Utilization of Liquid Biopsy in Clinical Practice of Hematological Diseases

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Advances in next generation sequencing have revealed many genetic alterations in malignant lymphoma (ML). The importance of genetic analysis is being recognized not only in molecular diagnosis but also in the selection of targeted therapy. Tumor biopsy is essential for genetic analysis of ML, but there are some cases in which the tumor biopsy is difficult. In this issue, I would like to outline the current status of "liquid biopsy", a genetic analysis strategy using body fluids of patients, and discuss the usefulness in the clinical setting of ML.

Key Words：Liquid Biopsy, cfDNA, Genetic Alterations, MRD, DLBCL

Current Status of Genetic Analysis in the Treatment of Malignant Lymphoma

Advances in comprehensive gene mutation analysis have revealed genetic abnormalities in various types of malignant lymphoma[1–3]. Lymphocytes differentiate, proliferate and mature by antigen stimulation over the period when they reside in the bone marrow, the first lymphoid tissue, and in periphery lymph nodes, the secondary lymphoid tissue. The accumulation of specific genetic aberrations at each of these stages has been recognized to underlie differences between lymphoma types. Molecular-targeted agents with efficacy according to the profile of genetic aberrations are currently being developed[2].

Biopsy of tumor tissue and histopathological diagnosis are essential for the definitive diagnosis of malignant lymphoma. Since gene mutation analysis has recently been considered important for understanding the pathogenesis of lymphoma, some research institutions are attempting to bank biopsied tumor tissues and genomic DNA extracted from the tissues. **Fig 1** shows useful methods for collecting samples from malignant lymphoma in a clinical setting. It is desirable to extract DNA from fresh tumor tissues obtained by biopsy to ensure high-quality genomic DNA is obtained. However, since trace amounts of tissue samples, such as those by needle and endoscopic biopsies, are currently often used in the diagnosis of lymphoma, it is difficult to obtain residual fresh specimens for genetic analysis in many cases. In addition, although the extraction of genes from formalin-fixed paraffin-embedded (FFPE) sections for pathological diagnosis is an option, the quality and quantity of extracted DNA are insufficient in many cases, which is an immediate issue to be discussed. The analysis of minimal residual disease (MRD) has already been performed for the clinical evaluation of leukemia. This analysis requires the collection of tissues including the tumor over time. In cases of lymphoma, tumor tissue cannot be identified even by imaging studies, which makes it

