databases and by using a three-step algorithm designed to predict the somatic origin of the mutations (Supplementary Materials and Methods).

Molecular groups classification

The LymphGen probabilistic classification tool was used to classify our DLBCL cases into the recently described genetics subtypes (5). To this aim, we used the mutations identified in 79 cfDNA samples as well as *BCL2* and *BCL6* translocations. Because the A53 subtype is defined primarily by CNA, this subtype was excluded from the LymphGen classification algorithm in our series.

ctDNA quantitation

ctDNA levels were reported as haploid genome equivalents per mL of plasma (hGE/mL), determined as the product of total cfDNA concentration (fluorometry by Qubit, Thermo Fisher Scientific) and the mean allele fraction of somatic mutations (Supplementary Materials and Methods). This value was expressed as a base-10 logarithm (log hGE/mL). We used the 2.5 hGE/mL threshold to classify patients into low or high ctDNA amount as published previously (12).

2[18F]Fluoro-2-deoxy-D-glucose–PET/CT parameters

TMTV was calculated by adding up the metabolic volumes of all nodal and extranodal lesions. TLG was calculated as the sum of the product of the metabolic volume of each local tumor based on its SUVmean. Quantitative analysis of TMTV was performed using the semiautomatic MIM software and supervised by X. Setoain, S. Rodríguez, M. Simó, and S. Casanueva-Eliceiry, with a fixed SUV > 2.5 thresholding method for segmentation. According to previous publications, the optimal cutoff to classify into low and high TMTV was established as 400 cm^3 (20, 21).

Statistical analysis

We used standard definitions for complete response (CR), PFS, and OS (18). χ^2 method was used for categorical variables and Student *t* test for continuous variables. Nonparametric tests were applied when necessary. Logistic regression was used to select the best variables predicting CR. Actuarial survival analysis was performed by the Kaplan–Meier method and differences were assessed by the log-rank test. The optimal cut-off point for TLG for PFS was determined by using the maximally selected rank statistics (maxstat R package). Multivariate Cox regression analysis was used to assess the independent prognostic impact of different variables in terms of PFS and OS. Only patients treated with curative intent were included in the prognostic analyses. $P < 0.05$ was considered statistically significant. Statistical analyses were carried out using R (version 3.6.2; R Foundation).

Results

Clinical features, treatment, and outcome of the patients

cfDNA could be obtained from 85 of the 100 (85%) patients prospectively diagnosed with DLBCL during the period of the study. The failure to obtain DNA was due to start of treatment prior to sample extraction ($n = 13$) and concomitant diagnosis of a second neoplasm ($n = 2$). Moreover, in six additional cases, the mutational profile could not be assessed because of the low amount of cfDNA ($n = 2$) or other technical issues ($n = 4$; Supplementary Fig. S1). Finally, the mutational landscape was assessed in 79 patients, whose main clinicobiological characteristics are listed in **Table 1**. Fifty-four percent of patients had stage IV disease, including 27% with bone marrow infiltration, and 48.5% showed high-intermediate- or high-risk IPI. After first-line treatment, 59 (75%) patients achieved a CR, 4 (5%) patients achieved a partial response, and 16 (20%) were refractory, including 7 early deaths. Among CR patients, 6 of 59 (7%) eventually relapsed at a median of 14 months from CR achievement (range, 10–20 months). Patients not included in the cfDNA analyses showed significant differences in terms of initial features (they were older, had a higher risk IPI, and a poorer PS) and outcome, with a lower CR rate and shorter PFS and OS (Supplementary Table S2).

Mutational profile assessed in cfDNA

The mean coverage of the cfDNA samples was 329 × (range, 91 ×–737 ×) with more than 80% of the target regions covered at >100 × in 75% of the samples. At least one mutation was detected in 69 of 79 cases (87.3%; Supplementary Table S3). The median number of mutations per sample was six (range, 0–41) and the mean allele fraction was 26% (range, 2.4%–58.6%; Supplementary Table S4). **Figure 1** shows the mutational profile of the series, restricted to genes mutated in more than 5% of the cases. The most frequently mutated genes were *KMT2D, BCL2, TP53, TNFRSF14, MYD88, CREBBP, EP300, SOCS1, MYC*, and *PIM1* (the complete list of mutations is detailed in Supplementary Table S3). The distribution according to COO, MYC and BCL2 double expression, *MYC, BCL2*, and *BCL6* rearrangement, and double-hit status is also shown in **Fig. 1**. Moreover, we were able to classify 43% of the cases according to the genetic subtypes proposed by Wright and colleagues (5), as detailed in Supplementary Fig. S2.

