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Review Article

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FISH or not to FISH - Therapeutic Implications of sequential FISH studies in diagnosing B-cell "Double-Hit" and "Triple Hit" lymphomas

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Introduction

Why FISH…

Patients with High Grade B Cell Lymphomas / Double Hit or Triple Hit or HGBL-DH/TH comprise around 10% of newly diagnosed Diffuse Large B Cell Lymphoma (DLBCL) and typically demonstrate poor response to standard initial therapy (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone; RCHOP). They are also shown to have limited survival when compared to those without HGBL-DH/TH. There has been a retrospective analysis on 394 patient samples and detected 19 cases of HGBL-DH (12%). After treatment with anthracycline-containing regimens, the HGBL-DH patients had a significantly shorter median OS of 8.2 months compared with 56.8 months in non- HGBL-DH patients [1, 2].

After RCHOP therapy, these patients had exceedingly poor 5-year overall survival (OS) and progression-free survival (PFS) rates of 27% and 18%, respectively.

This data led investigators to question whether more aggressive induction therapy would be more effective than RCHOP for the treatment of patients with HGBL-DH. The MD Anderson group reported their experience with 129 HGBL-DH cases. The 2-year event-free survival was much lower than historically reported outcomes of patients with DLBCL and was observed at 25%, 32%, and 67% in patients who received RCHOP, R-hyperCVAD/MA (rituximab, cyclophosphamide, vincristine, doxorubicin, dexamethasone, methotrexate, cytarabine) and REPOCH (rituximab, etoposide, prednisone, vincristine, cyclophosphamide, and doxorubicin), respectively. A large retrospective multicenter study reviewed 311 HGBL-DH patients who received induction treatment with RCHOP, REPOCH, R-hyperCVAD/MA, or CODOX-M-IVAC (cyclophosphamide, vincristine, doxorubicin, methotrexate, ifosfamide, etoposide, and cytarabine). Patients who received REPOCH had the highest response rate. Multivariable analyses demonstrated significantly improved PFS and OS for those patients who received a more intensive induction therapy compared to R-CHOP (hazard ratio, 0.5). To date, the "best" induction regimen for patients with HGBL-DH/TH remains controversial, but most clinicians prefer to use a more intensive regimen than RCHOP in patients who can tolerate the therapy based on these retrospective series.

In addition to poor response and survival following RCHOP therapy, it is important to understand that patients with HGBL-DH/TH have an increased risk of CNS relapse. In a retrospective study of 135 patients with DLBCL, 9% were found to have a MYC translocation and the presence of this translocation held an increased risk of CNS relapse independent of all studied risk factors [3]. Additional study of patients with dual expression of MYC and BCL2 proteins demonstrated a near 10% risk of CNS relapse. Therefore, many experts feel that HGBL-DH/TH should be offered CNS prophylaxis with initial therapy.

Objectives

Standard chemoimmunotherapy for patients with high-grade B-cell lymphomas harboring rearrangements of *MYC* and *BCL2* and/or *BCL6* (HGBL-DH/TH) often yields poor prognosis, and it has been well established that less than 20% of such patients are estimated to survive long-term following standard therapies.

Incidentally, the frequency of HGBL-DH/TH in unselected aggressive B-cell lymphomas is relatively uncommon and estimated at 10% of all cases. These double- and/or triple-hit lymphomas are often, associated with a clinically aggressive presentation, high-grade morphologic features, or increased protein expression of the corresponding genes. However, a substantial number of patients have no clear indicators of underlying DH/TH. The paradox of an exceedingly poor prognosis coupled with a relatively uncommon frequency raises the practical challenge of determining which patient warrants FISH testing and is an area of substantial controversy and emerging data. The clinical consequence of missing HGBL-DH/TH is dire, as these patients are likely undertreated by standard chemoimmunotherapy (RCHOP).

We live in a cost-conscious age - therefore routine and widespread testing for biologic determinants of outcome may not be practical therefore and a critical evaluation of prognosticators is necessitated. This review addresses the clinical implications of

these rearrangements in aggressive B-cell lymphomas and the potential clinical, pathologic, or biologic predictors of underlying HGBL-DH/TH biology.

Results

Cytogenetic analysis, based on banding techniques, has historically proved to be invaluable for the detection of chromosomal abnormalities in tumor samples and is still considered to be the "gold standard" technique in tumor cytogenetics because it is the only technique providing a complete overview of all chromosomal changes within a tumor cell. However, the lack in availability of fresh material, the low mitotic index and/or percentage of neoplastic cells, the cytogenetic complexity, and the time-consuming nature of analysis all impose restrictions on the use of this technology for routine diagnosis [4-12].