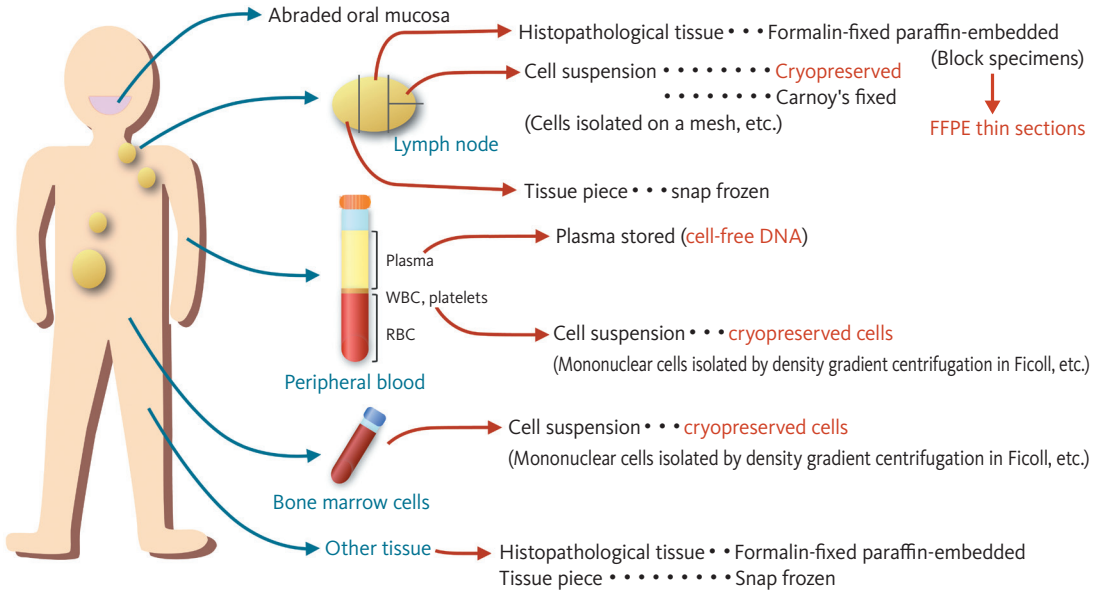

Fig 1. Methods of collecting samples for the diagnosis of malignant lymphoma

The figure illustrates potential clinical samples containing tumor components of malignant lymphoma. In patients with tumor masses in lymph nodes, the mass is biopsied or excised and sent for histopathological examination, human leucocyte antigen testing, chromosome analysis, and other appropriate analysis. The remaining samples will be stored frozen in liquid nitrogen as a whole or in cell suspension. These stored samples can be used to extract genomic DNA and RNA as well as proteins at a later date. Tumor cells may be present in the bone marrow (as an infiltrate) and peripheral blood. Tumor genes can be extracted from the cellular components of pleural effusion and ascites if tumor cells are present in them. Tumor-derived cfDNA may be detectable in plasma and other fluid components (see **Fig 2**). DNA extracted from abraded oral mucosa typically serves as a germline control.

impossible to collect the tumor over time for the MRD analysis.

Another challenge inherent in malignant lymphoma is heterogeneity depending on the site of origin. Lymphoma lesions are often found at multiple locations in the body. In clinical practice, it has been recognized that the degree of progression and response to treatment varies by site, indicating tumor heterogeneity in the same body. Tissue biopsy is usually performed at a single site due to its highly invasive nature, and thus does not ensure the accurate evaluation of the disease status other than that at the biopsy site.

With the background described above, it is desired to establish a minimally invasive methodology for examining over time genetic abnormalities in malignant lymphoma as a whole within an individual, to facilitate the detection of genetic abnormalities and to realize MRD analysis in the malignant lymphoma.

What is Liquid Biopsy?

Liquid biopsy is a new diagnostic whereby various tumor-derived components are extracted from patient's body fluids (**Fig 2A**)[4].

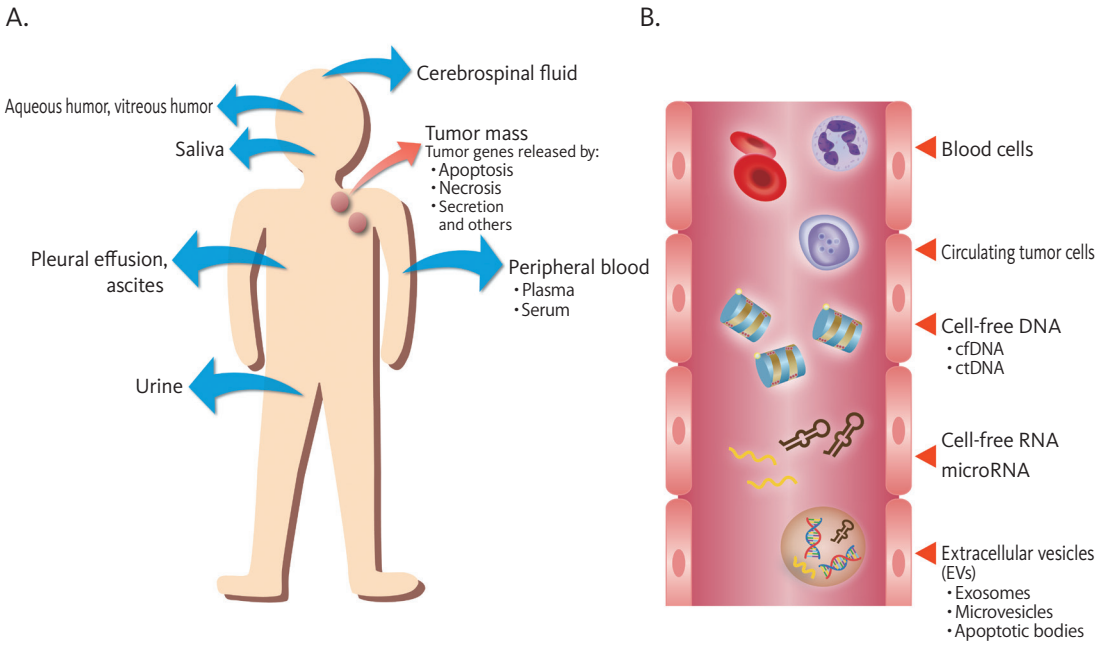

Fig 2. Overview of liquid biopsy

A: Different types of samples for liquid biopsy.
B: Cellular and other components circulating in the peripheral blood that may be used for liquid biopsy analysis. CTCs, ctDNA, cfDNA, cell-free RNA, microRNA, EVs (including exosomes and microvesicles) are gaining particular interest as potential targets for analysis.

