



RESEARCH ARTICLE

Liquid Biopsy Based Approaches for Subtyping Non-Hodgkin Lymphomas: A Proposal to Address an Important Unmet Clinical Need

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ABSTRACT

There are over 30 subtypes of non-Hodgkin lymphoma (NHL), each with distinct clinical and pathological features, prognoses, and treatment. Diagnosis of NHLs is made primarily through tissue biopsy. These procedures are invasive and carry risk, in addition to providing limited sampling from one portion of a single tumor lesion. Differentiating between subtypes of NHL can prove to be difficult, and incorrect diagnoses are not uncommon, which leads to delays in appropriate treatment. Thus, there is an unmet clinical need in the field, which we suggest can be improved by clinical implementation of liquid biopsy biomarkers. Liquid biopsies, including analysis of blood plasma for circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), and extracellular vesicles, are an emerging tool for diagnosis of tumors through blood or urine samples. In this review, we discuss the classification and significant features of the various subtypes of NHL, including their pathologic, clinical, and diagnostic criteria, and the latest progress on liquid biopsy biomarkers. The groundwork for studying and applying liquid biopsy biomarkers in non-Hodgkin lymphoma will aid in forming more accurate, less invasive diagnosis, which may ultimately help to guide earlier treatment and decrease disease burden.

Keywords: Non-Hodgkin lymphoma, liquid biopsy, extracellular vesicles, exosomes, biomarkers

Introduction

Non-Hodgkin Lymphomas (NHLs) are the most prevalent hematopoietic neoplasms in the world, and account for 4.3% of all cancers in the United States¹. NHL is an

umbrella term for over 30 different lymphomas, primarily of B-cell origin (85%), but occasionally of T-cell origin (15%)². They are frequently also divided into indolent (low-grade) and aggressive (high-grade) forms (Figures 1 and 2).

Figure 1. Frequencies of B-cell Non-Hodgkin Lymphomas.

Indolent B-cell lymphomas are in blue shades, while aggressive B-cell lymphomas are signified by red shades. The N.O.S. categorization here includes B-cell subtypes not otherwise specified (7%) and precursor forms of NHL (6%). Data is based on percentages from the National Cancer Institute’s Surveillance, Epidemiology and End Results (SEER) data, 2008-2017¹.

