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Cell-free DNA sequencing as a potential screening tool for phase I targeted treatment in refractory/relapse diffuse large B-cell lymphoma

Diffuse large B-cell lymphoma (DLBCL) represents the most common subtype of non-Hodgkin lymphoma (NHL).¹ With standard of care first line treatment, namely rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone (R-CHOP), 35% of patients will relapse or present a refractory disease (rrDLBCL).² For these patients, salvage options include high-dose chemotherapy, chimeric antigen receptor T cells and at advanced stage (≥ 3 lines), novel approaches with targeted agents. Given the low number of treatment options approved for such patients,³ clinical trials evaluating new drugs can provide access to more treatment options. The need for molecular orientation before inclusion in clinical trials and targeted treatment strategies is increasingly highlighted in the solid tumor and lymphoma fields.⁴ In order to do so, the ability to provide molecular characterization at the time of relapse, and during a period compatible with the aggressiveness of the disease, is mandatory. We previously reported on the feasibility of a “real-time” targeted screening platform based on tissue mutations analysis.⁵ However, this screening process might be associated with failure due to biopsy-related technical limits and/or legitimate patient refusal to undergo another biopsy. Using cell-free tumoral DNA (ctDNA) to provide mutation profiling would increase the rate of molecularly-oriented inclusion.⁶ In this study, we show that using a targeted panel to characterize the potential oncogenic driver in rrDLBCL for a molecularly-oriented treatment, ctDNA-based sequencing could identify 80% of the tumor variants, with a high sensitivity and an excellent coefficient of concordance per gene, providing here a proof-of-concept for molecular orientation based on ctDNA only, in rrDLBCL.

In this study, a real-life series of 53 rrDLBCL patients for which a targeted molecular characterization was performed at time of relapse, both in the tumor and in ctDNA is presented. All patients signed a written informed consent prior to tumor biopsy, which was consistently performed before ctDNA sampling. Eight to 10 mL of plasma per patient were isolated from blood sampled into EDTA tubes (centrifuged within 3 hours after drawing) and frozen before subsequent ctDNA extraction (using Maxwell-RSC Instrument and RSC-LV kit, Promega). Multiplex polymerase chain reaction was performed on 5-10 ng DNA using a customized 152 kb-panel (IAD137284) covering exonic regions of 44 genes.⁵ The 114 bp libraries were prepared according to the manufacturer’s recommendation

for Ion AmpliSeq deep-targeted sequencing on Ion-S5 System (LifeTechnology). A mean sequencing depth of 3,900 \times was achieved for tumoral biopsies and 3,500 \times for ctDNA samples. Variant calling of GRCh37/hg19-aligned reads were performed using an institutional pipeline aggregating results from three variant callers (IonTorrent_Suite, GATK HaplotypeCaller, FreeBayes), run with low-stringency default setting parameters: minimum base_calling_score=4, min relative_reads_quality=11, maximum strand-bias=0.95, min position_depth=40 \times , min either_strand_mutated_reads=2 \times , minimum variant allele frequency (VAF) =0.001. For subsequent analysis, ctDNA and tumor biopsy sequencing results were then filtered out based on VAF threshold (0.1% for ctDNA variants, and 1% for tumor biopsy variants [TB variants]); a minimum number of mutated reads of 4 \times for ctDNA variants, and 6 \times for TB variants; a minimum depth of 300 \times for ctDNA variants and 100 \times for TB variants; a strand-bias >80% (or >70% considering insertion/deletion polymorphism [indel]). Remaining called variants were validated thanks to integrative genomics viewer (IGV) visualization to (i) exclude artifacts according to misestimated strand-bias, context-dependent errors (homopolymers/repeated sequences) or read-relative positional errors (i.e., focusing on amplicon’s end artifact), and to (ii) evaluate background error noise (considering occurrence of other single nucleotide variants [SNV]/indel than the variant call) allowing true low allele frequency (AF) ctDNA mutations to be considered. CD3⁺-sorted lymphocytes germline DNA were used as a control to filter out patient-specific polymorphisms for n=25 patients. For the other patients, known polymorphisms and variants described in general population at frequency >0.5% (according to 1000Genomes Project or NHLBI-GoExome Sequencing Project public databases) were excluded, as well as remaining variants classified as “benign” or “likely benign” using VarSome online resource. The TB variants detection was considered the gold standard to perform a comparison with ctDNA variants detection. Cohen's κ coefficient⁷ was calculated per gene to analyze the concordance between both methods and interpreted with the Landis-Koch scale.⁸

