Somatic mutations of cell-free circulating DNA detected by next-generation sequencing reflect the genetic changes in both germinal center B-cell-like and activated B-cell-like diffuse large B-cell lymphomas at the time of diagnosis

Diffuse large B-cell lymphoma (DLBCL) is the most common form of lymphoma, accounting for 30-40% of newly diagnosed cases of non-Hodgkin lymphomas. The molecular heterogeneity of DLBCL has been deciphered by gene expression profiling, and DLBCL have been divided into three main molecular subtypes: the germinal center B-cell-like (GCB) subtype, the activated B-cell-like (ABC) subtype, and the primary mediastinal B-cell lymphoma subtype with distinct clinical outcomes and responses to immunochemotherapy. Next-generation sequencing (NGS) technologies, which allow for massive, parallel, high-throughput DNA sequencing, have emerged over the past decade and have provided new insights into the genomic characterization of DLBCL. Recurrent single nucleotide variants (SNV) are now well defined and provide new therapeutic opportunities for the three molecular subtypes. The SNV target genes play a crucial role in several pathways, including B-cell receptor signaling (*CD79A/CD79B*), NFκ-B (*CARD11*), Toll-like receptor signaling (*MYD88*), immunity (*CD58, TNFSRF14, B2M*), cell cycle/apoptosis (*TP53, BCL2*) and epigenetic regulation (*EZH2,CREBBP, MLL2*).[1,2]

Recently, whole exome sequencing in breast cancer has shown that mutations observed in the tumor could also be detected in circulating, cell-free DNA (cfDNA) and could be used to detect genetic changes during treatments and relapse, defining the concept of "liquid biopsy".[3] In DLBCL, whereas tumor circulating cells or leukemic phase are not usually detectable, clonotypic sequences have been constantly detected in cfDNA extracted from serum/plasma or peripheral blood mononuclear cells.[4-8]

In this study we sought to determine, by routinely applicable NGS technology, whether the pattern of acquired SNV observed in tumor DNA could also be detected in cfDNA in DLBCL patients at the time of diagnosis. For this purpose, we analyzed 12 DLBCL cases with available matched tumor DNA and plasma collected at the time of diagnosis. Patients harboring typical GCB/ABC-related mutations targeting CD79A/B, *EZH2, CARD11* or *MYD88* genes, previously identified by the Sanger method, were selected.[9] This study was approved by the regional ethical committee (numbered as CPP N°01/006/2014).

The main clinical features of the patients are summarized in Table 1. None of the selected cases harbored detectable circulating lymphoma cells by routine blood smear examination. Of note, no peripheral blood cytometry was performed, in accordance with our center's initial staging procedures for DLBCL patients. Tumor DNA was extracted from frozen lymph node samples by standard methods. cfDNA was extracted from archived EDTA-anticoagulated plasma aliquots (1 mL) stored at -80°C using the QIAamp® Circulating Nucleic Acid Kit (Qiagen) (with the QIAvac 24 Plus vacuum manifold, following the manufacturer's instructions), and concentrations were measured using a fluorometric assay (Qubit® dsDNA HS Assay Kit, Life Technologies). The mean cfDNA concentration in plasma was 1.65 ng/μL (range, 0.46-11.2 ng/μL) (Table 1). The cell-of-origin signature was determined by cDNA-mediated annealing, selection, extension, and ligation technology based on the expression of 19 genes, as previously reported.[10] Among the 12 cases analyzed, five belonged to the ABC subgroup, six to the GCB subgroup and one case was unclassified (Table 1).

Tumor DNA was sequenced using an Ion Torrent Personal Genome Machine (Life Technologies). Ten