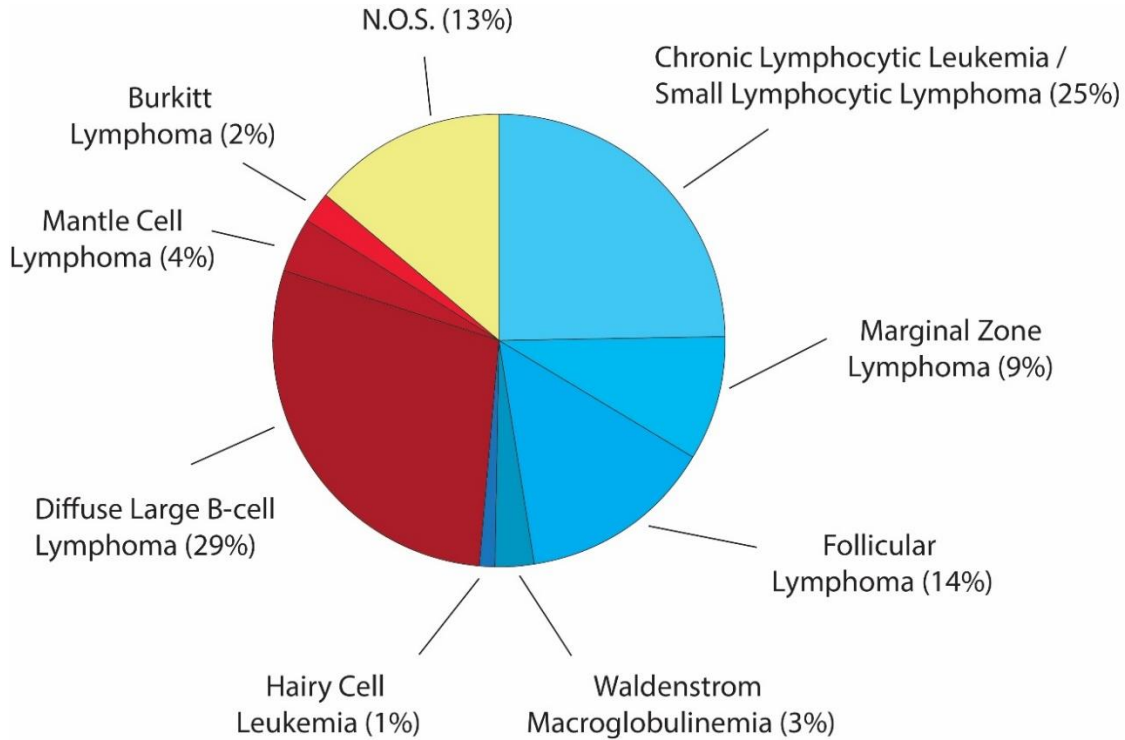
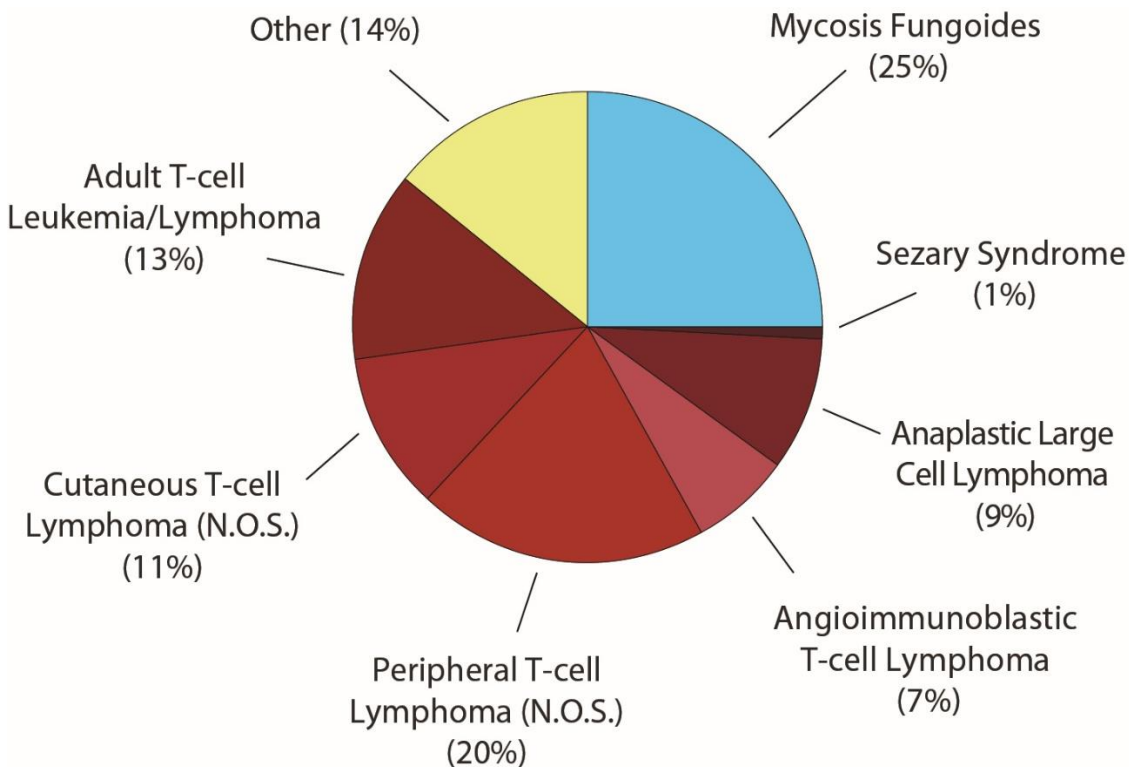


Figure 2. Frequencies of T-cell Non-Hodgkin Lymphomas.

Indolent T-cell lymphomas are in blue shades, while aggressive T-cell lymphomas are signified by red shades. The “other” categorization includes precursor forms of NHL and T-cell lymphomas under 1% prevalence. Data is based on percentages from the National Cancer Institute’s Surveillance, Epidemiology and End Results (SEER) data, 2008-2017¹.



Diagnosis of NHLs is typically made through tissue biopsy. Such procedures are invasive and carry risk, in addition to offering only a limited sample, from one portion of a single tumor lesion. Liquid biopsies are an emerging tool for generating a molecular profile of lymphomas. This can involve evaluation of mutations through molecular subtyping, development or prognosis, or prediction of treatment response. Analysis of circulating tumor DNA (ctDNA) and cell-free DNA fragments (cfDNA) derived from blood plasma are the most commonly used liquid biopsy approaches in lymphoma³. Extracellular vesicles (EVs) and their respective protein biomarkers have also emerged as important tools for predicting chemotherapy outcomes.

Ultimately, these methods can be used as either diagnostic or prognostic markers, and sometimes can serve both purposes. They may be able to provide a non-invasive means to determine the subtype of NHL, or to determine whether a particular type of lymphoma will be aggressive or indolent. Progress in this realm can fulfill the unmet clinical need of having easier, more rapid, non-invasive ways of evaluating lymphomas. In this review, we propose the utility of liquid biopsy biomarkers in differentiation between the numerous types of NHLs. We start by describing the various B-cell and T-cell lymphomas before discussing the advances and future potential of liquid biopsy-based approaches as biomarkers for NHL subtypes.

B-cell Lymphomas

BURKITT LYMPHOMA

Burkitt lymphoma (BL) is a highly aggressive form of NHL that is classically seen in children. There are strong associations with a t(8;14) translocation, seen in 75% of cases, and involving a reciprocal translocation of the *c-myc* gene on chromosome 8 with heavy-chain Ig on chromosome 14. This results in an overexpression of *c-myc*, and ultimately, subsequent increased expression of several genes involved in cellular proliferation. BL has been associated with a nearly 100% Ki-67 proliferation index, and as such, has the shortest doubling time of any human neoplasm⁴.