Validation of mutations in tissue biopsies

To validate the cfDNA mutational analysis, we performed targeted NGS in 45 paired FFPE samples. Mean percentage of tumor content was 80% (range, 30%–100%). The mean coverage in these samples was 509 × (range, 77 ×–1,050 ×). In 28 of 45 cases (62%), the majority (≥69%) of the mutations were observed both in the cfDNA and FFPE samples. In the remaining 17 cases, the number of mutations identified in cfDNA was lower than that observed in the paired FFPE samples. In 10 cases, additional mutations were only detected in cfDNA compared with gDNA (**Fig. 2**). The sensitivity of cfDNA to detect the mutations present in paired FFPE samples was 68% [95% confidence interval (CI), 56.2–78.7]. When we considered mutated genes instead of individual mutations, cfDNA genotyping was able to detect 71% of mutated genes. When taking into account only the mutations present with >20% allelic frequency in the FFPE samples, we detected up to 77% of the mutations in cfDNA. Of note, most cases in which mutations could not be detected in the cfDNA corresponded to localized stages [mutations were detected in 3/14 (21%) localized stages vs. 24/30 (80%) of disseminated stages; $P < 0.001$]. In addition, among the four cases in which cfDNA sample was obtained after excisional biopsy of the primary tumor, in two of these cases less than 50% of mutations observed in the paired FFPE sample could be detected in the cfDNA, while none of the mutations were detected in the remaining two cases.