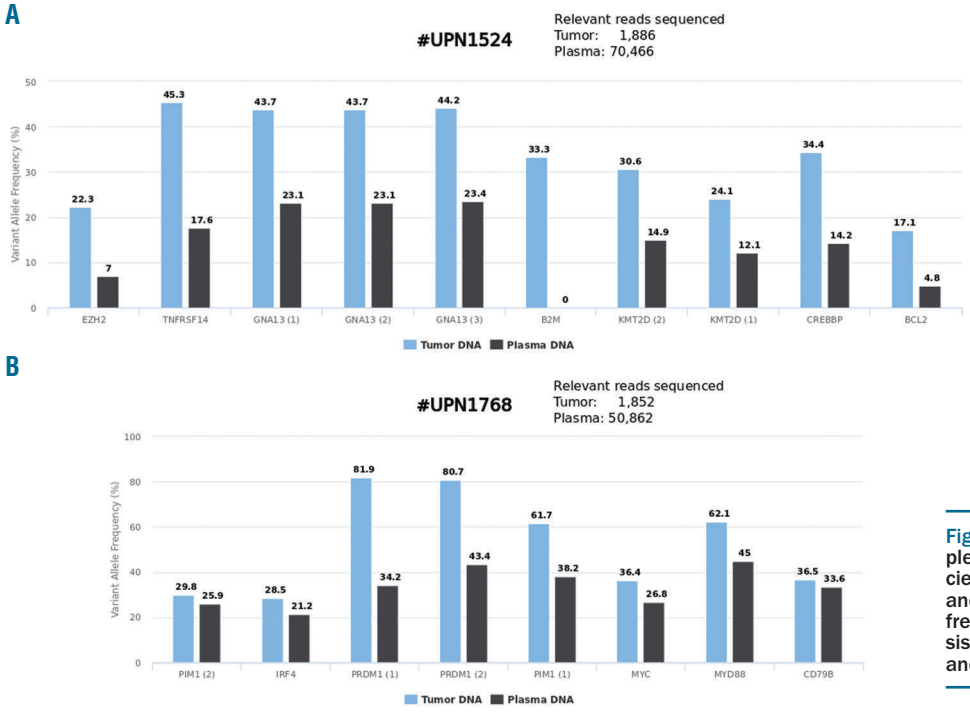

Figure 1. Representative examples of variant allele frequencies observed in tumor DNA and matched circulating cell-free DNA at the time of diagnosis in two different patients (A and B).

Table 1. Clinical characteristics and list of somatic variants (insertion/deletion/ single nucleotide variant) detected by sequencing in tumor DNA and cell-free, plasma circulating DNA. Details of the locations of the mutations are indicated in *Online Supplementary Table S1*.