Peripheral blood is most commonly used for liquid biopsy studies. Tumor-derived components demonstrated using peripheral blood cells and fluid components[4] include circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), cell-free DNA (cfDNA), cell-free RNA, microRNA, and extracellular vesicles (EVs), including exosomes and microvesicles (**Fig 2B**)[5]. cfDNA refers to the entire fragmented DNA present in the fluid, and may include both tumor- and normal tissue-derived DNA. In addition to plasma, body fluids, such as saliva, stool, urine, and cerebrospinal fluid, as well as pleural fluid and ascites, may contain tumor-derived DNA (ctDNA), and they are attracting attention as samples of minimally invasive biopsy for tumor-derived components[4]. Genomic DNA fragments are useful for detecting genetic abnormalities specific to tumors, such as mutations, translocations, and epigenomic abnormalities.

Properties of Cell-free Nucleic Acids

Genomic DNA is wrapped around a histone octamer to form a nucleosome structure. The nucleosome structure is tightly regulated on a

cell-by-cell and gene-by-gene basis by linker histones, DNA methylation, transcription factors and regulators, and histone acetylation and methylation. Once genomic DNA in the nucleus is released into tissues or the blood by cellular apoptosis or necrosis, DNAase degrades the unwrapped DNA, that is not involved in the nucleosome structure[6,7]. The size of cfDNA observed as ladder bands in a gel electropherogram ranges from about 167 bp, equal to the length of a DNA fragment wrapped around one chromosome (nucleosome + linker histones) to multiple times this bp (**Fig 3A and 3B**)[6–9]. cfDNA concentration is generally low in normal individuals and seems to increase in patients with tissue necrosis (e.g., myocardial infarction)[10], those with inflammation (e.g., autoimmune diseases)[11], and those with significant tumor cell destruction (e.g., fast-growing lymphoma)[12,13], although differences in properties of cfDNA between individuals remain unclear. cfDNA in normal tissues is believed to be derived mainly from hematopoietic cells that have undergone apoptosis. This is supported by the fact that the donor's single nucleotide polymorphisms (SNP) accounted for a significantly higher proportion than the recipient's SNP in the *CDKN2A* polymorphism in cfDNA samples from a patient who underwent allogeneic hematopoietic stem cell transplantation (**Fig 4**)[8,14]. A comparison of cfDNA in plasma and serum indicates that the detection of genetic mutations may be more sensitive in plasma[8,9].

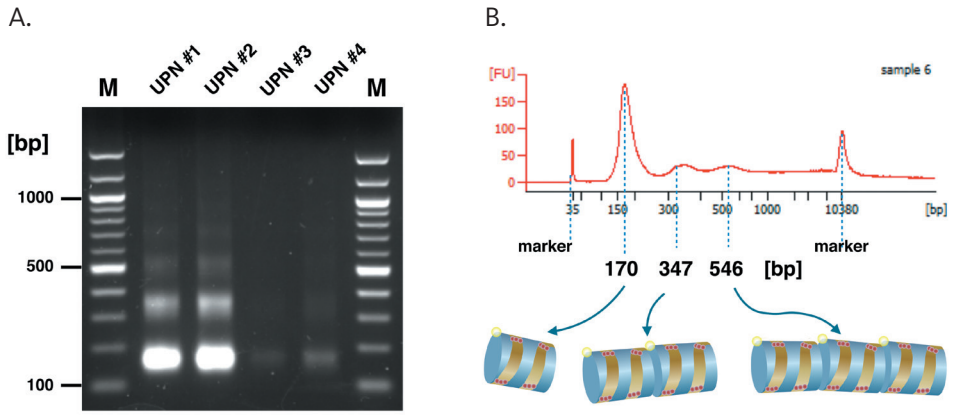

Fig 3. cfDNA in plasma

Adopted from Suzuki Y, et al. Peripheral blood cell-free DNA is an alternative tumor DNA source reflecting disease status in myelodysplastic syndromes. Cancer Sci. 2016; Supporting information with partial modifications

A: cfDNA was isolated from plasma samples of four patients and electrophoresed in 1% agarose gel. The cfDNA appears as ladder bands of small fragments at different concentrations between the samples.
B: The analysis using an Agilent 210 Bioanalyzer revealed cfDNA fragments at a position of approximately 170 bp and its multiple numbers, suggesting that DNA is wrapped around a histone octamer to form a nucleosome structure, and unwrapped DNA, not involved in this structure, is digested by DNase.

