Dynamic Monitoring of Circulating Tumor DNA in Non-Hodgkin Lymphoma

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Abstract

Disease surveillance and response assessment in lymphoma relies on imaging by computerized tomography and positron emission tomography. Imaging scans, however, do not capture dynamic biologic processes at the molecular level and there is a need to more precisely direct treatment decisions. High-throughput next-generation sequencing-based assays detect circulating tumor DNA (ctDNA) in the blood allowing assessment of tumor dynamics and molecular heterogeneity at low thresholds. Monitoring ctDNA that encodes immunoglobulin receptors detects recurrent disease prior to scans in diffuse large B-cell lymphoma. “Liquid biopsies” of ctDNA for somatic mutations can also address tumor heterogeneity, clonal evolution, and mechanisms of resistance to guide precision treatment. To maximize clinical utility, pre-analytic collection and processing procedures need to be validated and standardized. In this Spotlight, we describe emerging clinical and research applications of ctDNA monitoring in lymphoma including real-time analysis of tumor dynamics, pre-clinical disease detection, and precision-directed treatment paradigms.
Introduction

Monitoring treatment response in non-Hodgkin lymphoma relies on computerized tomography (CT) and FDG-positron emission tomography (PET) scans. Imaging scans provide macro estimates of tumor volume and location, but do not significantly improve survival and are limited by cost and risk of ionizing radiation.\textsuperscript{1-3} Because disease recurrence originates from persistent tumor below limits of clinical detection, imaging scans are suboptimal for surveillance monitoring in curable lymphomas such as diffuse large B-cell lymphoma (DLBCL).\textsuperscript{1} Lack of specificity limits the accuracy of PET scans for response-adapted approaches and surveillance, despite improved sensitivity over CT scans.\textsuperscript{4,5} Imaging scans are “snapshots” of clinically detectable tumor and cannot capture dynamic processes such as tumor response kinetics, clonal evolution and cellular resistance.

With emergence of targeted therapy, clinically validated technology is needed to temporally assess the molecular heterogeneity across disease sites.\textsuperscript{6,7} Tissue biopsies determine molecular features of a tumor, but are prone to sampling bias and difficult to obtain serially. Circulating tumor DNA (ctDNA) can provide an average of the overall clonal heterogeneity and noninvasively assess molecular changes over time. Free from sampling bias of singular site biopsies, ctDNA integrates all genetic lesions and provides a more detailed view of the tumor.

Assessment of ctDNA is powerful technology that overcomes fundamental limitations of imaging scans and molecular analysis via tissue biopsies. In this review, we discuss the technical issues and present the promises of ctDNA for clinical application and novel research design.
Technical aspects of ctDNA

Fragmented DNA from normal and diseased tissue is constantly shed into the bloodstream as cell-free DNA (cfDNA) through processes of apoptosis, necrosis, and secretion.\textsuperscript{8,9} Cancer patients have higher overall levels of cfDNA than healthy people, but the fraction of DNA from malignant cells, captured as ctDNA, may be as low as 0.01%.\textsuperscript{10-12} Since ctDNA derives from tumor tissue, it’s a highly specific tumor biomarker.\textsuperscript{13} It is present without detectable circulating tumor cells, and correlates with tumor burden in early and late-stage malignancies.\textsuperscript{14,15} Assays designed for molecular monitoring must accurately discriminate ctDNA from DNA that originates in non-malignant tissue.\textsuperscript{16}

Molecular monitoring of lymphoma by ctDNA has gained momentum due to technical advances in detection capability and processing speeds (Table 1). Quantification of ctDNA in B-cell lymphomas using polymerase chain reaction (PCR) analysis of rearranged immunoglobulin heavy chains are subject to relatively low sensitivity and artifact, limiting their clinical utility.\textsuperscript{17} Modern platforms combine universal PCR primers for the variable-dense-joining (VDJ) regions of immunoglobulin receptors with next-generation sequencing (NGS) resulting in highly sensitive and specific detection of ctDNA in multiple B-cell lymphomas.\textsuperscript{15,18,19} As a surrogate for the entire tumor genome, ctDNA may also be analyzed for tumor-specific mutations, commonly referred to as a “liquid biopsy.”\textsuperscript{20,21} Modern NGS techniques have the necessary specificity and bandwidth to identify genetic aberrations circulating at low allele frequencies.\textsuperscript{22,23}
Methods to identify specific ctDNA sequences perform best on tumor biopsies. Clonal VDJ sequences can be determined from baseline tumor tissue in over 85% of DLBCL cases but with a significantly lower yield from blood samples.\textsuperscript{15,19,24} Targeted re-sequencing mutational panels for ctDNA are now emerging, but also require baseline biopsy samples to confirm tumor origin.\textsuperscript{22,23,25} Hence, quality DNA extraction from pretreatment tissue is important and is affected by low tumor content and necrosis.