UPN	Sex	Age (years)	Stage	IPI (score)	LDH (xUNL)	Bone marrow involvement	Phenotype	Tumor DNA Gene	VAF (%)	read number	mean VAF (%)	Circulating DNA VAF (%)	read number	mean VAF (%)	allele call	concentration (ng/μL)
964	F	17	IV	3	5	0	GCB	*ITPKB (1)*	40.9	79/193	40	2.5	158/6313	2	Heterozygous	2.02
								ITPKB (2)	40.6	78/192		2.4	152/6260		Heterozygous	
								CARD11	17.1	13/76		0.0	0/54		Absent	
								EZH2	26.3	76/289		2.8	126/4435		Heterozygous	
								B2M	75.6	102/135		4.0	130/4016		Heterozygous	
1003	F	75	III	3	2.5	0	ABC	*ITPKB*	35.3	36/102	40	1.4	22/1540	5	Heterozygous	1.94
								MYD88	26.5	67/253		0.0	0/21		Absent	
								CIITA	51.9	14/27		16.7	1/6		Heterozygous	
								CD79B	46.3	93/201		2.1	369/17260		Heterozygous	
1251	M	42	IE	0	0.8	0	GCB	*TNFRSF14*	52.5	74/141	31	0.0	0/1113	0	Absent	0.46
								EZH2	9.8	38/389		0.0	0/2519		Absent	
								SOCS1	30.4	7/23		0.0	0/1053		Absent	
1437	M	76	III	4	5.85	0	ABC	*CD79A (1)*	17.0	53/318	17	89.1	344/386	89	Heterozygous	11.2
								CD79A (2)	17.0	53/318		89.1	344/386		Heterozygous	
1524	F	45	III	2	1.52	0	GCB	*TNFRSF14*	45.3	77/170	34	17.6	539/3064	14	Heterozygous	1.48
								EZH2	22.3	106/475		7.0	818/11751		Heterozygous	
								KMT2D (1)	24.1	64/266		12.1	1269/10509		Heterozygous	
								KMT2D (2)	30.6	33/108		14.9	1015/6818		Heterozygous	
								B2M	33.3	44/132		0.0	0/4555		Absent	
								CREBBP	34.4	45/131		14.2	47/331		Heterozygous	
								GNA13 (1)	43.7	52/119		23.1	1909/8254		Heterozygous	
								GNA13 (2)	43.7	52/120		23.1	1920/8314		Heterozygous	
								GNA13 (3)	44.2	53/120		23.4	1946/8312		Heterozygous	
								BCL2	17.1	42/245		4.8	409/8558		Heterozygous	
1528	F	66	III	4	1.39	0	GCB	*ITPKB (1)*	9.3	14/151	31	0.0	0/1713	3	Absent	1.94
								ITPKB (2)	15.2	19/125		0.0	0/3582		Absent	
								ITPKB (3)	15.2	19/125		0.0	0/3577		Absent	
								ITPKB (4)	15.5	19/123		0.0	0/3570		Absent	
								MYD88	37.8	74/196		8.5	161/1888		Heterozygous	
								TNFAIP3	45.7	32/70		5.6	177/3152		Heterozygous	
								EZH2	5.9	44/741		2.1	152/7401		Heterozygous	
								KMT2D	36.1	122/338		5.7	207/3620		Heterozygous	
								B2M	50.0	63/126		3.2	78/2465		Heterozygous	
								CIITA (1)	39.0	30/77		0.0	0/2130		Absent	
								CIITA (2)	7.7	14/183		0.0	0/9150		Absent	
								CIITA (3)	7.6	14/184		0.0	0/9162		Absent	
								GNA13 (1)	71.0	105/148		0.0	0/2695		Absent	
								GNA13 (2)	71.0	105/148		3.1	82/2672		Heterozygous	
								BCL2(1)	34.1	47/138		4.3	214/4929		Heterozygous	
								BCL2 (2)	32.9	45/137		4.2	209/5013		Heterozygous	
								MEF2B	33.3	137/411		5.9	654/11153		Heterozygous	
1559	F	83	IE	1	0.8	0	ABC	*CD58*	92.9	92/99	64	0.0	0/13231	<1	Absent	0.618
								MYD88	51.8	187/361		0.0	0/11427		Absent	
								CREBBP	46.3	154/333		0.0	0/5663		Absent	
								TP53	89.0	105/118		0.0	0/13012		Absent	
								CD79A (1)	52.0	122/235		0.5	20/3930		Absent	
								CD79A (2)	52.0	122/235		0.5	20/3932		Absent	
1586	F	62	III	2	0.85	0	NA	*TNFRSF14*	13.8	44/318	20	2.1	6/284	<1	Heterozygous	1.31
								EZH2	13.8	41/298		0.0	0/6575		Absent	
								STAT6	18.9	83/439		0.0	2/5517		Absent	
								CREBBP	33.6	49/146		1.3	65/4894		Absent	
								GNA13	17.7	11/62		0.0	0/4391		Absent	
								TNFRSF14	34.8	146/419		19.3	694/3587		Heterozygous	
								EZH2	9.9	57/575		1.8	62/3480		Heterozygous	
								STAT6	15.4	79/514		5.1	200/3889		Heterozygous	
1623	M	53	IV	2	1.03	0	GCB	CREBBP *(1)*	23.2	166/717	18	11.6	489/4222	11	Heterozygous	0.95
								CREBBP (2)	14.0	54/387		10.1	328/3239		Heterozygous	
								CD79B	17.1	83/486		18.0	839/4651		Heterozygous	
								MEF2B	11.8	74/626		9.9	1283/12969		Heterozygous	

Table 1 (continued on the next page).