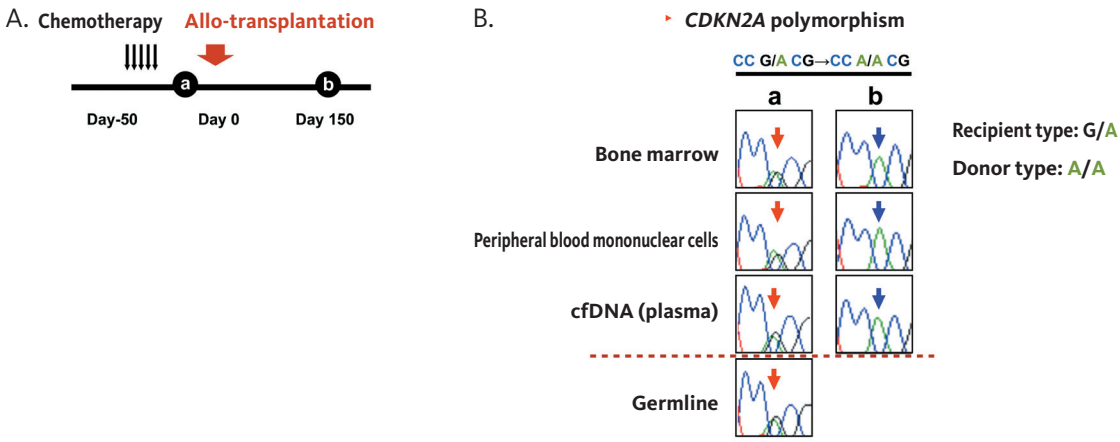

Fig 4. Genetic mutation analysis of peripheral blood cfDNA

Adopted and translated from Suzuki Y, et al. Peripheral blood cell-free DNA is an alternative tumor DNA source reflecting disease status in myelodysplastic syndromes. Cancer Sci. 2016 with partial modifications

A: Clinical course of a patient with myelodysplastic syndrome who underwent allo-transplantation after chemotherapy. **a** and **b** indicate the sample collection time.
B: Results of CDKN2A gene polymorphism analysis using cfDNA.

The gene mutation analysis of cfDNA from a patient with hematological malignancy who underwent allo-transplantation showed that the SNP G/A was present in the *CDKN2A* gene in samples of different sites collected before allo-transplantation. This SNP was a genetic polymorphism because it was also observed in normal cells (germline). Analysis using the samples collected at the time of disease remission after allo-transplantation detected the donor-derived sequences but not the SNP that was detected at the corresponding collection sites before transplantation. This suggests that cfDNA is primarily derived from bone marrow cells in the normal state.

Clinical Applications of Liquid Biopsy

Tumor-derived ctDNA can be detected by gene mutation analysis of cfDNA. Liquid biopsy allows the collection of samples over time in the clinical course due to its minimally invasive nature. MRD analysis is possible by examining the variant allele frequency (VAF) in liquid biopsy samples. In addition, the mutation profile analysis over time makes it possible to evaluate disease progression based on clonal switching, and to determine if drug resistance has developed. However, it should be noted that levels of ctDNA released in the peripheral blood may vary among disease types, and that the clinical utility of liquid biopsy remains under investigation for different disease types.

The quality of cfDNA in blood samples may change with temperature and time. Plasma tends to contain higher cfDNA concentrations than serum (**Fig 5A**)[13,15]. Plasma collected from EDTA-treated blood is commonly used to isolate cfDNA to prevent contamination of genomic DNA from leukocytes destroyed in the blood collection

tube. To obtain better quality cfDNA, dedicated collection tubes, such as PAXgene® DNA collection tubes (BD)[16], Streck® blood collection tubes (Veritas), and Cell-Free DNA Collection Tubes® (Roche), are available. In samples collected in these tubes, cfDNA is maintained stable for approximately one week at room temperature. Plasma can be stored at -80°C, and DNA can be extracted using dedicated extraction kits for DNA/RNA from plasma. The chromatin structure is destroyed by protease treatment to extract linear nucleic acids. The extracted DNA can be used for gene sequence analysis by PCR or the Sanger method, quantitative analysis of known gene mutations by droplet digital PCR (ddPCR), and genetic analysis by next-generation sequencing (NGS), including targeted sequencing and whole exon analysis (WES)[8,9,13,17,18]. The extracted DNA can also