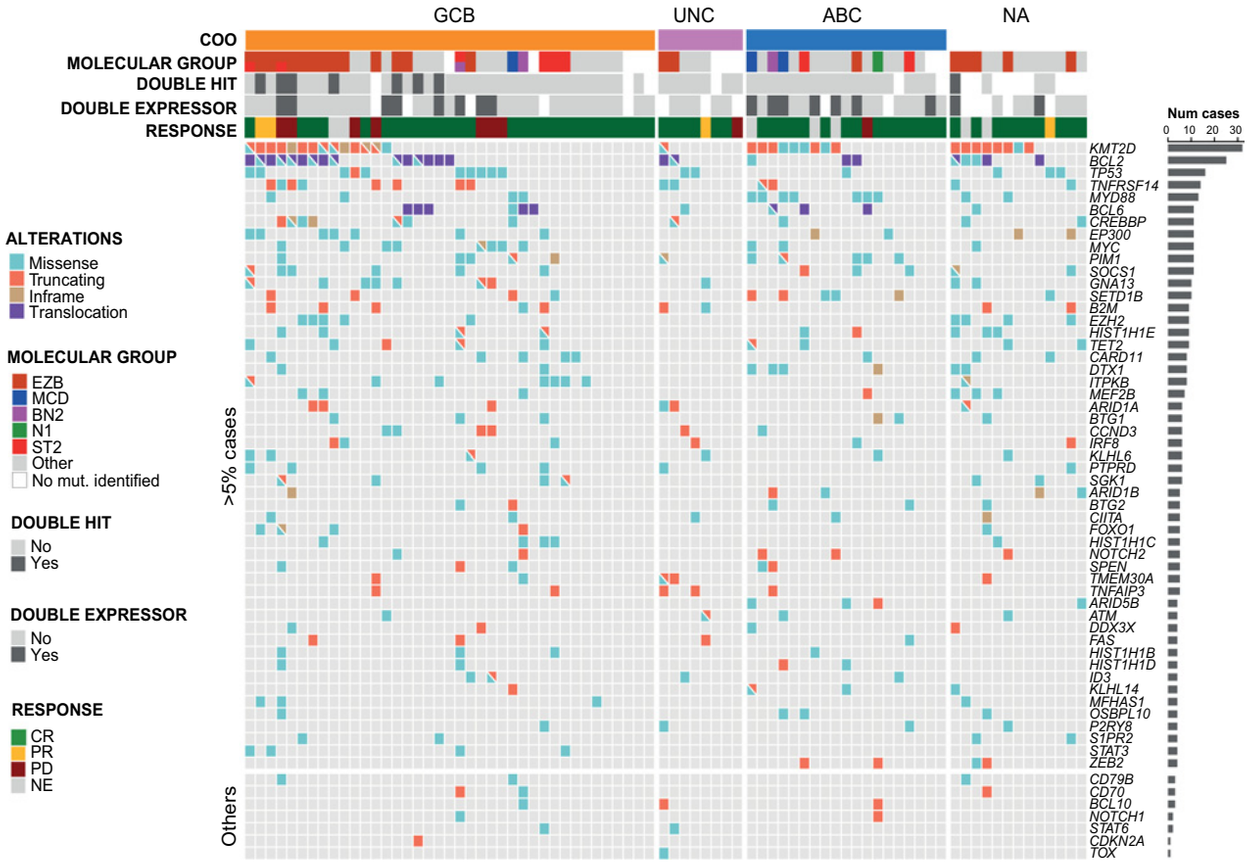

Figure 1.

Mutational profile in cfDNA of the 79 patients with DLBCL. Each column represents one tumor sample, and each row represents one gene. Molecular groups; MYC and BCL2 double expression; *MYC, BCL2*, and *BCL6* rearrangement; double-hit status; and response to first-line treatment are also shown. Cases are grouped by COO.

Tumor burden assessment

ctDNA

The median amount of ctDNA was 2.64 log hGE/mL (range, 1.29–4.27). Higher quantity of ctDNA significantly correlated with the presence of B symptoms, elevated LDH and β2-microglobulin serum levels, advanced Ann Arbor stage, and high-risk IPI ($P < 0.05$ in all cases; **Fig. 3A**; Supplementary Table S5). However, there was no significant correlation between the amount of ctDNA and the presence of bulky mass or primary extranodal disease. Of note, the number of detected mutations was not related to the amount of ctDNA (mean, 7.2 vs. 7.8 for low and high ctDNA, respectively).

PET/CT

Volumetric PET/CT determinations could be assessed in 63 cases. Median pretreatment TMTV was 207 cm^3 (range, 0–4,171 cm^3), whereas median TLG was 1,525 (range, 0–26,295). As expected, high TMTV and TLG correlated with bulky disease, presence of B symptoms, elevated LDH serum levels, β2-microglobulin, advanced stage, and IPI ($P < 0.05$ in all the cases; Supplementary Table S5). The ctDNA concentration significantly correlated with the TMTV ($R = 0.56$; $P < 0.001$) and TLG ($R = 0.43$; $P < 0.001$), confirming that ctDNA measurements are related to the lymphoma tumor burden (**Fig. 3B**). The optimal cutoff for PFS as determined by maxstat for TLG was 7,898.

Impact of initial variables, including mutational profile and tumor burden, on response and outcome

Initial variables predicting the achievement of a CR in the 69 patients treated with curative intent included poor ECOG PS, advanced stage (III–IV), elevated LDH and β2-microglobulin, and high-intermediate- or high-risk IPI (all $P < 0.05$; Supplementary Table S6). The following single mutations predicted a low CR rate: *SETD1B, CIITA*, and *FOXO1* ($P < 0.03$). The previously described genetic subtypes and the number of mutations did not predict CR. We also examined the impact of pretreatment ctDNA levels on outcome, using 2.5 log hGE/mL of ctDNA as threshold. Patients with high ctDNA levels had a significantly lower CR rate than those with low ctDNA levels (65% vs. 96%, respectively; $P < 0.004$). Patients with high TMTV and TLG also had a significantly lower CR rate (TMTV, 56% vs. 97%; $P < 0.001$ and TLG, 36% vs. 94%; $P < 0.001$). In a multivariate analysis, including IPI, ctDNA amount, and TMTV, only TMTV (HR, 0.56; $P = 0.009$) maintained its predictive value for CR achievement in the final model with 50 cases.