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UPN	Sex	Age (years)	Stage	IPI (score)	LDH (xUNL)	Bone marrow involvement	Phenotype	Tumor DNA Gene	VAF (%)	read number	mean VAF (%)	Circulating DNA VAF (%)	read number	mean VAF (%)	allele call	concentration (ng/µL)
1631	M	64	I	2	1.54	0	ABC	*MYD88*	49.8	220/442	35	11.7	655/5586	5	Heterozygous	1.1
								PIM1	20.6	14/68		2.7	130/4797		Heterozygous	
								CARD11 (1)	47.1	99/210		5.1	154/3028		Heterozygous	
								CARD11 (2)	47.9	68/142		5.5	344/6300		Heterozygous	
								STAT6	18.1	96/532		4.3	570/13250		Heterozygous	
								TP53	32.4	72/222		3.9	522/13317		Heterozygous	
								CD79B	26.7	52/195		3.3	386/11853		Heterozygous	
1639	M	69	IV	2	2.4	0	GCB	*TNFRSF14*	75.8	47/62	39	82.3	2400/2915	34	Heterozygous	10.2
								MYD88	27.5	133/483		7.1	1616/22541		Heterozygous	
								EZH2	24.0	126/526		32.8	3259/9939		Heterozygous	
								KMT2D (1)	33.8	187/554		35.0	5878/16784		Heterozygous	
								KMT2D (2)	28.3	13/46		22.9	671/2929		Heterozygous	
								CREBBP	37.0	17/46		30.6	2918/9530		Heterozygous	
								BCL2 (1)	45.8	11/24		31.5	223/709		Heterozygous	
								BCL2 (2)	53.0	98/185		28.2	2267/8032		Heterozygous	
								EP300	29.6	34/115		38.3	4597/11990		Heterozygous	
1768	M	83	IV	3	4.2	0	ABC	*MYD88*	62.1	216/348	52	45.0	5936/13194	34	Heterozygous	1.82
								IRF4	28.5	75/263		21.2	929/4374		Heterozygous	
								PIM1 (1)	61.7	145/235		38.2	1541/4030		Heterozygous	
								PIM1 (2)	29.8	34/114		25.9	987/3812		Heterozygous	
								PRDM1(1)	81.9	149/182		34.2	1273/3724		Heterozygous	
								PRDM1(2)	80.7	221/274		43.4	5076/11689		Heterozygous	
								MYC	36.4	102/280		26.8	262/979		Heterozygous	
								CD79B	36.5	57/156		33.6	3041/9060		Heterozygous	

ABC: activated B-cell-like; GCB: germinal center B-cell-like; In/del: insertion/deletion; LDH: lactate dehydrogenase; IPI: International Prognosis Index; SNV: single nucleotide variant; UPN: unique personal number; ULN: upper limit of normal value; VAF: variant allele frequency.

nanograms of genomic DNA were submitted to NGS using a laboratory-developed Lymphopanel set, designed to identify mutations in 34 genes relevant to lymphomagenesis (*Online Supplementary File S1A*). This design covers 87,703 bases and generates 872 amplicons. Amplified libraries (Ion AmpliSeq™ Library Kit 2.0) were submitted to emulsion polymerase chain reaction with the Ion OneTouch™ 200 Template Kit (Life Technologies) using the Ion OneTouch™ System (Life Technologies) according to the manufacturer's instructions. The templated Ion Sphere™ Particles were enriched with the Ion OneTouch™ Enrichment System and loaded and sequenced on an Ion 316™ v2 Chip (Life Technologies).

After alignment to a reference genome sequence (hg19) and a variant calling procedure, variants were filtered through a dedicated bioinformatic pipeline that eliminated synonymous variants and variants with a variant allele frequency (VAF) greater than 1% in the 1000 genome database (considered as polymorphisms). Only non-synonymous SNV/In/Del with a quality score >22 and/or confirmed by a Sanger experiment were retained as acquired somatic mutations in lymphoma cases and were used for the subsequent sequencing of the matched cfDNA (for the detailed pipeline see *Online Supplementary File S1B*). Somatic mutations identified in tumor DNA were used to build a hot-spot file enabling higher sensitivity tracking of somatic mutations in circulating DNA.

In order to increase the sensitivity and specificity of variant detection in cfDNA, we exclusively amplified amplicons targeting mutations detected in the corresponding tumor DNA by the complete Lymphopanel by performing a dedicated sequencing procedure with a pool of oligonucleotide primers selected among the 872 pairs provided by Life Technologies. The procedure to make libraries and sequence amplicons was the same as that for tumor DNA but used a 314™ v2 Chip. When possible, circulating DNA was extracted and sequenced from two different aliquots of plasma.

The SNV/In/Del detected in both tumor and circulating DNA are indicated in Table 1 with their corresponding VAF. As expected, we identified a typical SNV pattern in the five ABC DLBCL cases, including mutations targeting *MYD88, CD79A/B, PIM1, PRDM1, CARD11* or *IRF4*, whereas *EZH2, BCL2, GNA13*, or *TNFSRF14* were mutated in the unclassified and GCB DLBCL cases. *MLL2 (KMT2D)*, *CREBBP* or *ITPKB* were targeted by somatic mutations shared in the two cell-of-origin subtypes. The sequencing depths obtained for each sample and targets are indicated in Table 1.

The mean number of reads targeting the mutated regions for tumor DNA was 241 (range, 23-741) as compared to 5,987 (range, 6-22,541) for plasma DNA, indicating a 24-fold mean depth sequencing increase. The mean VAF in the tumor DNA was 35% (range, 17-64%) as compared to a mean of 11% for plasma DNA (range, 2-89%) (Table 1).