Burkitt lymphoma can be further subdivided into three variants: (1) sporadic, which occurs worldwide, (2) endemic, which occurs predominantly in sub-Saharan Africa and is strongly EBV-associated, and (3) immunodeficiency-associated, which occurs primarily in the setting of HIV. Each of the three clinical variants are at risk for seeding the CNS and bone marrow, which are associated with worse outcomes. The sporadic form, which classically presents with a mass in the abdomen, is more commonly seen in adults and may have features similar to diffuse large B-cell lymphoma (DLBCL). The endemic form is most commonly seen in children and is associated with involvement of the mandible and maxilla. Unlike the other two clinical variants, endemic BL has an exquisite sensitivity to chemotherapy, with children having localized disease achieving a >90% 5-year survival rate⁵. While sporadic and immunodeficiency-associated subtypes share a similar genetic profile, whole genome sequencing has revealed that endemic-type BL has more frequent mutations in *BCL7A* and *BCL6*, and less frequent mutations in *DNMT1*, *SNTB2*, and *CTCF*⁶. Further, gene

expression profiling techniques have revealed nearly identical profiles in sporadic and immunodeficiency-associated BL, but notable differences from sporadic BL, particularly in pathways B-cell receptors, TNF- α and interleukin-dependent cascades, and the RBL2-associated gene network⁷.

Patients with any variant of BL generally present with a rapidly growing mass, elevated lactate dehydrogenase (LDH), and high uric acid levels⁸. Diagnosis of BL in the acute setting is primarily done using imaging studies (ultrasound and CT), while tissue biopsy of the mass is seen as the gold standard for diagnosis⁹. A study in endemic Uganda, however, demonstrated only a 75% diagnostic accuracy due to inconsistency of local pathology procedures¹⁰. Furthermore, in BL endemic areas such as sub-Saharan Africa, where the capacity for invasive tissue biopsies is severely limited, there would be an important clinical utility of ctDNA to improve the speed of diagnosis¹¹. This highlights the importance of the development of more standardized diagnostic tools, especially in resource-constrained settings.

The primary differential diagnosis of Burkitt lymphoma is diffuse large B-cell lymphoma (DLBCL). This is a more pertinent problem in adults rather than children, due to the higher prevalence of DLBCL in adults. While Burkitt lymphoma has been classically associated with *c-myc* rearrangement, up to 15% of DLBCL has been shown to also share this similar rearrangement¹². The three subtypes of BL have been shown to share a homogenous microRNA (miRNA) profile that is distinct from DLBCL¹³.

FOLLICULAR LYMPHOMA

Follicular lymphoma (FL) is a highly common, generally indolent form of NHL. The majority of follicular lymphomas are characterized by a t(14;18) translocation, involving *bcl-2* on chromosome 18 with heavy-chain Ig on chromosome 14. This results in overexpression of Bcl-2 protein, which prevents release of cytochrome c from mitochondria, and ultimately results in decreased apoptosis. FL typically presents as an asymptomatic peripheral lymphadenopathy, and thus can go unnoticed for long periods of time. Diagnosis of follicular lymphoma is preferably through biopsy of a lymph node, where immunocytochemical staining is positive for CD19, CD20, CD10, monoclonal immunoglobulin, and *bcl-2*¹⁴.

EZH2 is one of the most commonly mutated epigenetic modifiers, present in up to 27% of follicular lymphomas¹⁵. While *EZH2* mutations have not been observed to have a prognostic impact, the frequencies of *EZH2* mutations are decreased or eliminated following successful treatment of FL, and persistently elevated *EZH2* levels are associated with treatment failure. A recent study demonstrated the efficacy of circulating tumor DNA mutation analysis of *EZH2* as a rapid, real-time monitoring tool for detecting levels of *EZH2* mutations¹⁶.

A majority of follicular lymphomas are indolent, and exhibit an excellent response to rituximab in combination with chemotherapy¹⁷. However, some follicular lymphomas can transform into DLBCL, which has a much poorer prognosis¹⁸. A number of prognostic markers have

been associated with the transformation of FL to DLBCL, including mutations in p53, MYC, BCL-2, and BCL-6¹⁸.

Studies on the utility of liquid biopsy in FL are still emerging. Hatipoğlu et al. suggested that taking pre-treatment concentrations and genotyping cfDNA could have clinical utility in FL for risk stratification and prediction for transformation. In addition, they showed that the presence of BCL2 mutation in cfDNA is associated with poor survival in treatment naïve patients¹⁹.

Recently, Jimenez-Ubieto et al. evaluated minimal residual disease (MRD) in FL based on tumor cfDNA detection using an ultra-deep sequencing approach and found that positive cfDNA following first-line therapy correlated with increased risk of progression²⁰. Martin Munoz et al. reported that a positive LiqBio-MRD test within the first 6 months of FL patients who underwent chimeric antigen receptor (CAR) T-cell therapy was a useful approach to identify patients with high risk of relapse²¹.