Within peripheral blood samples, high rates of DNA fragmentation and low ratios of tumor DNA are methodologically challenging barriers. Normal DNA contamination from white blood cells is typically higher in the serum than plasma, and delay and temperature of blood samples before centrifugation affect DNA concentration.\textsuperscript{26} Specialized collection tubes (Streck\textsuperscript{®}) can reduce DNA degradation by nucleases and contamination from white blood cell DNA.\textsuperscript{27} While successful analysis of ctDNA can be performed on stored serum samples, standardized collection procedures will optimize the analysis, particularly for detection of tumor mutations.\textsuperscript{26,28}

**Monitoring tumor genotype**

The complete molecular heterogeneity of a tumor cannot be adequately assessed by single or even multiple biopsies, whereas a “liquid biopsy” captures genetic information shed from all sites of disease.\textsuperscript{16} Recent reports demonstrate high concordance between mutations within tissue and targeted re-sequencing panels from ctDNA\textsuperscript{23,25} Analysis of ctDNA can also detect somatic mutations not identified in tumor biopsies. However, mutations with low allele frequencies are difficult to detect in the blood, suggesting that the different methods provide complementary information. Liquid
biopsies of ctDNA can also be effective when needle biopsies are challenging, such as isolated CNS disease.\textsuperscript{15,19,29} Recent reports have demonstrated that tumor-associated mutations are detectable in the plasma and cerebral spinal fluid of patients with primary central nervous system lymphoma (including authors unreported data).\textsuperscript{30}

In addition to spatial heterogeneity, lymphomas exhibit temporal heterogeneity and continuously evolve over time, particularly under treatment selection pressure (Figure 1). In the case of DLBCL, evidence suggests recurrent disease is comprised of multiple genetically distinct sub-clones. Serial analysis of ctDNA for mutation allele frequency can analyze clonal evolution in consecutive samples and reveal a shift in the dominant sub-clone with potential implications on treatment.\textsuperscript{31-33} Monitoring ctDNA is a promising method for assessing sub-clone allele frequency to overcome both spatial and temporal tumor heterogeneity.\textsuperscript{7}

**Monitoring tumor VDJ sequence**

DLBCL is molecularly heterogeneous, but rearranged VDJ immunoglobulin receptor genes are unique to each patient’s tumor and readily detectable by modern NGS platforms.\textsuperscript{34} Quantitative high-throughput methods that combine PCR-based amplification of immunoglobulin gene segments and NGS can detect and quantify ctDNA from B-cell lymphomas in the blood with a detection limit of one tumor cell equivalent per $10^6$ diploid genomes.\textsuperscript{15,19}

A recent study in 126 patients with untreated DLBCL demonstrated that ctDNA of VDJ predicted early treatment failure and serial monitoring after therapy identified disease recurrence months before CT scans.\textsuperscript{15} Another study in a separate cohort of
DLBCL patients demonstrated that monitoring cell-free ctDNA of VDJ in plasma was more effective than monitoring circulating cells with the same assay.\textsuperscript{19} Notably, both studies showed a significant association between the quantity of pre-treatment ctDNA and indices of tumor mass.

**Applications of ctDNA monitoring**

Clinical applications of ctDNA depend on the assay performed, the timing of the assay, and the goal of therapy (Table 2). The therapeutic goal for aggressive B-cell lymphomas, such as DLBCL, is cure. This requires eradication of all tumor clones or disease recurrence is virtually inevitable. Molecular relapse of ctDNA heralds failure of curative treatment. The highly sensitive and quantitative characteristics of VDJ ctDNA leads to early detection of treatment failure compared with conventional imaging (Figure 2A).\textsuperscript{15,19} Indeed, identification of a molecular relapse allows institution of salvage treatment before the development of high disease burden that is required for clinical detection of recurrence.