Clinical variables associated with a shorter PFS in the univariate analysis were: high serum LDH and β2-microglobulin, double expression of MYC and BCL2, advanced stage, and high-risk IPI (**Table 2**; **Fig. 4A**). Mutations of the following genes were related to a poor PFS: *CIITA, SETD1B, OSBPL10*, and *MYC* ($P < 0.05$). Patients with high ctDNA levels had a significantly inferior 24-

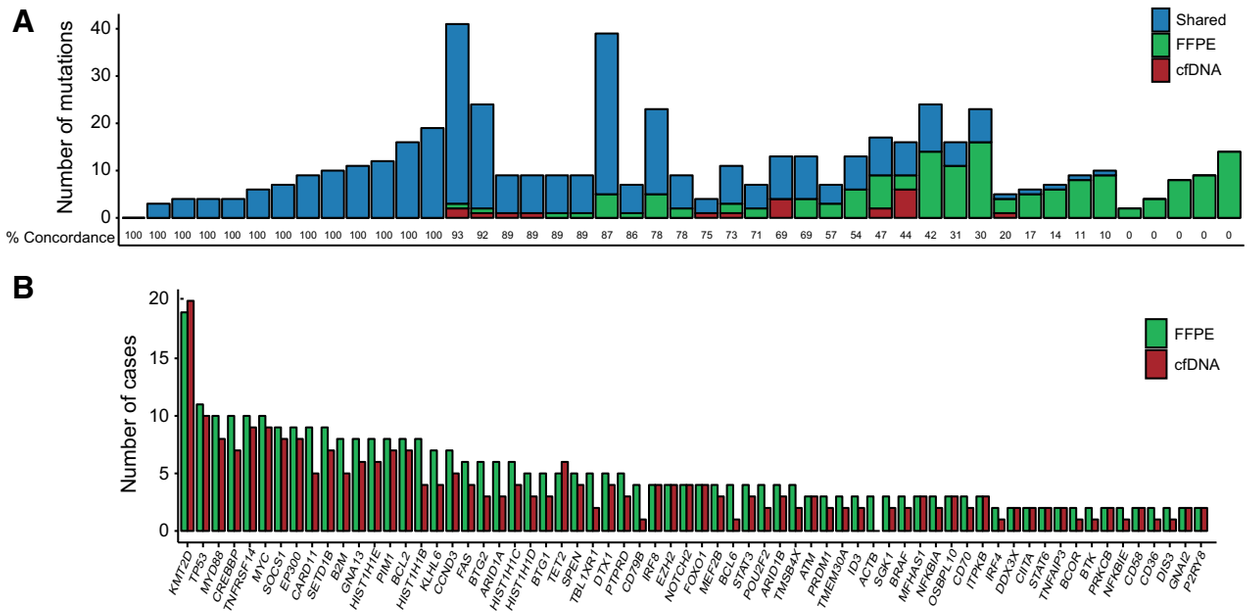

Figure 2.

Concordance between mutations detected in cfDNA and matched tumor gDNA. **A,** Number of mutations by case. Mutations are coded by color according to whether they were detected in both samples (blue), only in the FFPE samples (green), or in cfDNA (red). The percentage of concordance is shown for each case. **B,** Prevalence of somatic mutations detected by NGS in plasma and gDNA.

