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Liquid biopsy in lymphoma

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Background

The term "liquid biopsy" means accessing tumor DNA through a blood sampling, without the need of an invasive tissue biopsy. Cell-free fragments of DNA (cfDNA) are shed into the bloodstream by cells undergoing apoptosis and circulate at a low concentration in plasma as double-stranded DNA fragments that are predominantly short (<200 base pairs).[1] In healthy subjects, cfDNA primarily derives from the apoptosis of cells of hematopoietic lineage, with minimal contributions from other tissues, and circulates in concentrations of 1-10 ng/mL of plasma.[2-8] In lymphoma patients, a proportion of cfDNA derives from apoptotic tumor cells.[5] The total amount of cfDNA in lymphoma patients is always increased compared with age- and gender-matched healthy subjects, with a mean concentration of 30 ng/mL of plasma.[9-12] Levels of circulating tumor DNA (ctDNA) vary across different lymphoma subtypes, being higher in aggressive lymphomas than in indolent lymphomas. Beside lymphoma type, tumor volume also affects cfDNA levels, which are higher in advanced stage disease than in limited stage disease, and in overt progressive disease than in a disease that is clinically responding to treatment.[9,11]

This perspective aims at describing the unmet needs in the field of diagnosis, genotyping, and assessment of treatment response in lymphomas that can be addressed by ctDNA technologies, as well as current evidence, and/or further investigations or actions that would be needed before transferring ctDNA technologies into the clinic.

Technologies for ctDNA identification and measurement

By using the tumor mutation profile or the immunoglobulin gene rearrangement as lymphoma fingerprints, normal cfDNA can be discriminated from cfDNA derived from tumor cells, also called circulating tumor DNA (ctDNA).[9,12-15] ctDNA fraction in the pool of cfDNA originating from hematopoietic cells is frequently very small. Therefore, the test used for ctDNA detection and quantification must suppress both the technical noise (i.e. reduce the background errors) and the biological noise (i.e. suppress true mutations originating from an underlying clonal hematopoiesis by sequencing paired granulocytes genomic DNA) in order to reach the required analytical sensitivity and specificity.[15,16] Finally, sensitivity strongly relies on input material quantity and quality. For example, a single gene test can only achieve a sensitivity of 1 in 10,000 (i.e. 10^{-4}) if the input material matches or exceeds this threshold.

When the mutation profile is used as tumor fingerprint, the type of genetic aberrations being detected guide the choice of the molecular technique to be used for ctDNA identification and quantification. A single, trunk, fully clonal, stereotypic genetic variant, that occurs in most patients, characterizes a few lymphoma types [eg. the *MYD88* L265P mutation in lymphoplasmacytic lymphoma and primary central nervous system lymphoma (PCNSL)].[17] Such mutations can be detected and quantified by PCR-based methods like mutation-specific droplet digital PCR.[17]

Molecular aberrations of most lymphomas, however, are heterogeneous. Ultra-deep next-generation sequencing (NGS) methods can overcome the limitations of assays covering single somatic variants by detecting a large spectrum of genetic alterations, including single nucleotide variants, insertions/deletions, chromosomal rearrangements, and copy number changes.[18-20] The Cancer Personalized Profiling by Deep Sequencing (CAPP-seq) is a targeted capture ultra-deep NGS method for ctDNA detection and quantification in molecular heterogeneous tumors (Figure 1).[18,19] CAPP-seq utilizes a disease-specific "selector", which is a set of exonic and intronic targets chosen to cover regions of

known recurrent mutations for a particular cancer type. Those targets are then amplified and sequenced in a patient's cfDNA sample, allowing quantification of ctDNA based on the detection of tumor-specific mutations, and simultaneous determination of an individual's specific tumor mutation profile. This method can simultaneously assay all classes of mutations, including single nucleotide variants, insertion/deletions, copy number alterations and rearrangements.[18-20] The "selector" is tumor specific and requires detailed knowledge of the underlying genetic landscape of the tumor, a limitation that is currently overcome by the availability of from dozens to hundreds of genomes across all types of lymphoma.