Indolent lymphomas present a different scenario because they generally are not treated with curative intent. In this setting, monitoring of ctDNA provides a quantitative estimate of sensitivity to treatment and can be monitored serially to guide treatment decisions. The common practice to treat indolent lymphomas for prolonged and even indefinite periods of time offers opportunities for ctDNA monitoring (Figure 2B). In some cases, extended treatment may be unnecessary, such as in patients who become ctDNA negative (i.e. minimal residual disease (MRD) negative), whereas in other cases, resistant clones may accumulate below the level of imaging detection. In both cases,
serial monitoring of ctDNA could inform clinical decision-making including treatment duration, retreatment timing and guide precision treatment based on features of the dominant clones (Figure 1).

Pre-treatment Applications

ctDNA re-sequencing mutational panels (liquid biopsies) and quantification of pre-treatment VDJ ctDNA provide novel opportunities for precision treatment. Multiple studies have shown a correlation between clinical determinants of tumor burden and quantitative ctDNA. A few studies have reported that higher pretreatment concentrations of ctDNA are associated with a poorer prognosis, possibly due to higher tumor burden, and hence the potential for more aggressive treatment. Molecular features such as tumor metabolism and/or proliferation may also be captured by ctDNA analysis and provide new areas of investigation. The molecular information obtained from liquid biopsies may provide valuable information on targetable oncogenic pathways and mutations, which are heterogeneous in DLBCL and often track with the cell of origin.

Response Assessment

The current response criteria for lymphoma rely on CT and PET imaging, and the completeness of response to initial therapy is prognostic. Detection of subclinical disease by ctDNA, however, could identify patients not in complete molecular remission (CMR) (Figure 2A). A recent study of ctDNA for tumor VDJ in DLBCL showed that patients who did not achieve a CMR were not cured, indicating its importance in
aggressive lymphomas.\textsuperscript{15} For indolent lymphomas, achieving CMR may assist with treatment decisions regarding maintenance therapy and provide a novel endpoint for clinical trials and drug approval. In follicular lymphoma, PCR-based primers to detect BCL2 breakpoints have been used to assess molecular remission, which is associated with improved outcome.\textsuperscript{37,38} Similar findings have been reported in mantle cell lymphoma using PCR-assays to detect the CCND1/IgH translocations.\textsuperscript{39} These findings support the importance of molecular remissions.

\textbf{Interim Monitoring}

Interim ctDNA monitoring (i.e. during treatment), like interim PET scans, can be developed for risk-adapted treatment strategies including early treatment termination for patients in molecular remission or treatment modification for patients with poorly responsive tumors. While risk-adaptive strategies based on interim PET scans can identify DLBCL patients at risk of treatment failure, two recent prospective studies failed to show clinical benefit from switching therapy, indicating the need for more sensitive and specific selection methods.\textsuperscript{40-42} Interim ctDNA monitoring has potential advantages over PET scans due to high tumor specificity, quantitative analysis, and temporal kinetics. Indeed, quantitative response kinetics may improve upon response-adapted strategies in lymphomas. Our group studied the predictive significance of quantitative ctDNA for VDJ after each treatment cycle in previously untreated patients with DLBCL.\textsuperscript{15} Patients who cleared ctDNA after two cycles were significantly more likely to be progression-free at 5 years compared to patients who were ctDNA positive (80.2\% v. 41.7\%, \textit{p}<0.0001).\textsuperscript{15} Approximately half the patients cleared ctDNA after only one cycle
and 78% were negative after two treatment cycles. Various patterns of interim ctDNA kinetics were associated with early treatment failure, but absence of clearance was associated with the shortest survival.\textsuperscript{15} Other reports of interim ctDNA monitoring in DLBCL have also observed that ctDNA concentrations after only 1-2 cycles are predictive.\textsuperscript{22} Future studies are needed to validate the clinical utility of interim ctDNA and its role with interim PET scans.