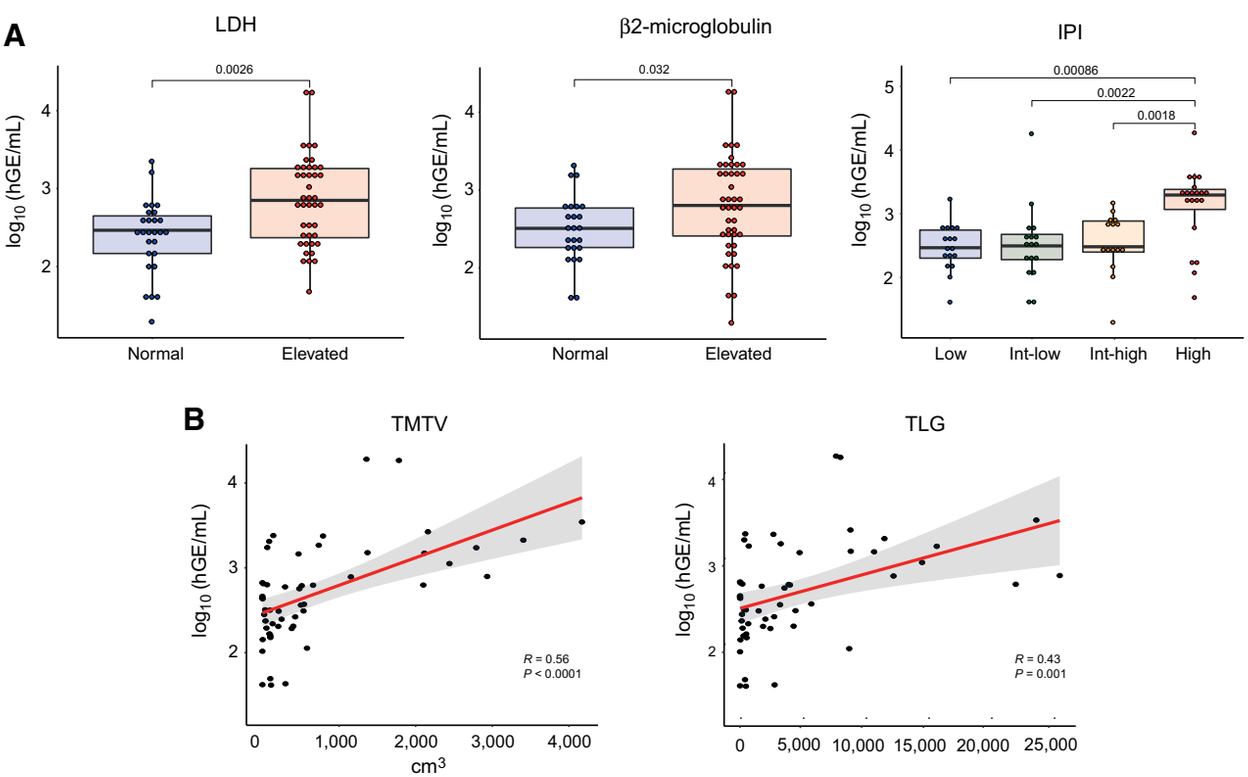

Figure 3.

Correlation between pretreatment ctDNA and tumor burden parameters. **A,** Box plot showing the relationship between pretreatment ctDNA levels and serum LDH, serum β2-microglobulin, and IPI. Each dot corresponds to one sample. **B,** ctDNA correlation with TMTV and TLG. Int, intermediate.

Table 2. CR rate, PFS, and OS according to parameters reflecting tumor burden in the 69 patients with DLBCL treated with curative intent.

Variable	*N*	CR (%)	2-year PFS	2-year OS
Bulky disease				
No	52	43 (83)	75	86
Yes	17	12 (71)	61	88
LDH				
Normal	30	29 (97)[a]	84[a]	97[a]
>ULN	39	26 (67)	63	79
β2-microglobulin				
Normal	30	30 (100)[a]	88[a]	100[a]
>ULN	39	24 (63)	61	76
IPI				
Low/intermediate-low	39	36 (92)[a]	83[a]	97[a]
Intermediate-high/high	30	19 (63)	60	73
ctDNA				
Low	25	24 (96)[a]	85[a]	100[a]
High	34	22 (65)	65	73
TMTV				
Low	35	34 (97)[a]	80[a]	94[a]
High	25	14 (56)	56	75
TLG				
Low	46	43 (94)[a]	81[a]	91
High	14	5 (36)	36	71

Abbreviation: ULN, upper limit of normal.
[a]$P < 0.05$.

month PFS than those with low levels (65 vs. 85%, respectively; $P = 0.038$; **Fig. 4B**). Higher TMTV and TLG predicted for a lower 24-month PFS (TMTV, 56% vs. 80%; $P = 0.012$ and TLG, 36% vs. 81%; $P < 0.001$; **Table 2**; **Fig. 4C**). A multivariate analysis was performed, including IPI, ctDNA (low vs. high), and TMTV (low vs. high). In the final model with 50 patients, only TMTV [HR, 3.32 (95% CI, 1.22–9.0); $P = 0.018$] retained independent prognostic value for PFS (Supplementary Fig. S3).

Nine patients eventually died during follow-up, with a 24-month OS of 86% (95% CI, 79%–93%). Initial variables predicting OS were older age (>60 years), poor ECOG PS, presence of B symptoms, extranodal involvement, advanced stage, high β2-microglobulin serum levels, higher IPI, double expression of MYC and BCL2, high ctDNA level, and TMTV (all $P < 0.05$). *PIM1, FOXO1, DTX1, CIITA, SETD1B, OSBPL10*, and *MYC* mutations were associated with poor OS ($P < 0.05$). Patients with high ctDNA levels had a significantly inferior 24-month OS than those with low levels (73% vs. 100%, respectively; $P = 0.007$; **Fig. 4B**). Higher TMTV, but not TLG, predicted for a poorer 24-month OS (TMTV, 75% vs. 94%; $P = 0.0478$ and TLG, 71% vs. 91%; $P = 0.071$; **Table 2**; **Fig. 4C**).