In a recent study by Bang et al., it was suggested that exosomal mRNA expression of BCL2 and BCL6 was correlated with disease progression both in FL and DLBCL²².

MARGINAL ZONE LYMPHOMA

Marginal zone lymphoma (MZL) is a generally indolent form of NHL with strong associations with numerous inflammatory states, including autoimmune disease²³ (Hashimoto's thyroiditis, Sjogren's syndrome) and chronic inflammation due to infection²⁴ (*H. pylori*, *B. burgdorferi*, *C. psittaci*, hepatitis C, Epstein-Barr virus). Commonly, marginal zone lymphoma, and particularly gastric mucosa-associated lymphoma tissue (MALT), is associated with t(11;18) translocations which result in formation of chimeric or fusions proteins that upregulate NF-κB, leading to transcription of multiple survival-related genes²⁵.

Marginal zone lymphoma is divided into three subtypes: nodal, extranodal, and splenic. Each subtype has specific diagnostic criteria, genetic characteristics and clinical pathogenesis²⁶. The diagnosis of MZL involves immunohistochemistry staining of tumor biopsy, including markers CD20, CD10, CD5, and cyclin D1²⁷. Importantly, each of the subtypes exhibit clinically distinct outcomes. Patients with nodal marginal zone disease present with more advanced-stage disease and have lower 5-year survival rates than patients with extranodal, gastric MALT lymphoma²⁸. Further, each of the variants have the potential to progress to DLBCL.

MANTLE CELL LYMPHOMA

Mantle cell lymphoma (MCL) is classically considered an aggressive, mature form of NHL with a historically poor prognosis. It is defined by a t(11;14) translocation in which *CCND1* on chromosome 11 is translocated with heavy-chain Ig on chromosome 14, leading to overexpression of cyclin D1 protein. This ultimately leads to promotion of transition from G1 to S phase through modulation of cyclin-dependent kinase proteins. Classically, the disease has been marked by rapid relapse and poor long-term outcomes. However, clinical

data has suggested considerable heterogeneity within this disease entity, with presentations ranging from indolent to highly aggressive disease²⁹. Diagnosis is established through histological analysis of a lymph node biopsy, including mandatory evaluation of cyclin D1 expression through immunohistochemistry³⁰. A number of markers have been suggested as prognostic indicators of long-term outcomes, including mantle cell international prognostic index (MIPI), Ki-67 proliferation index, and TP53 mutations^{31,32}. Differentiation of mantle cell from marginal zone lymphoma and chronic lymphocytic leukemia is challenging, due to significant overlap in the tissue immunophenotypes, including of CD5, CD19, CD20²⁹.

In a prospective study, Lakhotia et al. reported that detecting immunoglobulin receptor encoding ctDNA shows good correlation with tumor burden on PET scans and the clearance of ctDNA after first round of treatment was associated with improved progression free survival³³.

Plasma liposomes could also be considered as MCL biomarkers. In MCL, lipid metabolism via CD36 affects drug resistance and survival, and thus, ectopic expression of CD36 causes an increase in lipid droplets and is associated with bortezomib resistance in MCL cell lines³⁴.

CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) AND SMALL LYMPHOCYTIC LYMPHOMA (SLL)

CLL and SLL comprise the most common indolent forms of NHL. They are two very similar cancers, with the exception of site predominance. SLL cells are found in lymph nodes, while CLL cells are found in blood and bone marrow. Multiple causes of the disease have been suggested and evaluated, including chromosomal deletions and translocations, environmental exposures, and drugs, with numerous minor associations identified³⁵.

Diagnostic tools vary for CLL and SLL due to their site predominance. CLL diagnosis is performed primarily through flow cytometry of the blood, while lymph node biopsy is the gold standard for SLL diagnosis. The typical immunophenotype panel includes CD5, CD19, CD20, CD23, and CD19³⁶. Flow cytometry for cyclin D1 and fluorescence in situ hybridization (FISH) for t(11;14) are also often evaluated to rule out mantle cell lymphoma. Other important evaluations include TP53 mutation status and del(17p) by FISH as their presence guide selection of initial immunotherapy.

The cancers are characterized by the potential to transform into DLBCL through a Richter transformation resulting from a number of molecular events, including acquisition of TP53 mutations and 17p13 deletions³⁷. Clinical progression of SLL/CLL is heterogenous and depends on the presence of mutations in heavy-chain Ig variable regions, genomic changes, and comorbidities.

In a study from Yeh et al., it was demonstrated that CLL cells release abundant amounts of exosomes in plasma with a distinct CD37, CD9, and CD63 phenotype. They also provided evidence that members of miR-29 family such as miR-150, miR-155, and miR-223 are also associated with CLL³⁸.

De Luca et al. suggested that detection of serum microvesicles (MV) is a useful tool to follow disease. In their studies, the total number of MVs, mainly with CD19 and CD37 phenotype were significantly elevated compared to healthy donors and correlated with advanced clinical stage and poor prognosis³⁹.

