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 mL10 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× was achieved for tumoral biopsies and 3,500× 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×, min either_strand_mutated_reads=2×, 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× for ctDNA variants, and 6× for TB variants; a minimum depth of 300× for ctDNA variants and 100× 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-GO-Exome 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 coefficient[7] 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

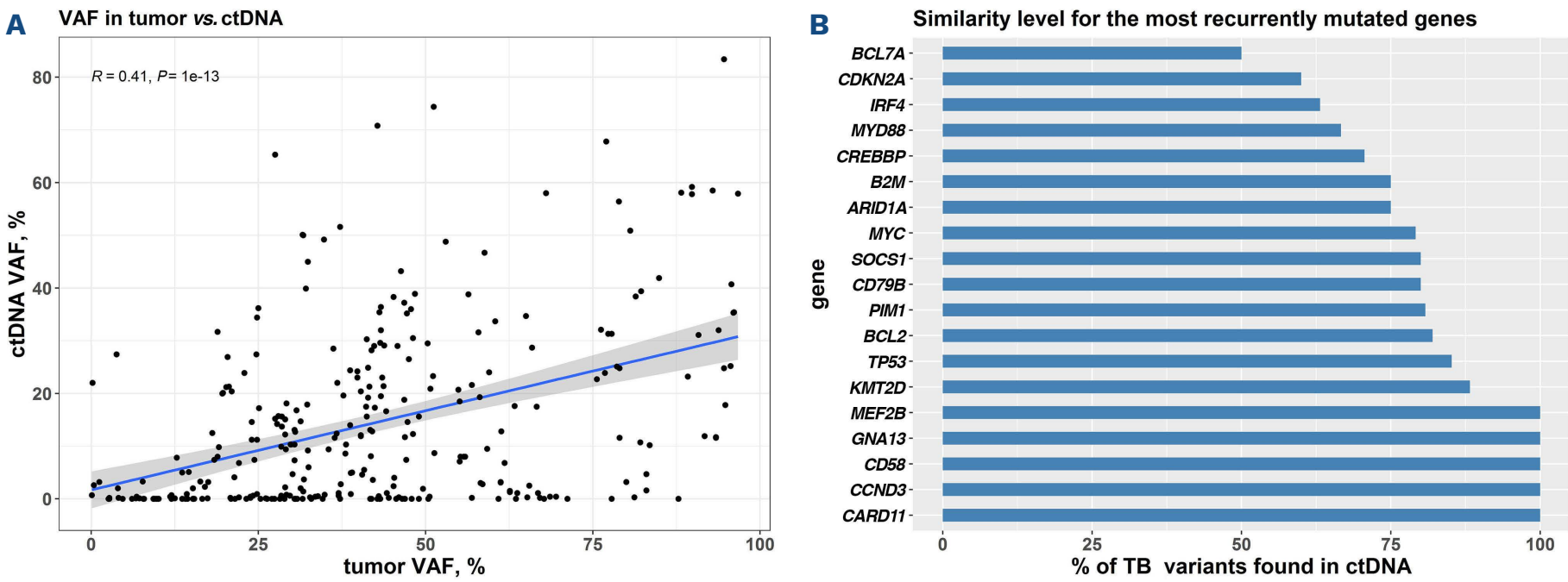

Figure 1. Detection of tumor mutations in cell-free tumoral DNA. (A) Correlation between variant allele fraction (VAF) as assessed in cell-free tumoral DNA (ctDNA) sequencing or genomic DNA sequencing extracted from tumor biopsy (TB), and (B) level of similarity within most recurrently mutated genes, 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 rrDLBCL[9,10].