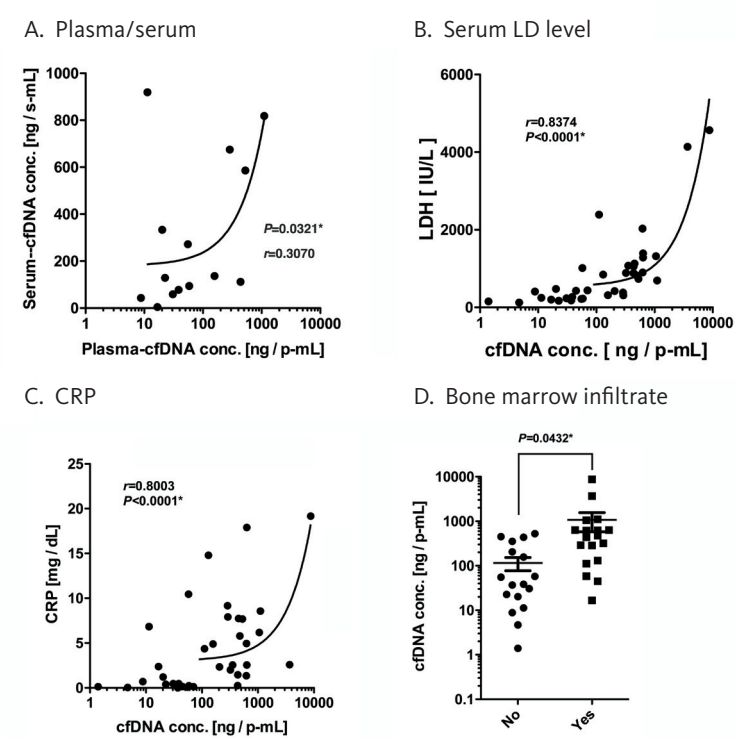

Fig 5. cfDNA concentrations in DLBCL

Adopted and translated from Shimada K, et al. Frequent genetic alterations in immune checkpoint-related genes in intravascular large B-cell lymphoma. Blood. 2021; Supplementary Material with partial modifications

A: Comparison of cfDNA concentrations in plasma and serum show that they significantly correlate with each other, and that dfDNA concentrations tend to be higher in plasma than in serum.
B: Plasma cfDNA concentrations correlate with serum LD levels.
C: Plasma cfDNA concentrations correlate with CRP.
D: cfDNA concentrations are significantly higher in patients who were positive for bone marrow infiltration than those who were negative.

be used for epigenomic analysis such as DNA methylation[19,20]. Care must be taken in constructing PCR primers for extracted DNA because of its short length (about 170 bp)[8].

The concentration of cfDNA varies greatly from a few ng/mL to several hundred ng/mL (amount of DNA in 1 mL of plasma) depending on the disease status and the number of days after treatment, even in the same patient[13]. Therefore, it is necessary to consider an feasible analysis method depending on the diagnosis and disease status and timing of analysis. The half-life of cfDNA in blood is extremely short[21], ranging from a few minutes to several tens of minutes. This means that genetic analysis using cfDNA is a method that reflects the disease status of the patient in real time. In addition, while genetic analysis using biopsy materials detects only abnormalities at the site of collection (due to the regional heterogeneity of tumors), analysis using cfDNA may detect abnormalities in all lesions throughout the body, which is also considered to be an important advantage.

Liquid Biopsy in the Clinical Management of Malignant Lymphoma

Reports of the utility of liquid biopsy in malignant lymphoma have increasingly been published. The following are some of the reports, including our experiences.

1. Diffuse large B-cell lymphoma (DLBCL)

DLBCL is the most common type of lymphoma, accounting for approximately 40% of all cases of lymphoma. cfDNA levels in patients with DLBCL tend to be higher than those in normal individuals and patients with follicular lymphoma (FL)[9,15,17]. The cfDNA level reflects the disease activity and may correlate with the serum level of lactate dehydrogenase (LD), the CRP, the level of bone marrow infiltration, and the disease stage (**Fig 5B–5D**)[9,12,22,23]. Possible cfDNA-based genetic analysis for DLBCL includes whole exon analysis, disease panel analysis, and ddPCR to detect VDJ gene rearrangements in immunoglobulin receptor genes (*IGHV* and *IGK*) (BCR repertoire analysis) and mutations and structural abnormalities in specific genes that are repeatedly found to be abnormal in DLBCL.