DIFFUSE LARGE B-CELL LYMPHOMA

Diffuse large B-cell lymphoma (DLBCL) is the most common form of NHL, comprising 30-40% of cases worldwide⁴⁰. It is an aggressive form of NHL with an overall 5-year survival rate of 64%¹. Numerous mutations have been implicated in the pathogenesis of DLBCL, including in *bcl-2*, *bcl-6*, and *p53*, among others. Further, associations have been made with infectious causes, including EBV, HHV-8, HIV, and *H. pylori*. DLBCL can arise sporadically, or via transformation from follicular lymphoma, marginal zone lymphoma, or CLL. Multiple variants of DLBCL exist, of which the most common include centroblastic, immunoblastic, and anaplastic variants. Further, DLBCL can also be classified by cell of origin, including activated B-cell (ABC) subtype, germinal center-B cell (GCB), and Type III, or unclassifiable by gene expression profile⁴¹.

The gold standard in DLBCL diagnosis is through histological examination of either a surgical excision or needle core biopsy. Immunophenotype evaluation is used for diagnosis of DLBCL, similarly to other NHL subtypes. A number of recent studies have emerged highlighting the utility of cfDNA as both a prognostic biomarker and a predictor of treatment response in DLBCL^{42,43} with high cfDNA levels being associated with more aggressive disease presentation and depletion of cfDNA levels being associated with better treatment response.

Molecular subtyping of DLBCL has been shown to have important clinical utility. For example, a large subset of patients with activated B-cell DLBCL develop resistance against ibrutinib, a commonly used tyrosine kinase inhibitor in DLBCL. Recent work has identified mutations in *BCL10* as the molecular mechanism which confers resistance against ibrutinib⁴⁴. Preemptive molecular identification of this mutation can thus guide treatment away from ibrutinib to other agents. Along this line, previous studies demonstrated that mutant L265P MyD88 is associated with ibrutinib resistance, therefore could be a useful marker to follow ibrutinib sensitivity/resistance in DLBCL patients⁴⁵.

The role of EVs also emerged as a biomarker to follow DLBCL patients. Metthiesen et al. showed that EVs could be used for DLBCL monitoring and early identification of residual disease as well as response to therapy⁴⁶.

Finally, Rinaldi et al. showed promising data that miR-22 could be used as a biomarker to predict poor outcome in DLBCL patients⁴⁷.

PRIMARY DLBCL OF THE CENTRAL NERVOUS SYSTEM (CNS)

According to the 4th edition of the WHO classification, primary DLBCL of the CNS is defined by lymphoma arising from the brain, spinal cord, leptomeninges, or eye with exclusion of lymphomas of the dura, intravascular large B-cell lymphomas, secondary spread of systemic

lymphomas, and all other lymphomas that are related to immunodeficiency. The clinical prognosis of this disease is poor⁴⁸. The current gold standard of the diagnosis is biopsy; however, the procedure carries a risk of surgical complication⁴⁹. Therefore, introduction of non-invasive methods has an important clinical significance.

Liquid biopsy of the cerebrospinal fluid (CSF) is a less invasive, non-surgical method that could potentially be used for the diagnosis of primary CNS lymphomas (PCNSL). Mutations of MYD88 gene, specifically, the p.L265P amino acid substitution can be found in up to 85% of cases, therefore it could be used as a potential biomarker to identify primary CNS lymphomas. In a study of Yamagishi et al., using digital PCR showed that MYD88 L265P mutation was successfully identified in CSF with high sensitivity and specificity⁴⁹. MYD88 mutation from intraocular fluid was successfully detected in a cohort of patients with vitreoretinal lymphoma as well⁵⁰. Besides MYD88 mutation identification in ctDNA, the diagnostic value of microRNA and inflammatory cytokines has emerged as well. miR-21, miR-19b, and miR-92a concentrations in the CSF were significantly elevated in PCNSL compared to other neurological diseases⁵¹. In addition, CSF concentrations of IL-10 and IL-6 were reported to be increased in PCNSL patients compared to other brain tumors^{52,53}. Recently, a pilot study from Downs et al. identified methylated markers that could distinguish PCNSL from other CNS tumors with high accuracy⁵⁴.

WALDENSTROM MACROGLOBULINEMIA

Waldenstrom macroglobulinemia (WM) is an indolent form of non-Hodgkin lymphoma that is characterized by a monoclonal IgM protein. The mean age of diagnosis is 71 years, with a favorable 5-year survival that is >80%⁵⁵. Diagnosis of WM is confirmed by presence of IgM monoclonal protein associated with >10% of clonal lymphoplasmic cells in bone marrow. Additionally, the L265P mutation in MYD88 is detectable in over 90% of patients with confirmed WM⁵⁶, and can be detected in peripheral blood using PCR⁵⁷. However, MYD88 L265P is also seen in 5% of marginal zone lymphomas, and cannot distinguish between WM and monoclonal gammopathy of unknown significance (MGUS), which is nearly always a preceding phase before the onset of WM in patients⁵⁸.