The clonoSEQ Assay is a diagnostic test validated and approved for measuring minimal residual disease (MRD) on genomic DNA from bone marrow samples in leukemias and myeloma.[21,22] In the assay, genomic DNA is amplified by a set of locus-specific multiplex PCR using V, D and J gene primers covering all possible rearranged IgH (VDJ), IgH (DJ), IgK, and IgL receptor gene sequences. The amplicon library is then subjected to ultra-deep NGS. The tumor-specific clonotype is first identified in a tumor-enriched biological sample and then tracked within the repertoire of IgH, IgK and IgL rearrangements amplified and sequenced in post-treatment samples. By leveraging on the advantage that the IgH, IgK and IgL rearrangements represent a stable and tumor specific fingerprint, the clonoSEQ Assay has also been applied to ctDNA quantification in lymphomas.[9,10,12] However, tracking IgH, IgK and IgL sequences has some shortcomings when applied to cfDNA, including the need for lymphoma clonotype assignment through the analysis of the tissue biopsy, limited sensitivity in low tumor burden settings, and reduced applicability because of somatic hypermutation (SHM), which is ongoing in some lymphoma types such as diffuse large B-cell lymphoma of the germinal center type and follicular lymphoma, leading to difficulties in identifying clonotypic sequences.

Overall, although methods for ctDNA identification and quantification are becoming more common, they are not yet widely used in clinical laboratories and are not, therefore, prominently featured in disease management guidelines. Methodological challenges, both in molecular biology and bioinformatics analyses, must be overcome, standardized and harmonized as these methods become more routinely used.