I and my colleagues extracted genomic DNA from biopsied tumor tissues and peripheral blood (tDNA and cfDNA, respectively) simultaneously collected from a patient with newly diagnosed DLBCL to conduct a whole-exon analysis[9]. As shown in **Fig 6**, some of the mutations detected using tDNA and cfDNA overlapped at a high frequency, but some were detected in either tDNA or cfDNA only. These findings suggest that tumors at other sites may harbor mutations other than those detected at the biopsy site and may reflect the regional heterogeneity of tumors in the same individual. Thus, liquid biopsy is a potential tool to detect genetic mutations that are present not only at the biopsy site but also throughout the patient's body, which may be more useful in selecting appropriate targeted therapy in clinical practice.

Peripheral blood collection is less invasive than biopsy and bone marrow puncture, allowing repeated collection over time. Therefore, cfDNA may be used to monitor genetic alternations detected at the time of

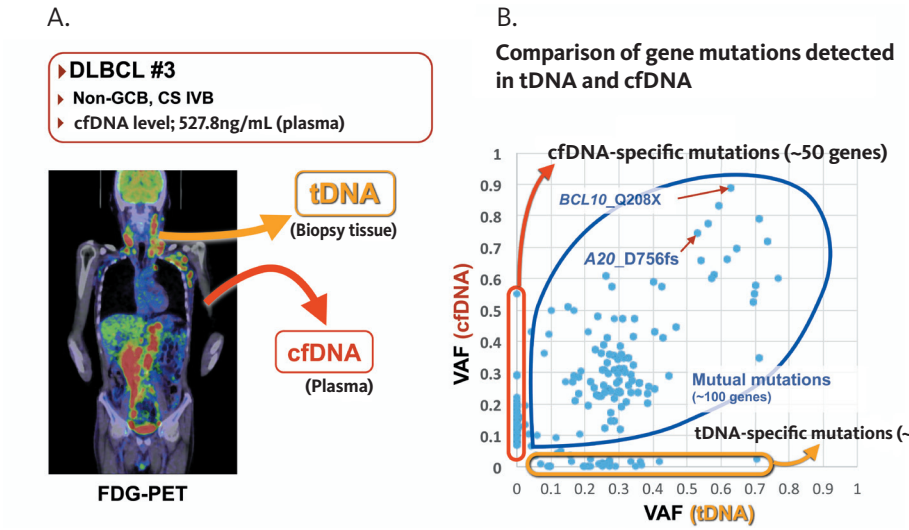

Fig 6. Comprehensive gene mutation analysis of plasma cfDNA and tumor DNA (tDNA) from biopsy tumor tissues

A: Genomic DNA (tDNA and cfDNA) was extracted from biopsy tissue and plasma samples from a DLBCL patient for whole exon analysis.

B: VAF of each mutant gene detected. Most mutant genes were detected in both tDNA and cfDNA. Mutations in the area enclosed by the red and yellow lines are those detected in either tDNA or cfDNA only. This finding may be due to the regional heterogeneity of tumors in the body.

diagnosis (i.e., MRD monitoring). **Fig 7A** and **7B** present the MRD analysis in one DLBCL patient remaining in remission and another whose disease relapsed after the initial treatment, respectively (our unpublished cases). In both patients, serum LD and plasma cfDNA levels increased at the initial presentation and decreased after the initial treatment. Both the serum LD and plasma cfDNA levels rose again in the patient with relapsed disease, while no significant increase in cfDNA level was observed in the patient remaining in remission. These results suggest that the cfDNA level itself may be a useful biomarker of disease progression[9,23]. The quantitative mutation detection using ddPCR revealed a re-elevation of the VAF of the *MYD88*^{L265P} mutation in the relapsed samples, suggesting that liquid biopsy in a combination with diagnostic imaging may be useful for MRD monitoring in the management of lymphoma.

There are increasing reports of NGS analysis of plasma cfDNA[12,15,18]. Rossi *et al*.[18] compared the detection of genetic mutations with a VAF ≥ 20% in plasma cfDNA against that of DNA extracted from biopsied tissues of DLBCL patients. The results showed that cfDNA genotyping correctly detected these mutations with a sensitivity of ≥ 90% and a specificity of 100%. They also performed a longitudinal analysis of the cfDNA and showed that the acquired mutations did not disappear, and new mutations developed in