Discussion

Liquid biopsy, particularly cfDNA, has been increasingly used for a wide variety of applications in oncology, including diagnosis, prognosis, and the identification of therapeutic targets (6). In addition, ctDNA provided information regarding tumor burden with a good correlation with other clinical parameters and the metabolically active tumor mass as assessed by PET (12, 22).

We have conducted a population-based prospective study showing that cfDNA is a reliable source for DLBCL genotyping. Although the study was designed to enroll all cases consecutively diagnosed with DLBCL at a single institution, 15% of the potential candidates could not be included, mostly due to the start of treatment before sampling. Of note, these cases corresponded to a high-risk population, with poor initial features, low CR rates, and unfavorable outcome. Obviously, this represents a bias in an attempted "real-life" study. Nevertheless, it is likely that this bias is also present in previous studies based on retrospective series (9, 12).

One of the aims of this study was the assessment of ctDNA to objectively estimate tumor burden. We have confirmed that baseline ctDNA levels are significantly associated with well-described clinical parameters of tumor burden, including serum LDH and IPI, and also with β2-microglobulin level and advanced stage, which were not clearly associated in previous series (7, 11, 12). The prognostic value of PET has been demonstrated in different lymphoma subtypes, particularly by using quantitative assessment of TMTV and TLG (13). We have confirmed the prognostic value of these two factors in this cohort. Furthermore, a correlation between baseline ctDNA levels and TMTV was observed, indicating that ctDNA levels might be a surrogate for tumor burden. Kurtz and colleagues (12) showed that pretreatment ctDNA levels and molecular responses both after first-line and salvage therapy were independent prognostic markers. Using the same cutoff, we observed that patients with high ctDNA levels had significantly inferior 24-month PFS (65% vs. 85%; $P = 0.038$) and OS rates (73% vs. 100%; $P = 0.007$) than those with low levels. To our knowledge, this is the first confirmation of this finding in a larger prospective single-center series. Finally, the multivariate analysis showed that TMTV, but not ctDNA, retained independent prognostic impact on PFS. Although this is somewhat different from previous studies (12, 22), it is not surprising because both ctDNA and TMTV likely reflect the active mass of the tumor. Considering the relatively small number of cases included in this multivariate analysis, larger studies are needed to confirm the potential independent prognostic value of these highly correlated measurements and to further clarify the role of ctDNA in the clinical setting.

Median TMVT was lower than in previous series (20, 21). The median value is crucially dependent on the segmentation method, the patient population characteristics, and the efficacy of treatment. There is no agreement on the best method. We employed the SUV ≥2.5 method, which according to previous reports has the best interobserver agreement and is the easiest to apply (20). Mikhaeel and colleagues (21) analyzed the prognostic value of quantitative PET measurements, particularly metabolic tumor burden, in a retrospective study including 147 consecutive patients treated with R-CHOP at a single institution. They used an in-house software to automatically segment tumor volumes with SUV ≥ 2.5. Median TMTV of this series was 592 cm^3; however 40% of the patients had bulky disease at diagnosis and 68% of patients had stage III–IV.

Previous studies have shown that cfDNA could provide an accurate picture of the genetic landscape of lymphoprolipherative disorders. This is of great interest since the expansion of NGS has highlighted the importance of gene mutations and CNA beyond the COO and FISH alterations. In fact, new genetic classifications that incorporate such data have been recently proposed, with the objective of grouping the patients according to common mechanisms of lymphomagenesis susceptible of potential specific target therapies (3–5). Indeed, cfDNA might be useful not only at diagnosis when tissue biopsy is mandatory, but also at relapse when excision biopsy is frequently unavailable.