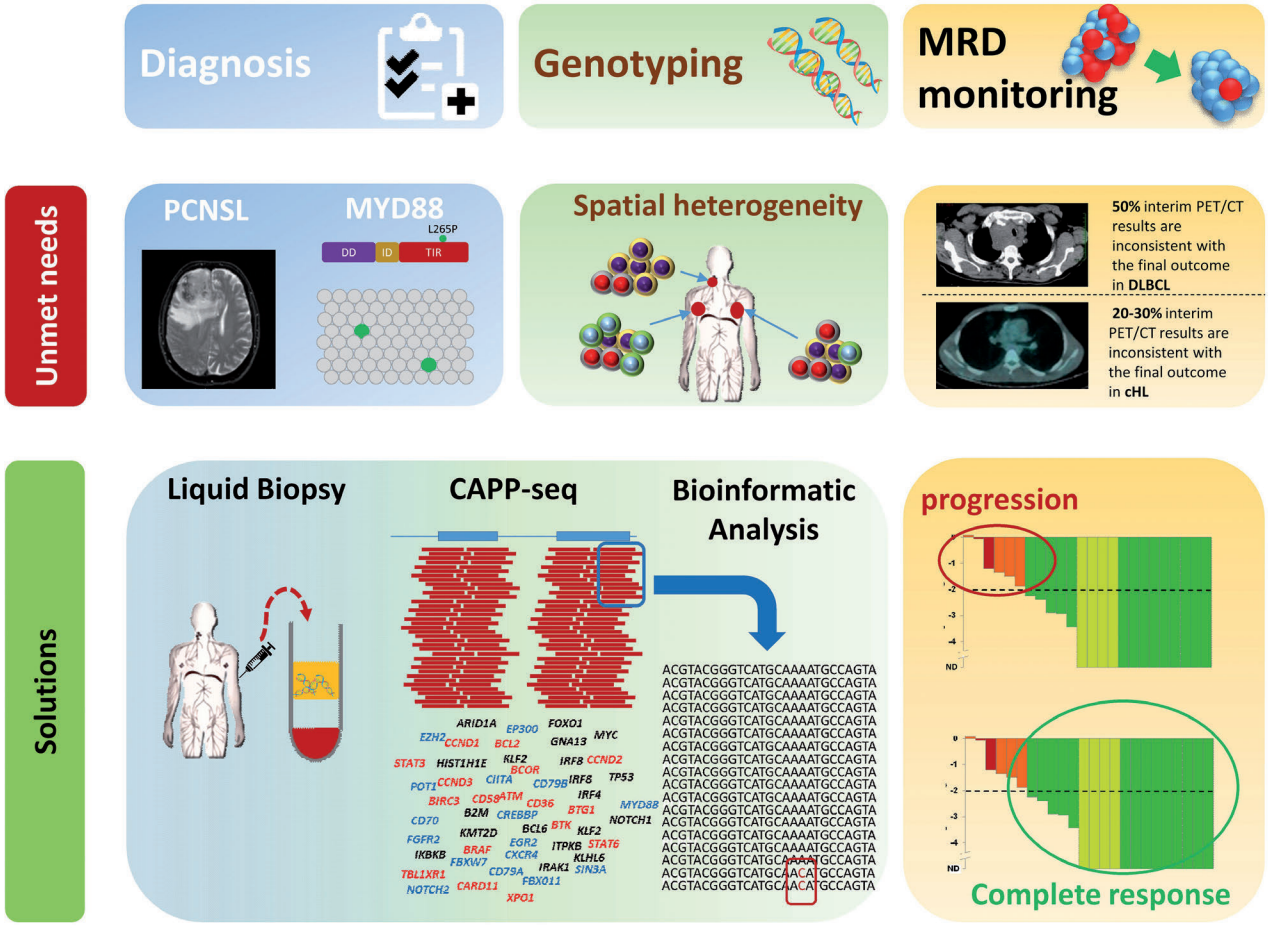

Figure 1. Schematic representation of liquid biopsy unmet needs in diagnostic, genotyping and minimal residual disease (MRD) monitoring fields with relative actions to overcome these limitations. PCNSL: primary central nervous system lymphoma; PET / CT: positron emission tomography/computed tomography; DLBCL: diffuse large B-cell leukemia; cHL: classic Hodgkin lymphoma; CAPP-seq: Cancer Personalized Profiling by Deep Sequencing.

Lymphoma diagnosis by ctDNA

ctDNA cannot substitute tissue biopsy for lymphoma diagnosis. Only one single, rare, special scenario, namely the non-invasive diagnosis of PCNSL in those patients whose brain masses are surgically inaccessible, might one day be able to take advantage of the diagnostic potential of ctDNA. The diagnostic procedure of choice to establish the diagnosis of PCNSL is a stereotactic biopsy; if ocular or cerebrospinal fluid (CSF) involvement is evident, vitrectomy or CSF cytology may be sufficient. If a biopsy of the brain lesion is not possible, and CSF or ocular involvement is ruled out, histological diagnosis can be difficult at both initial stages and at relapse. The *MYD88* L265P mutation occurs in up to 85% of tissue biopsies from PCNSL patients but never in those from non-hematologic brain tumors, suggesting that this mutation is a fairly sensitive and highly specific biomarker for differential PCSNL among central nervous system cancers.[23-30] Droplet digital PCR assays probing the *MYD88* L265P mutation in cfDNA samples from PCNSL patients known to harbor the *MYD88* L265P have a 60% true positive rate.[17] However, droplet digital PCR assays for detecting the *MYD88* L265P mutation in cfDNA are far from being a validated non-invasive diagnostic test of PCNSL. Indeed, apart from standardization of the technique to suppress the false positive rate originating from the methodology, there are no data on the biological false positive rate of this assay. The *MYD88* L265P mutation occurs in pre-malignant conditions such as monoclonal gammopathies of undetermined significance (MGUS) and monoclonal B-cell lymphocytosis (MBL). Both are relatively common in the older adult, and thus can co-occur by chance with a brain mass in the same subject, raising the issue of false positive results originating from a biological background (Figure 1).[31,32] Plasma samples from large cohorts of patients diagnosed with a brain mass should be tested with standardized droplet digital PCR assays for the *MYD88* L265P mutation to precisely define its diagnostic accuracy before bringing this test into diagnostic routine practice for PCNSL.