A total of 53 rrDLBCL patients were included in the study. The median age at the time of tumor biopsy was 68 years. The vast majority of the patients, at the time of tumor biopsy, were found to have a disseminated disease (70% of stage 3-4), lactate dehydrogenase (LDH) upper the normal

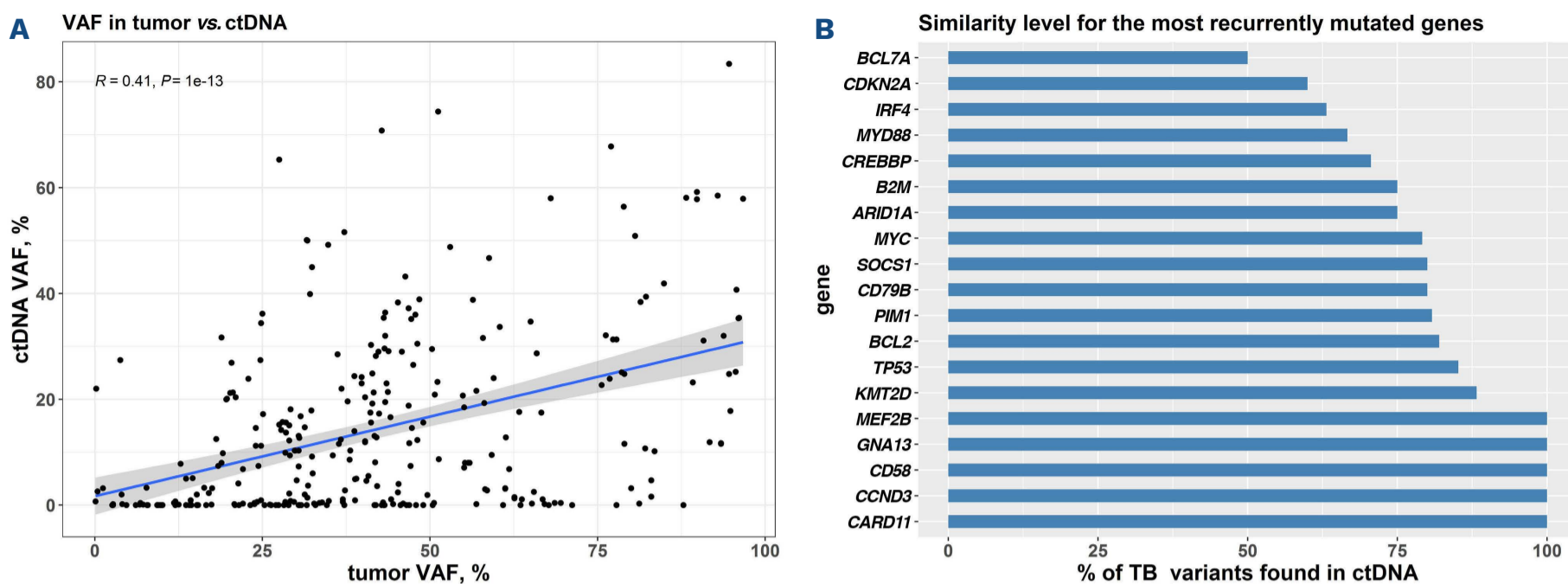


Figure 1. Detection of tumor mutations in cell-free tumoral DNA. (A) Correlation between variant allele fraction (VAF) as assessed in cell-free tumoral DNA (ctDNA) sequencing or genomic DNA sequencing extracted from tumor biopsy (TB), and (B) level of similarity within most recurrently mutated genes, calculated for each gene as the percentage of TB variants found in ctDNA.

limit (60%) and received in median two prior lines of therapies (range, 1-10) (*Online Supplementary Table S1*). The median time between tumor biopsy and ctDNA sampling was 24 days (range, 0-60). The turnaround time for ctDNA analysis was 7 to 10 days. Of note, in this real-life study, among the 53 patients, 28 received a short course of treatment between TB and ctDNA analysis (13 patients received a single short course/cycle of chemotherapy-based salvage and 15 an oral treatment). These patients had higher LDH levels than the others, however similar clinical characteristics overall. A total of 300 TB variants, within 34 genes, were found in the tumor, mean six per patient (median 6; range, 0-15), 241 (80%) were also present in ctDNA (ctDNA variants) (mean 4.8; median 5; range, 0-13), and eight variants were present in ctDNA only. Three patients were not mutated for any 44 targeted genes in both tumor biopsy and cell-free DNA (cfDNA). The incidence of genomic abnormalities is in line with previous reports in rDLBCL^{9,10}.