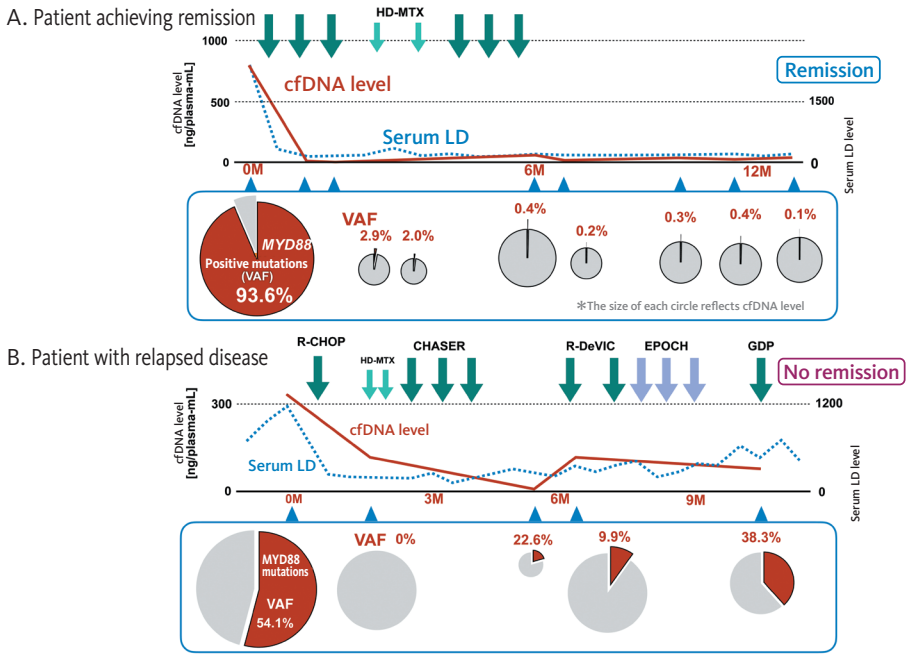

Fig 7. Longitudinal MRD analysis of cfDNA from patients with malignant lymphoma

17) Plasma cfDNA was collected over time from a DLBCL patient remaining in remission (A) and another with relapsed/refractory disease (B) after initial treatment. Changes over time in cfDNA and serum LD levels are shown with solid and dotted lines, respectively. Changes in the VAF of the *MYD88*^{L265P} mutation were also examined by ddPCR and are shown in the circle graphs. The size of each circle reflects the cfDNA level. cfDNA samples can be repeatedly collected during the course of treatment, and MRD analysis can be made possible by examining the VAFs of specific gene mutations in cfDNA (all data from our patients, unpublished data). R-CHOP, HD-MTX, CHASER, R-DeVIC, EPOCH, and GDP are agents included in their combined chemotherapy regimens.

treatment-resistant patients, indicating the potential application of this analytical approach to the study of MRD and clonal evolution.

Rushton *et al*.[24] performed target sequencing of 63 genes using cfDNA derived from peripheral blood of 117 patients with relapsed/refractory DLBCL, and found that the number of mutations, predominantly including *TP53* and *KMT2D* mutations, increased over time. They also identified mutations in *MS4A1 (CD20)* in some patients, indicating that liquid biopsy may help to detect acquired resistance to anti-CD20 antibody therapy.

cfDNA may also be used to analyze the epigenetic methylation status of gene promoters[20,25]. Kristensen *et al.*[20] extracted plasma cfDNA from 74 patients with newly diagnosed DLBCL to investigate DNA methylation in the promotor regions of *DAPK1*. The results showed that the group with methylated *DAPK1* had significantly poorer OS than that with unmethylated *DAPK1* ($P < 0.0007$). Other applications of liquid biopsy reported in DLBCL include the

analysis of microRNA in exosomes extracted from plasma. For example, Zare *et al.*[26] isolated exosomes from the plasma of DLBCL patients and healthy individuals by ultracentrifugation, and confirmed their presence by electron microscopy. Then, they extracted exosomal miR-146a and quantified its expression by real-time PCR. EV studies are paid attention as a new application field of liquid biopsy.