Tumor genotyping by ctDNA

Tumor genotyping of lymphomas lacking a leukemic phase has so far relied on the analysis of the diagnostic tissue biopsy. However, multiregional sequencing showed that the diagnostic tissue biopsy might be subject to a selection bias resulting from spatial heterogeneity and, therefore, might not be representative of all the tumor genetics.[33] Indeed, in follicular lymphoma, different areas of the same tumor may show different genetic profiles (i.e. intratumoral heterogeneity).[34] A biopsy from one part of a tumor may miss mutations occurring in subclones residing in anatomically distant sites, including clinically relevant genetic biomarkers for treatment tailoring or anticipation of resistance.[33] Furthermore, serial sampling of tumor material through repeat biopsies is not usually feasible in lymphomas lacking a leukemic phase, hampering efforts to understand patterns of genomic evolution during disease progression and the development of treatment emergent resistant mutations. On the basis of this, lymphoma genotyping on ctDNA can complement, though not entirely substitute, the analysis of the diagnostic tissue biopsy in order to deal with the clinical need of a comprehensive and easily accessible tumor genotyping. ctDNA is representative of the entire lymphoma heterogeneity, thus bypassing the bias imposed by tissue biopsies in the reconstruction of the entire cancer clonal architecture, and identifying resistant clones that are dormant in non-accessible tumor sites. Accessing the blood stream has also a clear advantage for sampling in the serial monitoring of treatment emergent resistant mutations in real time.[35]

Independent studies have assessed the sensitivity and specificity of targeted gene mutation analysis in ctDNA *versus* tumor biopsy as gold standard from untreated DLBCL patients by using CAPP-Seq (Figure 1).[15,36,37] The recovery rate of confirmed mutations (i.e. true positive rate) in the tumor biopsy ranges from 95% to 99%. The mutations confirmed by biopsy that were missed in ctDNA (i.e. false negative rate) range from 1% to 5% and are mostly of low allelic abundance in the tumor. After suppressing the biological background originating from clonal hematopoiesis by the sequencing of matched granulocyte DNA, such a false positive rate is represented by somatic variants recovered in cfDNA but absent in the tumor biopsy due to tumor mutations restricted to clones that are anatomically distant from the biopsy site.[15,36,37] CAPP-seq of ctDNA thus stands as a robust and validated technology for accurate DLBCL genotyping. Genotyping of ctDNA by CAPP-seq allows recovery of 100% of tumor biopsy-confirmed actionable mutations of DLBCL, like *EZH2*, *MYD88*, *CD79B*, and longitudinal monitoring in the blood of the emergence of ibrutinib-resistant mutations.[15,36-38] These data support the implementation in the clinic of this non-invasive technique in both settings. CAPP-seq standardization is, however, required before bringing this test into diagnostic routine practice for DLBCL (Figure 1).

ctDNA is an alternative source of tumor DNA when representation of lymphoma cells is insufficient in the tissue biopsy, as in classic Hodgkin lymphoma (cHL).[16,39] The rarity of neoplastic Hodgkin and Reed-Sternberg cells in the biopsies is a limit to the genetic characterization of cHL, which can only be overcome by complex techniques for tumor cell enrichment that are beyond the budget of a diagnostic lab. By CAPP-seq, biopsy-confirmed tumor mutations are detectable in ctDNA samples with a true positive rate of 87% in cHL patients.[16] Though clinical application is still a long way off, CAPP-seq of ctDNA opens up the opportunity of genotyping large cohorts of cHL patients for the identification of genetic prognostic biomarkers and, within clinical trials, for the identification of biomarkers predictive of response to treatment.

Residual disease quantification by ctDNA

Due to the lack of a leukemic dissemination, MRD monitoring has so far been limited to tissue-born lymphomas without bone marrow (BM) involvement, such as DLBCL and cHL. MRD monitoring in lymphomas is defined as any approach aimed at detecting, and possibly quantifying, residual tumor cells beyond the sensitivity level of routine imaging techniques. Whenever a patient achieves complete clinical remission, a number of different scenarios may actually be taking place, including full eradication of the neoplastic clone or persistence of residual tumor cells capable of giving rise to a full clinical relapse within months or years. According

to the Lugano criteria, positron emission tomography (PET)/computed tomography (CT) has become the recommended imaging strategy for sensitive disease response assessment in DLBCL and cHL.[40] The best classification of patients with good *versus* poor prognosis is reached by the end-of-treatment PET/CT. However, this timepoint would be rather late to adapt treatment strategies according to the quality and depth of response. Interim PET/CT performed after two cycles of treatment has been tested for the early identification of chemorefractory patients, as they are candidates for treatment intensification to maximize the chances of cure, as well as to identify good-risk patients early, as they are candidates for treatment de-escalation to avoid both short- and long-term complications of chemo-radiotherapy.[41] The accuracy of interim PET/CT has been considered adequate to inform early treatment intensification or de-escalation in both limited and advanced stage cHL.[42] However, even in the ideal technical and analytical setting, interim PET/CT results are inconsistent with the final outcome in approximately 20-30% of patients, who are thus still exposed to over- or under-treatment.[41,42]