At least one mutation was detected in plasma in the majority (87%) of the cases. This finding is consistent with previous publications, in which the rate of detection varied from 63% to 85% (7, 11). We also aimed to determine the reliability of the technique by comparing the

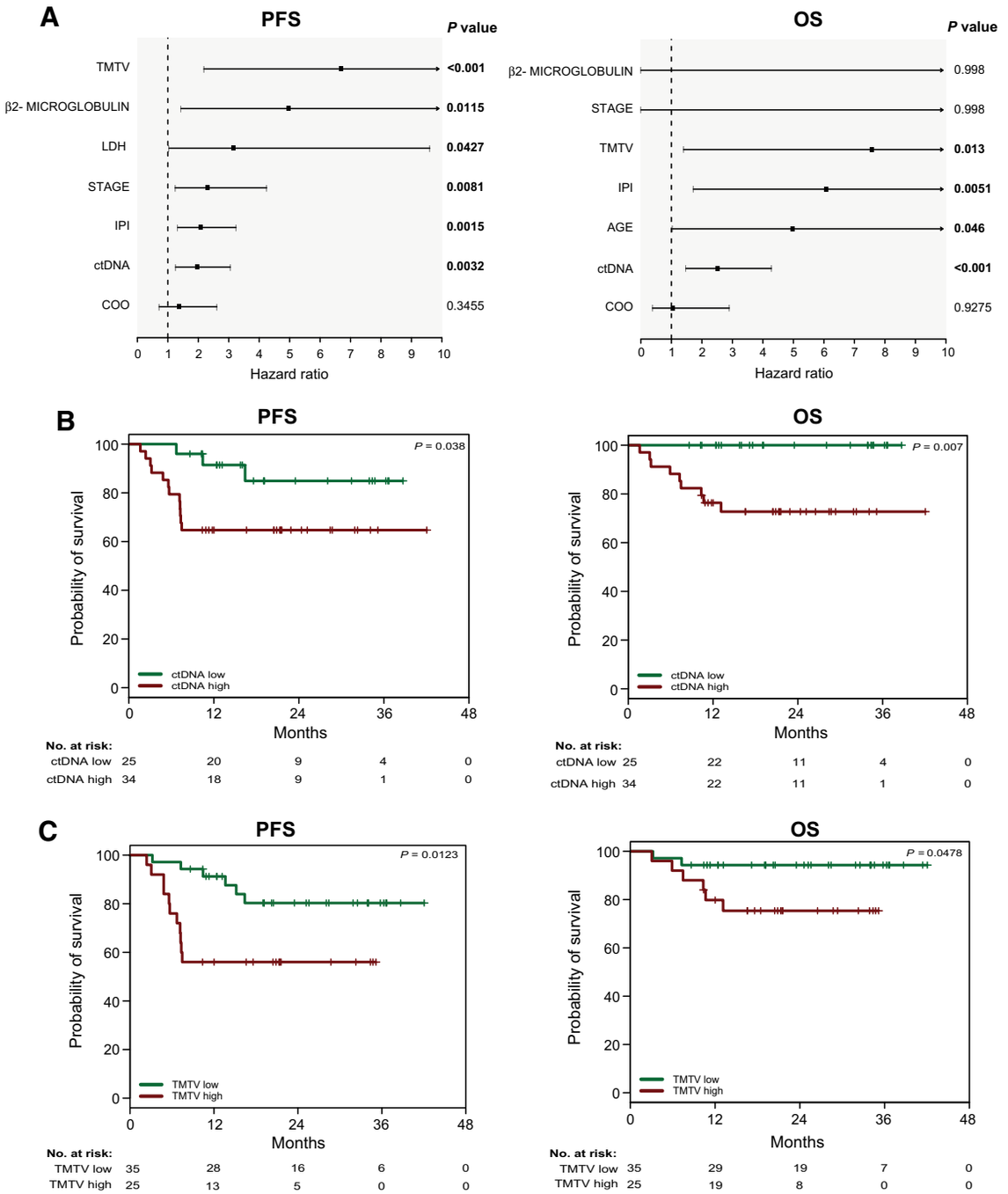

Figure 4.

Survival analysis of the 69 patients treated with curative intent. **A,** Results of the univariate analysis for PFS and OS. **B** and **C,** Kaplan–Meier estimates of PFS and OS according to pretreatment ctDNA levels and TMTV value.