**Post-Treatment Monitoring**

The ability to identify patients with aggressive lymphomas before they develop clinically detectable disease has significant appeal because some may yet be cured with further treatment (Figure 2A). While this has not been prospectively validated, it has been shown that patients with lower disease burdens at recurrence have better outcomes.\textsuperscript{43} The potential value of surveillance ctDNA monitoring lies in its low detection limit and ease of repetition. We showed that serial surveillance monitoring of ctDNA for VDJ after therapy in DLBCL detected recurrence a median of 3.5 (range 0-200) months prior to CT scans.\textsuperscript{15} In patients who relapsed greater than 6 months from the end of therapy, ctDNA was identified in 91\% (10/11), and 80\% (8/10) were detected prior to clinical disease on CT scans. In another series, similar rates of detection prior to relapse were observed.\textsuperscript{19} Paradigms for molecular surveillance monitoring will differ based on the lymphoma subtype and therapeutic goals. For indolent lymphoma, the usual clinical goal is disease control, although the potential for cure cannot be ruled out. In the case of long-term disease control despite the persistence of malignant clones, the
goal of surveillance monitoring is to assess the malignant clone kinetics clonal evolution (Figure 1).

cDNA and precision medicine

Identification of the molecular aberrations within an individual patient’s lymphoma will be needed for precision treatment decisions.\(^6\) Barriers to precision treatment include spatial and temporal tumor heterogeneity.\(^7\) Although in its infancy and unvalidated for clinical decision-making, the liquid biopsy has obvious advantages for selecting treatment based on the identification of dominant resistant clones (Figure 1). For example, increasing frequency of clones with myc translocations or TP53 mutations suggest emerging resistance, and may allow treatment modifications with targeted agents before the emergence of clinically detectable disease (Figure 1). Individual somatic mutations can also predict response to target agents as recently shown with CD79B and MYD88 mutations in DLBCL and response to ibrutinib.\(^{44}\) Such information is likely to significantly improve outcomes through rational timing of treatment and precision drug selection. A new generation of “smart trials” based on this technology may also lead to more rational and rapid drug development and approval.

Conclusions

Circulating tumor DNA has the potential to transform clinical care paradigms and future trial designs. The ability of tumor VDJ ctDNA to dynamically assess molecular tumor response and detect recurrence of occult disease in B-cell lymphomas will undoubtedly improve the care of these patients. For indolent lymphomas, ctDNA
provides a critical “look below the water line” that can aid clinical decisions on treatment timing and duration. Liquid biopsies of tumor genotypic ctDNA add a further dimension that can integrate all the genetic lesions within a tumor and optimally address traditional barriers to precision treatment such as tumor heterogeneity and clonal evolution. As we move forward, it will be imperative to standardize collection, storage, and processing procedures for ctDNA technology and validate its clinical and research utility.

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Contributions
All authors (MR, LMS, and WHW) made a substantial contribution to discussion of the content. MR wrote the first draft of the manuscript and WHW revised the final draft. All authors reviewed and edited the final manuscript prior to submission.

Conflicts of interest
The authors declare no competing conflicts of interest
REFERENCES


### Table 1. Methods for molecular monitoring of ctDNA for non-Hodgkin lymphoma

<table>
<thead>
<tr>
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<th>ctDNA of IgH</th>
<th>ctDNA of VDJ sequence</th>
<th>Tumor genotypic ctDNA</th>
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<tbody>
<tr>
<td><strong>Technique</strong></td>
<td>Allele-specific PCR</td>
<td>PCR + NGS</td>
<td>NGS</td>
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<tr>
<td><strong>Primary tumor required</strong></td>
<td>Yes</td>
<td>No (^1)</td>
<td>No (^2)</td>
</tr>
<tr>
<td><strong>Sensitivity(^3)</strong></td>
<td>1 in 10(^5)</td>
<td>1 in 10(^6)</td>
<td>Unknown</td>
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<tr>
<td><strong>Processing time</strong></td>
<td>Weeks</td>
<td>1-2 weeks</td>
<td>1-2 weeks</td>
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<tr>
<td><strong>Track clonal evolution</strong></td>
<td>No</td>
<td>Limited</td>
<td>Possible</td>
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<tr>
<td><strong>Track resistance</strong></td>
<td>No</td>
<td>Limited</td>
<td>Possible</td>
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<tr>
<td><strong>Potential</strong></td>
<td>Tumor specific Quantifiable</td>
<td>Tumor specific Quantifiable</td>
<td>Broad genomic coverage Track clonal evolution Track resistance mechanisms</td>
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<tr>
<td><strong>Limitations</strong></td>
<td>Not universal Specific primers required Limited foci assessed</td>
<td>Limited genotypic information</td>
<td>Low allele frequency Molecular heterogeneity of tumor</td>
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</tbody>
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\(^1\)Tumor clonotype can be determined without baseline tissue but the yield is lower  
\(^2\)Mutational panels common to lymphoma subtypes would obviate the need for baseline tumor  
\(^3\)Sample size required to detection one cellular equivalent  