Importantly, the TB variants VAF and ctDNA variants VAF were correlated (Figure1A; 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-Koch[8] 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 K coefficient[7]	Interpretation according to the Landis-Koch scale[8]
ARID1A	1	1	3	73%	good
ATM	0	1	1	66%	good
B2M	0	3	9	82%	excellent
BCL2	0	9	41	34%	weak
BCL7A	0	2	2	65%	good
CARD11	0	0	7	100%	excellent
CCND3	0	0	5	100%	excellent
CD58	0	0	5	100%	excellent
CD79B	0	1	4	88%	excellent
CDKN2A	0	2	3	73%	good
CIITA	0	0	2	100%	excellent
CREBBP	0	5	12	77%	good
CXCR4	0	0	2	100%	excellent
EED	0	0	1	100%	excellent
EP300	1	1	2	65%	good
EZH2	1	0	3	85%	excellent
FOXO1	0	0	4	100%	excellent
GNA13	0	0	7	100%	excellent
IRF4	0	7	12	69%	good
KMT2D	0	2	15	91%	excellent
MEF2B	0	0	6	100%	excellent
MYC	1	5	19	77%	good
MYD88	0	2	4	78%	good
NOTCH1	0	0	2	100%	excellent
PIM1	0	5	21	81%	excellent
PRDM1	0	1	0	0%	bad
PTPN1	0	0	1	100%	excellent
SF3B1	0	1	0	0%	bad
SOCS1	0	3	12	85%	excellent
TCF3	0	0	1	100%	excellent
TNFAIP3	0	1	3	85%	excellent
TNFRSF14	0	1	8	93%	excellent
TP53	4	4	23	70%	good
XPO1	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 Cohens's κ concordance coefficient was interpreted with the Landis-Koch scale[8]: 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%) (Table2). The sensitivity of ctDNA detection 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, %
ARID1A	75	98	75	98
ATM	50	100	100	98
B2M	75	100	100	93
BCL2	82	100	100	25
BCL7A	50	100	100	96
CARD11	100	100	100	100
CCND3	100	100	100	100
CD58	100	100	100	100
CD79B	80	100	100	98
CDKN2A	60	100	100	96
CIITA	100	100	100	100
CREBBP	71	100	100	88
CXCR4	100	100	100	100
EED	100	100	100	100
EP300	67	98	67	98
EZH2	100	98	75	100
FOXO1	100	100	100	100
GNA13	100	100	100	100
IRF4	63	100	100	83
KMT2D	88	100	100	95
MEF2B	100	100	100	100
MYC	79	97	95	85
MYD88	67	100	100	96
NOTCH1	100	100	100	100
PIM1	81	100	100	84
PRDM1	na	100	na	98
PTPN1	100	100	100	100
SF3B1	na	100	na	98
SOCS1	80	100	100	93
TCF3	100	100	100	100
TNFAIP3	75	100	100	98
TNFRSF14	89	100	100	98
TP53	85	85	85	85
XPO1	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 sequencing 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.

Authors

Cyril Quivoron,[1,2*] Anthony Tarabay,[3*] Jean-Marie Michot,[3,4*] Arnaud Pagès,[5,6] Hélène Lecourt,[1] Anne Aupérin,[5] Alina Danu,[3] Julien Lazarovici,[3] Julien Rossignol,[3] David Ghez,[3] Peggy Dartigues,[7] Véronique Vergé,[7] Christophe Massard,[4] Valérie Camara-Clayette,[1,8] Vincent Ribrag[1,2,3,4] and Clémentine Sarkozy[2,4]

[1]Hematology Translational Research Laboratory, AMMICa, INSERM US23 / CNRS UAR 3655, Gustave Roussy; [2]INSERM U1170, Gustave Roussy; [3]Hematology Department, Gustave Roussy; [4]DITEP, Gustave Roussy; [5]Department of Biostatistics and Epidemiology, Gustave Roussy; [6]INSERM U1018, Gustave Roussy; [7]Department of Medical Biology and Pathology, Gustave Roussy and [8]Plateforme de Recherche Gustave Roussy, Centre de Ressources Biologiques, UMS AMMICa, INSERM US23 / CNRS UMS 3655, Villejuif, France
**CQ, AT and JMM contributed equally as co-first authors.*

Correspondence:
C. SARKOZY - clementine.sarkozy@gustaveroussy.fr

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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.

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