In 11/12 DLBCL cases, we observed somatic mutations in cfDNA, similar or partially similar to those observed in the tumor (Figure 1, Table 1 and *Online Supplementary Figure S1*). We defined the concordance rate as the ratio of the number of mutated cfDNA genes and the number of mutated tumor DNA genes. This rate ranged from 33% to 100% (5 cases) in the 11/12 cases with detected mutated cfDNA. Overall, the median concordance rate between tumor DNA and cfDNA was 85%.

In one case (#1251), SNV observed in the tumor DNA were not detected in plasma DNA. In another case (#1559),

SNV were barely detectable (VAF of 0.5% for two variants). Of note, both cases displayed limited disease (stage I or II) and normal lactate dehydrogenase levels, indicating that the amount of tumor-specific circulating cfDNA is at least partially related to tumor burden. Despite a low amount of circulating DNA extracted from plasma for cases #1251 and #1559, we obtained adequate sequencing quality and depth (the overall number of reads sequenced with mutated targets was 4,685 and 51,195 respectively; Table 1), indicating that in some rare cases, tumor-specific cfDNA is absent or beneath the level of sensitivity of the NGS method used. Of note, in case #1631 characterized by limited stage I disease, SNV were detected with a mean VAF of 5.2% in plasma DNA, as compared to a mean VAF of 34.6% in the tumor DNA (Table 1). In contrast, cases #1639 and #1768 (both with stage IV disease and elevated levels of lactate dehydrogenase) displayed a high proportion of tumor-specific circulating DNA, as indicated by the high VAF observed (Table 1). Interestingly, in these two cases, the sub-clonal distribution of certain mutations, as indicated by the VAF distribution of each individual variant, was also observed in cfDNA, suggesting that sequencing cfDNA can reflect the SNV pattern observed in tumor cells in some instances (Figure 1 and *Online Supplementary Figure S1*). In case #1524, despite a sufficient number of relevant reads (> 4,000) we failed to detect the *B2M* SNV present in the lymph node biopsy. This result was confirmed by manual Integrative Genomics Viewer checking, suggesting that the *B2M* SNV is present only in a subclone caught in the biopsy sample but not highlighted by the cfDNA that reflects the entire tumor burden.

By contrast, in some cases, the number of target reads was clearly insufficient for adequate SNV detection (case #964, *CARD11*; case #1003, *MYD88*; Table 1), most likely reflecting the low amount of cfDNA available rather than a true clonal divergence between tumor DNA and cfDNA. This was not observed in cases with higher amounts of cfDNA.

Failure to detect SNV in cfDNA appears related more to the proportion of tumor-specific DNA than to the total amount or quality of total cfDNA. Of note, we failed to find any tumor-related SNV in DNA extracted from 11/12 samples of peripheral blood mononuclear cells using this approach (*data not shown*), indicating that serum or plasma is preferable for the detection of mutated circulating DNA. In a large cohort of cases of Hodgkin lymphoma, mantle cell lymphoma and DLBCL, increased levels of plasma DNA (determined using quantitative polymerase chain reaction for the β-globin gene) were associated with advanced stage disease, presence of B-symptoms, elevated lactate dehydrogenase levels, and age >60 years also indicating that the amount of circulating DNA is partially related to tumor burden.[8] Furthermore, it was shown in a cohort of patients with Epstein-Barr virus-positive lymphoma that serum and plasma were equivalent for detecting lymphoma-specific DNA but that only the lymphoma-specific DNA could be used to monitor disease response in lymphoma.[7]

To our knowledge this is the first report of the detection of non-immunoglobulin somatic mutations in DLBCL from circulating DNA by routine NGS, enabling the identification of lymphoma-specific cfDNA. Other quantitative approaches, including digital polymerase chain reaction, are also suitable and could be used in this setting for detecting recurrent translocations or mutations.[11] More recently LymphoSIGHT®, a high-throughput DNA sequencing method, was developed to detect and quantify circulating tumor DNA as minimal residual disease and was able to predict both early treatment failure and relapse in patients with newly diagnosed DLBCL, chronic lymphocytic leukemia or acute lymphoblastic leukemia.[12-15] This approach is based on tumor DNA amplification using locus-specific primer sets for the immunoglobulin heavy/light-chain which failed in a substantial number of cases of DLBCL. Importantly the Lymphopanel used in this study is able to detect at least one acquired SNV in 95% of DLBCL cases at initial diagnosis (*manuscript in preparation*) and may, therefore, constitute a simple, routinely applicable test to provide the cell-of-origin subtype or to detect targetable mutations at the time of diagnosis or relapse. However, its capacity to detect minimal residual disease with a high level of sensitivity remains to be determined and we can hypothesize that at least 5-10% of DLBCL cases will not display any SNV detectable by our Lymphopanel. Furthermore, cfDNA sequencing was successfully performed using the entire Lymphopanel (including the 34 targeted genes) in one case (*data not shown*), indicating that this approach is feasible without the knowledge of the tumor variant calling. However this requires an increase in the sequencing depth capacity and entails a substantial increase of costs.