**Abbreviations:** IgH, immunoglobulin heavy chain; VDJ, variable-dense-joining region of the immunoglobulin receptor; PCR, polymerase chain reaction, NGS, next-generation sequencing;
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<th>Pretreatment</th>
<th>On treatment</th>
<th>End-of-treatment</th>
<th>After treatment</th>
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<tr>
<td><strong>ctDNA of VDJ sequence</strong></td>
<td>Prognostic tool</td>
<td>Quantitate tumor kinetics</td>
<td>Molecular remission</td>
<td>Early recurrence detection</td>
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<td><strong>Tumor genotypic ctDNA</strong></td>
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<td>Early resistance mechanisms</td>
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<td>Late resistance mechanisms</td>
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<td>Select next therapy</td>
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<td>Response-adapted therapy</td>
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Figure 1. Serial monitoring with circulating tumor DNA to guide precision medicine. Lymphomas are composed of multiple tumor clones and sub-clones that serially evolve over time, especially under the selective pressure of therapy. Liquid biopsies of ctDNA detect molecular features of resistant disease at the molecular level and can noninvasively genotype ctDNA throughout the disease course. Serial monitoring of ctDNA may be a powerful tool to address temporal heterogeneity of tumors, to detect clonal evolution, and to study mechanisms of treatment resistance. The timing and nature the dominant relapsing clone may guide precision treatment at relapse. As examples, patients who relapse with a myc rearrangement (green) might be offered targeted therapy with a BET inhibitor, while patients who relapse with a TP53 mutation (magenta) could be offered an MDM2 inhibitor.

Figure 2. Monitoring circulating tumor DNA enhances detection of relapse and defines molecular remission. The lead-time offered by serial monitoring of ctDNA represents an opportunity for early intervention with minimal tumor burden. The clinical applications would differ when treating with curative intent (i.e. aggressive lymphomas) or more extended duration of therapy (i.e. indolent lymphomas). A. Monitoring therapy for with ctDNA for curative intent. The patient with no relapse (green) achieves a complete molecular remission and represents successful cure of lymphoma. The patient with late relapse (blue) initially achieves a complete molecular remission, but has ctDNA reappear prior to imaging, which creates a lead-time for possible intervention. The patient with early relapse (red) has rising levels of ctDNA shortly after completion of therapy with a narrower lead-time. The patient with primary refractory disease (brown) has persistence of minimal residual disease at the end of therapy that is undetectable by imaging. B. Monitoring therapy with ctDNA for extended duration. B. Indolent lymphomas are frequently treated for extended durations with maintenance therapy designed to prolong duration of remission.
Successful maintenance therapy (green) could be monitored with ctDNA and continued as long as disease remains undetectable. Patients who have the reappearance of ctDNA while on maintenance therapy (red) might be considered for alternative therapy before clinical effects. Patients who are not initially treated with maintenance therapy can be offered “delayed maintenance” at a time when disease is detectable by ctDNA but not yet detectable by imaging scans.
Circulating tumor DNA (ctDNA)

Initial therapy

Detection limit of imaging

Detection limit of ctDNA

Initial diagnosis

MYC+

BET inhibitor

TP53+

MDM2 inhibitor

Figure 1
Figure 2

(A) Circulating tumor DNA (ctDNA) over time. Different colored lines represent different stages of treatment and relapse:
- **Green line**: No relapse
- **Blue line**: Late relapse
- **Red line**: Early relapse
- **Orange line**: Primary refractory

Detection limits of imaging and ctDNA are indicated by dashed lines. Lead time is also marked.

(B) Circulating tumor DNA (ctDNA) over time for different maintenance therapy scenarios:
- **Green line**: Maintenance therapy, no progression
- **Red line**: Maintenance therapy with progression
- **Blue line**: Delayed maintenance therapy

Detection limits of imaging and ctDNA are also indicated.
Dynamic monitoring of circulating tumor DNA in non-Hodgkin lymphoma

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