Importantly, the TB variants VAF and ctDNA variants VAF were correlated (Figure 1A; $R=0.41$, $P<0.001$; *Online Supplementary Figure S1A* patient level). Furthermore, the ability to detect the TB variant in ctDNA was significantly correlated with TB variant VAF (Student *t*-test: $P<0.001$) (*Online Supplementary Figure S1B*).

Overall, of the 50 patients with at least one mutation detected in the tumor, mutation profiles were mostly similar between the two methods (mean level of similarity: 81% of TB variants per patients detected in ctDNA). Importantly, there was no significant distinction between the percentage of TB variants found in ctDNA in patients that received a short course of treatment and those that did not. Indeed, of the 25 untreated patients, 84% (0-100%)

of TB variants were detected *versus* 78% patients (0-100%) of the treated patients ($P=0.54$). There was also no significant correlation between the number of days elapsed between tumor biopsy and ctDNA sampling and the percentage of TB variants detected in ctDNA ($P=0.12$). Bulky disease was the only factor significantly associated with a higher percentage of TB variant detected in ctDNA for each patient ($P=0.005$) whereas there was only a non-significant trend for higher LDH and Ann Arbor stage ($P=0.26$, $P=0.21$, respectively) and no correlation with the number of lines of previous therapies ($P=0.8$).

In mean, for each mutated gene, 78% of the TB variants were found in ctDNA (median 81%, range, 0-100%). When looking at the most recurrently mutated genes (in more than 8% of the patients, or top-20 recurrently mutated genes in this cohort), the percentage of TB variants found in ctDNA was always greater than 50% (mean 81%, range, 50-100%) (Figure 1B). Within this list of recurrently mutated genes, *BCL7A* had the lowest similarity level. Furthermore, the incidence of top-20 mutations was fully comparable between tumor biopsy and ctDNA (Fisher's test: $P>0.3$) (as shown in the co-oncoplot, *Online Supplementary Figure S1C*).

In order to further validate ctDNA sequencing as a good alternative to tumor biopsy sequencing, we assessed, within the 53 patients, the κ coefficient of concordance between the two techniques and the sensitivity/specificity (per gene) of variants detection in ctDNA, taking the tumor sequencing as reference. For this analysis, 308 variants (241 present in both compartments, 59 in tumor biopsy only, 8 in ctDNA only) were considered. As defined by the Landis-Koch⁸ scale, the concordance was excellent or good in 30 of 34 mutated genes (88%), medium/weak in

Table 1. Concordance between tumor biopsy and cell-free tumoral DNA variants detection per gene.

Gene	False positive N	False negative N	True positive N	Cohen's κ coefficient ⁷	Interpretation according to the Landis-Koch scale ⁸
<i>ARID1A</i>	1	1	3	73%	good
<i>ATM</i>	0	1	1	66%	good
<i>B2M</i>	0	3	9	82%	excellent
<i>BCL2</i>	0	9	41	34%	weak
<i>BCL7A</i>	0	2	2	65%	good
<i>CARD11</i>	0	0	7	100%	excellent
<i>CCND3</i>	0	0	5	100%	excellent
<i>CD58</i>	0	0	5	100%	excellent
<i>CD79B</i>	0	1	4	88%	excellent
<i>CDKN2A</i>	0	2	3	73%	good
<i>CIITA</i>	0	0	2	100%	excellent
<i>CREBBP</i>	0	5	12	77%	good
<i>CXCR4</i>	0	0	2	100%	excellent
<i>EED</i>	0	0	1	100%	excellent
<i>EP300</i>	1	1	2	65%	good
<i>EZH2</i>	1	0	3	85%	excellent
<i>FOXO1</i>	0	0	4	100%	excellent
<i>GNA13</i>	0	0	7	100%	excellent
<i>IRF4</i>	0	7	12	69%	good
<i>KMT2D</i>	0	2	15	91%	excellent
<i>MEF2B</i>	0	0	6	100%	excellent
<i>MYC</i>	1	5	19	77%	good
<i>MYD88</i>	0	2	4	78%	good
<i>NOTCH1</i>	0	0	2	100%	excellent
<i>PIM1</i>	0	5	21	81%	excellent
<i>PRDM1</i>	0	1	0	0%	bad
<i>PTPN1</i>	0	0	1	100%	excellent
<i>SF3B1</i>	0	1	0	0%	bad
<i>SOCS1</i>	0	3	12	85%	excellent
<i>TCF3</i>	0	0	1	100%	excellent
<i>TNFAIP3</i>	0	1	3	85%	excellent
<i>TNFRSF14</i>	0	1	8	93%	excellent
<i>TP53</i>	4	4	23	70%	good
<i>XPO1</i>	0	2	1	49%	medium