HAIRY CELL LEUKEMIA

Hairy cell leukemia is an indolent form of non-Hodgkin lymphoma characterized by the identification of hairy cells and presence of a *BRAF* V600E somatic mutation⁵⁹. The presence of additional mutations within hairy cell leukemia have been shown to have prognostic implications⁶⁰. A mutated *IGHV* gene confers an improved prognosis⁶¹, while *VH4-34* positive cases have a higher disease burden, worse response to standard therapy, shorter overall survival, and the absence of a *BRAF*-V600E mutation⁶².

T-cell Lymphomas

PERIPHERAL T-CELL LYMPHOMAS

Peripheral T-cell Lymphomas (PTCL) are a heterogeneous group of T-cell NHLs that accounts for approximately 5-15% of cases⁶³. Cutaneous T-cell lymphoma (CTCL), Angioimmunoblastic T cell lymphoma (AITL), Anaplastic

Large Cell Lymphoma (ALCL), and PTCL not otherwise specified (PTCL-NOS) are the most frequent subtypes of PTCL. Clinically, these are aggressive lesions^{63,64}. Recent advances in liquid biopsy technologies for these entities are outlined below.

CUTANEOUS T-CELL LYMPHOMA

Cutaneous T-cell lymphomas (CTCLs) are primary malignancies of the skin, characterized by a cutaneous infiltration of monoclonal T cells. Mycosis fungoides, Sézary syndrome, and primary cutaneous PTCL-NOS are the most important subtypes of CTCLs⁶⁵. These lymphomas are defined classically by malignant, skin-homing CD4+ T cells (>90%)⁶⁶.

Mycosis fungoides presents clinically with erythematous patches and plaques, which may last for many years without clinical progression. However, in about 30% of patients, there is progression to advanced-stage disease within 10 years⁶⁷. Sezary syndrome, the more aggressive form of CTCL, is defined by circulating T-cells with cerebriform ("sezary") nuclei. It can result de novo, or via progression from mycosis fungoides.

The diagnosis of CTCL is based on clinical observations, histological examination of skin biopsies, and additional laboratory diagnostics including miRNA profiling and PCR of tissue samples, which has demonstrated high specificity and sensitivity in distinguishing CTCL from benign disorders⁶⁸. However, CTCL is commonly misdiagnosed, and patients can go many years with an incorrect diagnosis.

Poor disease outcomes have been associated with genes such as *TOX*⁶⁹, and miRNA profiles including *miR-155* and *miR-21* expression⁶⁸. Other biomarkers, such as LDH, are elevated in advanced disease, but are rather non-specific⁷⁰. However, there is currently a lack of prognostic biomarkers to predict disease progression from an indolent to an aggressive state⁷¹.

ANAPLASTIC LARGE CELL LYMPHOMA

Anaplastic large cell lymphomas (ALCLs) are a group of generally aggressive lymphomas that were discovered in the 1980s⁷². The key immunophenotypic markers of ALCL include CD30 and anaplastic lymphoma kinase (ALK)⁷³. Today, classification of ALCL involves two groups: (1) those that express ALK (ALK+), which are relatively homogenous and have chromosomal abnormalities involving 2p23; (2) the remaining, heterogenous group of ALK- immunophenotypic ALCL.

ALK+ ALCL is a disease entity that more commonly affects younger males, has a better prognosis than its heterogenous ALK- counterparts (79% vs 46% overall 5-year survival, respectively), and is more responsive to chemotherapy than ALK- disease, which is more likely to relapse⁷⁴. Thus, identification of ALK status is a critical step in determining the prognosis and treatment plan. Immunohistochemistry of biopsy samples is currently the gold standard used for diagnosis of tumors with ALK fusions⁷⁵. However, there is evidence that demonstrates the utility of anti-ALK antibody titers in the plasma as a diagnostic and prognostic indicator⁷⁶. Anti-ALK antibodies were identified in 92% of patients with confirmed disease. Further, higher antibody titers in

patients correlate with significantly lower incidence of relapses in response to chemotherapy, and with fewer circulating tumor cells⁷⁶.

Recent studies suggested a diagnostic role of extracellular vesicles in ALCL. Lovisa et al. found that RNY4 fragments in circulating exosomes are significantly elevated in pediatric ALCL⁷⁷. Increased tenascin C, osteopontin and hsp90 levels are also suggested as potential markers for ALK+ ALCL prognostic stratification⁷⁸.

ANGIOIMMUNOBLASTIC T-CELL LYMPHOMA

Angioimmunoblastic T-cell lymphoma (AITL) is a complex disease process with a poorly understood etiology and pathogenesis. The cell-of-origin has been identified as the follicular T helper cell, as demonstrated by molecular analyses⁷⁹. AITL often affects older adults >65 years, and has a poor prognosis with a 5-year median survival of 32%⁸⁰. Diagnosis of AITL is typically done by evaluation of a lymph node biopsy; however, the lack of specific hallmarks of the disease makes a definitive diagnosis difficult to achieve, and errors in initial diagnosis are common with AITL⁸¹. A recent study evaluated the immunophenotypic features of AITL using flow cytometry on tissue biopsy and peripheral blood samples⁸², and found an aberrant T-cell population in over 2/3 of patients with confirmed AITL, particularly with a loss of CD3 expression and positive CD10 expression. Another study demonstrated that higher serum levels of soluble IL-2R at diagnosis predicted poor outcomes in AITL⁸³.