In DLBCL, interim PET/CT does not correctly inform on the subsequent outcome in a larger number of patients than in cHL. Indeed, the positive predictive value of interim PET/CT in DLBCL is 50%.[43] This means that half DLBCL patients are misclassified by interim PET/CT as being R-CHOP resistant, but ultimately are converted to a negative PET/CT at the end of treatment and cured by R-CHOP. The negative predictive value of interim PET/CT is 70%.[43] This means that 30% of DLBCL patients are misclassified by interim PET/CT as R-CHOP sensitive, but ultimately relapse after R-CHOP. On the basis of this, interim PET/CT can not yet be adopted for clinical use to guide treatment decisions in individual DLBCL patients and remains a subject for research.

Minimal residual disease can be measured in tissue-born lymphomas without BM involvement and lacking a leukemic component by using ctDNA technologies. Compared to genomic DNA extracted from circulating mononuclear cells, plasma cfDNA harbors a 150-fold higher representation of tumor DNA, which makes cfDNA more reliable than genomic DNA from circulating cells for MRD monitoring in DLBCL.[12] By using the immunoglobulin gene rearrangements to quantify ctDNA in plasma, DLBCL patients with undetectable ctDNA after two chemotherapy courses show a superior progression-free survival compared with patients with positive ctDNA.[37] Despite its value as a prognostic tool, using the immunoglobulin gene rearrangement to quantify ctDNA in DLBCL has several shortcomings. This includes limited sensitivity in low tumor burden settings and reduced applicability because of the somatic hypermutation process, leading to difficulties in identifying clonotypic sequences.[12] In addition, as for PET/CT, also for high throughput sequencing of the immunoglobulin genes the best informative timepoint is end of treatment.[37]

By covering a large spectrum of genetic lesions, ctDNA quantification by CAPP-seq is cross-validated by multiple tumor tags, and avoids false negative results caused by treatment-induced clonal shift. In both DLBCL and cHL, the change in ctDNA measured by CAPP-seq after two cycles of therapy associates with both event-free and overall survival.[15,16,44] A drop of 100-fold (or 2-log drop) in ctDNA levels after two chemotherapy courses is associated with an eventual complete response and cure. Conversely, a drop of less than 2-log in ctDNA after two treatment courses is associated with an eventual progression.[16,44] Quantification of ctDNA coupled with PET/CT improves the accuracy of residual disease assessment at the interim time compared to the sole PET/CT in both DLBCL and cHL. Indeed, patients inconsistently judged as interim PET/CT positive, but having a negative (i.e. >2-log drop in ctDNA) liquid biopsy, are actually cured, while patients inconsistently judged as interim PET/CT negative, but having a positive (i.e. <2-log drop in ctDNA) liquid biopsy, are actually not cured.[16,44] These results generate the hypothesis that ctDNA may complement interim PET/CT in informing on DLBCL and cHL patients' outcome (Figure 1). Before translating this technology into the management of DLBCL, the precise cumulative sensitivity and specificity of PET/CT and ctDNA monitoring in anticipating the clinical course of patients should be precisely defined in clinical trials.

Further investigations

At the clinical level, it is critical that well-designed trials validate current concepts and further explore applications of ctDNA for interim monitoring, surveillance monitoring, and response assessment in lymphomas. The most immediate implementation of ctDNA technology in lymphoma clinical trials includes: i) non-invasive diagnostics of PCNSL; ii) baseline screening for the identification of patients harboring actionable mutations; iii) early and accurate identification of non-responding patients; iii) monitoring the development of resistance mutations against targeted agents (Figure 1).

At the technological level, standardization and harmonization projects, like those performed before the implementation of clinical MRD assessment in leukemias, should be designed and implemented also in lymphoma in order to meet clinical standards, and allow accurate, robust and reproducible results of ctDNA genotyping and quantification.

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