True positive: number of tumor biopsy tumor biopsy (TB) variants also found in cell-free tumoral DNA (ctDNA); false negative: number of TB variants not found in ctDNA; false positive: number of variants found in ctDNA but not reported in biopsy. The Cohen's κ concordance coefficient was interpreted with the Landis-Koch scale⁸: concordance is excellent if coefficient is in between: 1-0.81; concordance is good if coefficient is in between: 0.81-0.61; concordance is medium if coefficient is in between: 0.60-0.41; concordance is weak if coefficient is in between: 0.40-0.21; concordance is bad if coefficient is in between: 0.20-0.00.

two (6%) genes (*XPO1*-E571K and *BCL2*) and bad in two genes being mutated only once (*PRDM1*, *SF3B1*), potentially explaining this result (Table 1). The mean and median sensitivity of ctDNA detection per gene was 83% and 84% respectively (range, 33-100%), and specificity 99% and 100% (range, 98-100%) (Table 2). The sensitivity of ctDNA detec-

tion reported here is in line or even slightly better compared to the literature data published so far,^{10,11} reporting on smaller cohort of patients at time of diagnosis.¹¹ Finally, in this real-life cohort, 18% of patients with a molecular alteration could be molecularly oriented for a targeted treatment.⁵ This is in line with other studies in solid

Table 2. Sensitivity and specificity assessment per gene.

Gene	Sensitivity, %	Specificity, %	PPV, %	NPV, %
<i>ARID1A</i>	75	98	75	98
<i>ATM</i>	50	100	100	98
<i>B2M</i>	75	100	100	93
<i>BCL2</i>	82	100	100	25
<i>BCL7A</i>	50	100	100	96
<i>CARD11</i>	100	100	100	100
<i>CCND3</i>	100	100	100	100
<i>CD58</i>	100	100	100	100
<i>CD79B</i>	80	100	100	98
<i>CDKN2A</i>	60	100	100	96
<i>CIITA</i>	100	100	100	100
<i>CREBBP</i>	71	100	100	88
<i>CXCR4</i>	100	100	100	100
<i>EED</i>	100	100	100	100
<i>EP300</i>	67	98	67	98
<i>EZH2</i>	100	98	75	100
<i>FOXO1</i>	100	100	100	100
<i>GNA13</i>	100	100	100	100
<i>IRF4</i>	63	100	100	83
<i>KMT2D</i>	88	100	100	95
<i>MEF2B</i>	100	100	100	100
<i>MYC</i>	79	97	95	85
<i>MYD88</i>	67	100	100	96
<i>NOTCH1</i>	100	100	100	100
<i>PIM1</i>	81	100	100	84
<i>PRDM1</i>	na	100	na	98
<i>PTPN1</i>	100	100	100	100
<i>SF3B1</i>	na	100	na	98
<i>SOCS1</i>	80	100	100	93
<i>TCF3</i>	100	100	100	100
<i>TNFAIP3</i>	75	100	100	98
<i>TNFRSF14</i>	89	100	100	98
<i>TP53</i>	85	85	85	85
<i>XPO1</i>	33	100	100	96
Range	33-100	98-100		

PPV: positive predictive value; NPV: negative predictive value; na: non-calculable values (no positive test).

tumors,⁴ suggesting that molecular orientation remains a work in progress and is highly dependent on availability of clinical trials at time of inclusion. Our analysis has some limitations, including the time difference between tissue and plasma collection, and receipt of intervening treatment for some patients, even though these two parameters were not correlated with the ability to detect ctDNA mutations.

In conclusion, our study shows that using a targeted panel to characterize the potential oncogenic driver in rrDLBCL for a molecularly-oriented treatment, ctDNA-based se-

quencing could identify 80% of the tumor variants, with a high sensitivity and an excellent-to-good κ coefficient of concordance per gene. Importantly, the number of lines of previous therapy as well as the number of days between tumor biopsy and ctDNA sampling had no impact on the level of similarity between the two techniques, whereas the VAF or tumor bulk were associated with a greater similarity, by patient. All in all, this technology seems appropriate in routine to screen rrDLBCL patients for inclusion in clinical trials with a molecular orientation process.

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Disclosures

No conflicts of interest to disclose.

Contributions

CQ, AT, J-MM, PD, VC-C, VR and CS developed the concept and designed the study; CQ, AT, J-MM, HL, AD, JL, JR, DG, PD, VV, CM, VR and CS acquired data; CQ, AT, AP, HL, AA, VR and CS analyzed and interpreted data. All authors reviewed and approved the manuscript.