Mutation in *RHOA* was found in high frequency in AITL and showed significant correlation with disease progression and relapse^{84,85}.

PTCL-NOS

PTCL-NOS is a heterogenous group of this entity established by diagnosis of exclusion⁸⁶. Based on gene expression studies it can be divided into two main categories. The first involves upregulation of *GATA3* and its downstream targets (e.g., *CCR4*, *CXCR7*), and is generally associated with a poor prognosis. The second category is the *TBX21* subgroup which shows upregulation of the NF- κ B pathway, and has more favorable outcomes. The *TBX21* group is associated with Tfh phenotype, and therefore is likely to be related to AITL at the molecular level^{85,87}.

The most common mutations and copy number variations in PTCL-NOS affects the TCR and NF- κ B pathways, including in *PLCG1*, *CARD11*, *CD28*, *VAV1*, *FYN*, *PTPRC*, and *TNFAIP3*⁸⁸.

Studies on the utility of liquid biopsy in PTCL-NOS are limited. Ottolini et al. found evidence of recurrent *RHOA* mutations in a study of 25 patients diagnosed with AITL and PTCL-NOS⁸⁵. Miljkovic et al. used next generation sequencing of the TCR gene in PTCL patients' serum and concluded that PTCL-NOS patients usually harbors more than one dominant TCR clone. In addition, they found that the overall sensitivity and specificity of surveillance ctDNA were lower compared to patients with DBCL⁸⁹.

ADULT T-CELL LEUKEMIA/LYMPHOMA

Adult T-cell leukemia/lymphoma (ATL) is a rare, aggressive malignancy caused by infection with human T-lymphotrophic virus 1 (HTLV-1). HTLV-1 is endemic in parts of the world including Japan, Africa, South America, and the Middle East. ATL has several subclassifications, including acute, lymphoma-type, chronic, and smoldering. Acute and lymphoma-type ATL are aggressive with a poor prognosis, while chronic and smoldering subtypes are indolent, responding well to zidovudine and interferon- α treatment⁹⁰.

Serum levels of interleukin-2 receptor alpha (sIL-2Ra) are known to reflect tumor burden and are often used to evaluate progression of ATL⁹¹. However, sIL-2R levels become elevated with inflammatory responses as well, which can be seen following certain treatments for ATL⁹².

Cell adhesion molecule 1 (CADM1), which was initially isolated as a tumor suppressor gene in non-small cell lung cancer, has been found to be highly expressed in HTLV-1 infected T cells, and aids in adhesion of these cells into different organs⁹³. Further, CADM1 is specific to ATL lymphomas, and is not expressed in most of non-ATL lymphomas⁹³. A recent study demonstrated that a soluble, extracellular domain of CADM1 (sCDAM1), can be detected in the serum of acute-type and smoldering-type ATL patients using an immunosorbent assay, and is highly correlated with clinical parameters and s-IL2R levels⁹⁴.

NK/T-CELL LYMPHOMA

NK/T-cell lymphoma (NKTCL) is a rare EBV-related non-Hodgkin lymphoma of T-cell origin. It is prevalent in Asia and Latin-America⁹⁵. The most common predilection site is the nasal cavity but can occur at any location⁹⁶. Recently several frequent somatic mutations such as STAT3, JAK3, DDX3X are identified in NKTCL, which could be used as valuable diagnostic and therapeutic biomarkers⁹⁷.

Adequate molecular evaluation of NKTCL lesions is usually hampered by large areas of necrosis which precludes adequate molecular evaluation of the tissue. Furthermore, given the invasiveness of NKTCL, tissue sampling puts the patients at risk of complications, and therefore development of non-invasive methods of molecular profiling would have an important clinical utility.

However, the use of liquid biopsy genotyping has not been examined in NKTCL in a large patient cohort. Qi et al. performed NGS on tumor tissues and longitudinally collected plasma samples obtained from a cohort of high-risk NKTCL patients undergoing systemic chemotherapy and demonstrated that plasma cfDNA reflects tumor genome DNA of NKTCL-associated mutations. In addition, they suggested that blood cfDNA could be a promising non-invasive approach to follow treatment response and survival in NKTCL⁹⁷.

Liquid Biopsy

Liquid biopsy approaches include the analysis of circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), and extracellular vesicles. These approaches are minimally invasive, and have been proposed as valuable tools for diagnosis and for extraction of information from the tumor in real time, allowing for

prognostic evaluation along with treatment monitoring⁹⁸. In this review, based on the heterogeneity of several NHL subtypes, we suggest that liquid biopsy can also be used as a tool to differentiate between these subtypes.