2. Intravascular large B-cell lymphoma (IVLBCL)

IVLBCL is a type of B-cell lymphoma characterized by localization of tumor cells primarily within blood vessels. In early stages of the disease, lymphadenopathy is absent, and many patients only experience a fever and disturbance of consciousness as initial symptoms. Accordingly, diagnosis of IVLBCL is challenging in a routine clinical setting. A histological diagnosis of IVLBCL may be made in suspected patients based on random skin biopsy, although its sensitivity is insufficient[27]. It is therefore desirable to establish a diagnostic method of the early-stage disease with high sensitivity. Our research group has been investigating the utility of liquid biopsy, focusing on the fact that cfDNA levels in IVLBCL are relatively high compared to other lymphoma types. We have performed whole-exon analysis using cfDNA from patients with newly diagnosed IVLBCL, resulting in successful mutation analysis in all patients studied. In addition, we investigated cfDNA isolated from bone marrow samples in patients with tumor cells present in the bone marrow. The VAF of mutated genes in the cfDNA samples was significantly higher than that of the bone marrow-derived samples, and the number of detectable genes was also higher. The difficulty of efficiently obtaining tumor cells has hampered genetic mutation studies in IVLBCL. However, by using the liquid biopsy technique, we were able to detect characteristic genetic abnormalities accumulated in the MCD subtype of DLBCL, such as those in *CD79B*, *MYD88*, *TBL1XR1*, and *PIM1*[1,2]. We also identified genetic abnormalities in *PD-L1* and other genes involved in immune evasion. Our research group is conducting a prospective clinical study to investigate the *MYD88/CD79B* mutation in peripheral blood cfDNA samples from patients with an unknown fever in whom malignant lymphoma is included in differential diagnosis, to evaluate whether liquid biopsy is helpful for the detection and diagnosis of lymphoma that poses a diagnostic challenge.

3. Central nervous system lymphoma (CNSL)

Malignant lymphoma also occurs in the central nervous system (CNS), including the cerebrum and spinal cord. CNSL presents with neurological symptoms, including disturbance of consciousness and muscle weakness, which makes it difficult to distinguish from inflammatory diseases of the CNS of early-stage CNSL. Biopsy is necessary for the definitive diagnosis of CNSL; however, brain biopsy is often difficult to be indicated for early stage CNSL due to its highly invasive nature. Reports have been accumulating regarding liquid biopsy of cerebrospinal fluid to detect genetic mutations characteristic of CNSL[28–30]. We have been working to explore a sensitive procedure for detecting *MYD88*^{L265P} and *CD79B*^{Y167} mutations in the cerebrospinal

fluid by ddPCR[9,31]. Both or either *MYD88*^{L265P} or *CD79B*^{Y167} were found in 90% of CNSL patients studied, although the number of patients was small. In contrast, these mutations were not detected in patients with demyelinating or infectious diseases (unpublished data by Murate, Iriyama, *et al.*). Since cerebrospinal fluid is often less abundant in cfDNA than the plasma of DLBCL patients, spinal fluid samples needs to undergo cfDNA amplification before the detection procedure, thus requiring special caution regarding its detection sensitivity[9]. In addition, we found that concurrent mutation analysis using cfDNA samples from the cerebral fluid and peripheral blood may be more preferable in some disease types. There is still no consensus on whether a diagnosis of CNSL can be made when the above genetic abnormalities are detected, and further investigation is needed for the clinical application of cerebrospinal fluid cfDNA.

4. Liquid biopsy in other types of lymphoma

In classical Hodgkin's lymphoma, it has been reported that cfDNA may be useful for mutation analysis in *STAT6*, *TNFAIP3*, *ITPKB*, *GNA13*, *B2M, ATM*, disease typing, disease activity assessment, and MRD analysis[32,33].

The cfDNA concentration is generally lower in FL than in DLBCL, and there are limited studies of the utility of liquid biopsy in FL.

In spite of the fact that it is clinically important to determine the presence or absence of histological transformation in FL, conventional biopsy, which is taken only at a single tissue site, provides only limited information on histological transformation. Since cfDNA reflects the tumor status throughout the body, elevated cfDNA levels and mutation analysis using cfDNA may be helpful in detecting FL transformation. Further studies are expected to use cfDNA in the disease.

RHOA^{G17V}, *TET2*, and *IDH2* mutations are frequently found in angioimmunoblastic T-cell lymphoma (AITL), which is often difficult to diagnose, and some other subtypes of peripheral T-cell lymphomas (PTCL)[34]. These mutations have been demonstrated to be detected using targeted sequencing and ddPCR, which are expected to be applied in a clinical setting[35–37].

Conclusion

The utility of liquid biopsy in malignant lymphoma has been confirmed in the last few years. This procedure is minimally invasive and has the potential to change the practice of lymphoma in the future through its application to early diagnosis and MRD analysis. What remains to be established includes the selection of the appropriate disease type for clinical application and interpretation when a genetic abnormality is detected. The accumulation of knowledge on liquid biopsy is expected through the collection of more cases.

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