information obtained from cfDNA with that from the tumor tissue. In our hands, the sensitivity of cfDNA to detect mutations present in the paired FFPE samples was 68%. This proportion increased to 71% when the number of mutated genes was taken into consideration, instead of the number of mutations. In a previous study, Rossi and colleagues (7) determined the basal genetic profile of DLBCL by ultra-deep targeted NGS of 50 diagnostic plasma samples. They were able to detect in plasma, 83% of the mutations seen in the tissue biopsy in 18 paired cases. This slight difference could be explained by the lower sequencing coverage of our series (mean depth, 329 × vs. >1,000 ×), together with a substantially higher number of tested genes ($N = 112$ vs. 59). In this regard, most of the biopsy mutations not detected in the cfDNA had a

low representation in the tissue suggesting that they could be present below the limit of detection in the cfDNA due to the low sequencing depth. In fact, when we considered only mutations with >20% allelic frequency in the tumor biopsy, we were able to detect up to 77% of the mutations in cfDNA. On the other hand, we observed that in localized stages or after excisional diagnostic procedures, the sensitivity of cfDNA to detect tumor mutations was significantly lower.

The mutational landscape described from cfDNA in our study was highly consistent with that previously published in different DLBCL series, including a different cohort from our institution (3, 4, 7, 23). It included genes with prognostic impact and related to targeted drugs, such as *TP53, MYD88, EZH2, NOTCH1, CD79B*, or *CREBBP* that could be relevant in the near future in the treatment of these patients. Note that we did not analyze CNA because of our NGS panel was not designed to capture regions effected by chromosomal alterations and the lack of baseline cfDNA samples to be used in the analyses. Recently, Wright and colleagues (5) proposed a new genetic system able to classify up to 63.1% of DLCBL cases (47.6% core cases, 9.8% extended cases, and 5.7% genetically composite cases). Applying this algorithm with the mutations identified in the cfDNA, we were able to classify 43% of our cases, providing evidence to use cfDNA as a source for molecular classification. The lower percentage of cases classified in our series could be explained by the limited number of genes studied together with the lack of information regarding CNA, which impaired the assessment of the entire classification proposed. This also might explain the fact that molecular classification had no significant impact on the response to treatment.

The identification of predictive biomarkers is an urgent need to allow a rational selection of the most effective therapies in the future clinical practice. It is proposed that the DLBCL genetic subtypes differ strikingly in their response to standard chemoimmunotherapy and may also respond differently to targeted therapies (5). In the last years, different agents have been combined with standard chemoimmunotherapy without an improvement in response or survival (24–26), although only COO classification was used. The incorporation of this molecular classification for treatment selection into the design of clinical trials, and eventually in the real-life setting, is a first step of improvement in the era of personalized medicine.

In summary, cfDNA was easily accessible and useful for estimating the tumor burden and tumor mutational profile in our prospective cohort of patients with DLBCL. Evaluating its relationship with the mutational burden or particular genetic profiles could provide decisive information to tailor therapeutic approaches.

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Authors' Contributions

A. Rivas-Delgado: Conceptualization, resources, data curation, formal analysis, funding acquisition, investigation, visualization, methodology, writing-original draft, project administration, writing-review and editing. **F. Nadeu:** Data curation, software, formal analysis, investigation, visualization, methodology, writing-original draft, writing-review and editing. **A. Enjuanes:** Conceptualization, formal analysis, supervision, validation, methodology, writing-original draft, writing-review and editing. **S. Casanueva-Eliceiry:** Data curation, software, investigation, methodology. **P. Mozas:** Data curation, investigation, writing-review and editing. **L. Magnano:** Resources, investigation, methodology, writing-review and editing. **N. Castrejón de Anta:** Resources, investigation, methodology. **J. Rovira:** Resources, data curation. **I. Dlouhy:** Resources, data curation. **S. Martín:** Resources, investigation. **M. Osuna:** Investigation. **S. Rodríguez:** Resources, methodology. **M. Simó:** Resources, formal analysis. **M. Pinyol:** Formal analysis, investigation, methodology. **T. Baumann:** Resources, data curation, methodology, writing-review and editing. **S. Beà:** Resources, data curation, methodology, writing-review and editing. **O. Balagué:** Validation, methodology. **J. Delgado:** Resources, methodology, writing-review and editing. **N. Villamor:** Supervision, visualization, methodology, writing-review and editing. **X. Setoain:** Data curation, software, validation, writing-review and editing. **E. Campo:** Supervision, funding acquisition, methodology, writing-review and editing. **E. Giné:** Conceptualization, resources, data curation, supervision, funding acquisition, visualization, methodology, writing-original draft, writing-review and editing. **A. López-Guillermo:** Conceptualization, resources, supervision, funding acquisition, visualization, writing-original draft, writing-review and editing.

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