To conclude, our results indicate that cfDNA can also be used in DLBCL to detect somatic variants, validating the concept of "liquid biopsy" in this type of tumor.[3] These preliminary results have prompted us to start a prospective study with the aim of serially sequencing cfDNA during DLBCL treatment and follow-up (registered at *clinicaltrials.gov* as NCT02339805). If these preliminary results are confirmed by a prospective study, new strategies should be proposed for both diagnosis and treatment tailoring based on the simple detection and quantification of SNV in plasma.

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References

1. Morin RD, Gascoyne RD. Newly identified mechanisms in B-cell non-Hodgkin lymphomas uncovered by next-generation sequencing. Semin Hematol. 2013;50(4):303-313.
2. Roschewski M, Staudt LM, Wilson WH. Diffuse large B-cell lymphoma-treatment approaches in the molecular era. Nat Rev Clin Oncol. 2014;11(1):12-23.
3. Diaz LA, Jr., Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. J Clin Oncol. 2014;32(6):579-586.
4. He J, Wu J, Jiao Y, et al. IgH gene rearrangements as plasma biomarkers in Non- Hodgkin's lymphoma patients. Oncotarget. 2011; 2(3):178-185.
5. Hosny G, Farahat N, Hainaut P. TP53 mutations in circulating free DNA from Egyptian patients with non-Hodgkin's lymphoma. Cancer Lett. 2009;275(2):234-239.

6. Mussolin L, Burnelli R, Pillon M, et al. Plasma cell-free DNA in paediatric lymphomas. J Cancer. 2013;4(4):323-329.
7. Jones K, Nourse JP, Keane C, et al. Tumor-specific but not nonspecific cell-free circulating DNA can be used to monitor disease response in lymphoma. Am J Hematol. 2012;87(3):258-265.
8. Hohaus S, Giachelia M, Massini G, et al. Cell-free circulating DNA in Hodgkin's and non-Hodgkin's lymphomas. Ann Oncol. 2009; 20(8):1408-1413.
9. Bohers E, Mareschal S, Bouzelfen A, et al. Targetable activating mutations are very frequent in GCB and ABC diffuse large B-cell lymphoma. Genes Chromosomes Cancer. 2014;53(2):144-153.
10. Lanic H, Mareschal S, Mechken F, et al. Interim positron emission tomography scan associated with international prognostic index and germinal center B cell-like signature as prognostic index in diffuse large B-cell lymphoma. Leuk Lymphoma. 2012;53(1):34-42.
11. Shuga J, Zeng Y, Novak R, et al. Single molecule quantitation and sequencing of rare translocations using microfluidic nested digital PCR. Nucleic Acids Res. 2013;41(16):e159.
12. Armand P, Oki Y, Neuberg DS, et al. Detection of circulating tumour DNA in patients with aggressive B-cell non-Hodgkin lymphoma. Br J Haematol. 2013;163(1):123-126.
13. Logan AC, Vashi N, Faham M, et al. Immunoglobulin and T cell receptor gene high-throughput sequencing quantifies minimal residual disease in acute lymphoblastic leukemia and predicts post-transplantation relapse and survival. Biol Blood Marrow Transplant. 2014;20(9):1307-1313.
14. Logan AC, Zhang B, Narasimhan B, et al. Minimal residual disease quantification using consensus primers and high-throughput IGH sequencing predicts post-transplant relapse in chronic lymphocytic leukemia. Leukemia. 2013;27(8):1659-1665.
15. Roschewski M, Dunleavy K, Pittaluga S, et al. Monitoring of circulating tumor DNA as minimal residual disease in diffuse large B-cell lymphoma. Blood. 2014;124(21):139 Abstr.