During the formation and growth of a tumor, circulating tumor cells (CTCs) and shed into the bloodstream and can be detected through different technologies. A bead-based Immunoassay utilizing antibodies that target epithelial cell adhesion molecule (EpCAM), as employed in the CellSearch system, has become the standard for detection and enumeration of CTCs in peripheral blood⁹⁹. CTC detection and quantification has drawn considerable attention in solid tumors, including lung, breast, prostate and colon cancer, as both a real-time biopsy and a surrogate marker for prognosis¹⁰⁰. However, the focus of liquid biopsies in lymphoma has primarily utilized molecular analyses using ctDNA and small extracellular vesicles.

Analysis of ctDNA has emerged as a valuable detection mechanism in lymphoma. ctDNAs originate from tumor cells that have undergone apoptosis or necrosis. They generally have short half-lives ranging from minutes to hours in the blood. The importance of the utility of ctDNA lies in its ability to carry genetic information from different disease sites, providing a comprehensive picture of the heterogeneity of the lymphoma, as compared to sampling from a single site. Two main methods exist for analysis of ctDNA: PCR-based assays, involving a targeted approach in which one must know the sequence being evaluated, or next-generation sequencing (NGS) assays, which provide an untargeted and unbiased approach to sequencing genes¹⁰¹. Studies have demonstrated the utility of ctDNA analysis in NHL, particularly in determining DLBCL cell of origin¹⁰². DLBCL can arise from transcriptionally distinct subtypes depending on their cell of origin: (1) activated B cell-like (ABC), (2) germinal center B cell-like (GCB), or (3) neither ABC/GCB. The subtypes are prognostic and may predict sensitivity to therapy¹⁰³. Further, other studies have demonstrated the utility of ctDNA in predicting outcomes in DLBCL¹⁰⁴. In treatment responders, levels of ctDNA were dramatically reduced within one week of commencing treatment, long before clinical outcomes had been demonstrated¹⁰⁴. Additionally, while levels of ctDNA are generally higher in aggressive compared to indolent NHL, there are still limitations in differentiating types of NHL using a liquid biopsy approach.

Exosomes, a class of extracellular vesicles, are another emerging diagnostic tool in non-Hodgkin lymphoma. Exosomal cargo includes proteins, nucleic acids, lipids, and metabolites. They are found in multiple body fluids including blood and urine, and have a relatively long half-life, making them good candidates as non-invasive diagnostic biomarkers. What was previously referred to as the exosome fraction from a particular sample is now known to contain a heterogeneous group of multiple types of different vesicles, including large exosomes, small exosomes, and exomeres, with distinct internal composition¹⁰⁵. An emerging technique, asymmetric-flow field-flow fractionation (AF4), has demonstrated the ability to perform separation and characterization of exosome fractions¹⁰⁶⁻¹⁰⁸. In several studies, exosomes have emerged as potential prognostic markers, post-

therapy surveillance markers, and as targets for new therapies. In a study of canine multicentric lymphoma, higher levels of small extracellular vesicles correlated with worse survival and a higher rate of disease relapse¹⁰⁹. Further, exosomes have been suggested as a prognostic marker in human DLBCL, with circulating exosomal levels of miR-99a-4p and miR-125b-5p being significantly higher in chemoresistant DLBCL¹¹⁰. Other studies have also demonstrated a number of exosomal-derived biomarkers are useful in post-treatment surveillance of DLBCL relapse¹¹¹. Exosomal CD20 in DLBCL has been shown to protect the cells against anti-CD20 immunotherapy, conferring treatment resistance¹¹².

At the current time, there are no clinical-grade liquid biopsy tests used in the management of patients with lymphoma. Potentially, such testing could have multiple applications, including diagnosis, prognosis, prediction of therapeutic response, detection of recurrence, subtyping, and risk stratification. There are numerous benefits to use of liquid biopsy approaches in lymphoma, including non-invasive assessment of disease, longitudinal monitoring, and ability to sample multiple disease sites within an individual, among others. Lymphomas, specifically non-Hodgkin's lymphomas, are a diverse group of cancers which have historically required histological analysis for

their diagnosis and management. We propose a new approach of using liquid biopsy testing for the management of lymphoma patients.

Conclusion

There are numerous subtypes of non-Hodgkin lymphomas (NHLs), each with distinct pathological and clinical traits. This heterogeneity highlights the need for sophisticated diagnostic tools by making diagnosis and therapy more difficult. Since liquid biopsies offer real-time, minimally invasive insights into tumor biology, they represent a paradigm leap in the treatment of lymphomas. Liquid biopsies can be used to accurately classify subtypes, predict treatment outcomes, and provide longitudinal disease monitoring by utilizing CTCs, ctDNA/cfDNA, and EVs.

Future studies should prioritize the standardization of liquid biopsy methods and validation of their applicability across various NHL subtypes. Advances in proteomic analysis and next-generation sequencing hold promise for expanding the repertoire of actionable biomarkers. Ultimately, integrating liquid biopsies into routine clinical practice could revolutionize NHL diagnosis and treatment, paving the way for improved outcomes and personalized care strategies.

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