The updated 2017 World Health Organization (WHO) classification requires the identification of all aggressive mature B-cell lymphomas with *MYC* and *BCL2* and/or *BCL6* rearrangements as a single category designated HGBL-DH/TH, with the goal of improving our understanding of the disease and to facilitate the development of alternative therapies.2 Thus, the former B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and Burkitt lymphoma category is now replaced by 2 new categories designated HGBL-DH/TH and HGBL-NOS. HGBL-DH/TH represents an aggressive mature B-cell lymphoma with *MYC* and *BCL2* and/or *BCL6* rearrangements that may display DLBCL, intermediate, or blastoid morphological features. HGBL-NOS is restricted to tumors with intermediate or blastoid morphology, but without the DH lymphomas, and excludes DLBCL with sole *MYC* or *BCL2* or *BCL6* breaks.

The updated WHO classification has significant consequences for the diagnostic workup of DLBCL in daily practice because DH/TH DLBCLs do not necessarily display aggressive morphological and/or immunohistochemical features, like starry sky pattern, high mitotic rate, or MYC protein overexpression. This raises the question whether every DLBCL should be referred for FISH testing for *MYC*, *BCL2*, and *BCL6* rearrangements to detect DH status.

Interphase FISH on formalin-fixed paraffin-embedded tissue section is a robust technique, but is time consuming, expensive, and not widely available. However, FISH techniques in recent years have greatly improved with automatization and development of digital imaging technologies. Various strategies have been proposed to restrict FISH testing to GCB subtype, or according to Ki67 proliferative index or MYC protein expression. Some authors suggested limiting FISH to GCB and DPE DLBCLs, which would reduce FISH analysis to 15% of cases. However, no consensus has been reached to date, the main reason being the lack of large cohorts of DLBCL patients with COO and FISH data to test various screening strategies.

Scott et al provide data from a large cohort of 1228 de novo DLBCLs, identified in 3 international clinical trials and a population-based registry, to evaluate the incidence of HGBL-DH/TH and the effects of screening strategies based on COO (Lymph2Cx gene expression assay and/or Hans's algorithm) and/or DPE. *MYC* rearrangement (*MYC-R*) was observed in 12.2% of DLBCLs and included mostly, but not exclusively GCB DLBCLs. *MYC* as sole genetic alteration and MYC/BCL6 HGBL-DH included both ABC and GCB DLBCLs, whereas *MYC*/BCL2 and MYC/BCL2/BCL6 HGBL-DH/TH were exclusively GCB. In total, HGBL-DH/TH represented □8% of tumors with DLBCL morphology (see figure).

According to the study by Scott et al (14), the best method for detecting all HGBL-DH/TH among tumors with DLBCL morphology is to screen all DLBCLs for *MYC* breaks. When the tumor is positive, it should be further tested for *BCL2* and *BCL6* gene alterations, which would require that the FISH technique be in pathology laboratories and that reliable *MYC* probes are used. Alternatively, restricting FISH testing to GCB DLBCLs would reduce FISH testing to half of DLBCLs and would still detect ≥99% HGBL-DH/TH with *BCL2* rearrangements. This approach is acceptable for *MYC*/*BCL2* HGBL-DH detection but would miss a considerable number of the uncommon *MYC*/*BCL6* HGBL-DH, where the prognostic value is still controversial [5, 8]. In addition, this approach would miss DLBCLs with isolated MYC rearrangement and ABC/non-GCB phenotype. A major point of the study is to show that selecting DLBCLs on DPE status and/or COO subtyping results in missing □35% of all HGBL-DH [13].

In summary, the study of Scott et al presents data on the impact of various FISH testing strategies to identify HGBL-DH/TH in tumors with DLBCL morphology [14]. FISH testing for *MYC, BCL2*, and *BCL6* should be incorporated in the routine diagnostic workup of all DLBCLs in an integrated approach together with gene expression assays and next-generation sequencing. If not possible, the optimal strategy is a 2-step approach with testing for *MYC* first and to perform FISH for *BCL2* and *BCL6* if there is *MYC* rearrangement. Other screening strategies to limit the costs should be discussed in each institution depending on the local resources and with the knowledge of the limitations of each strategy as reported in this study.

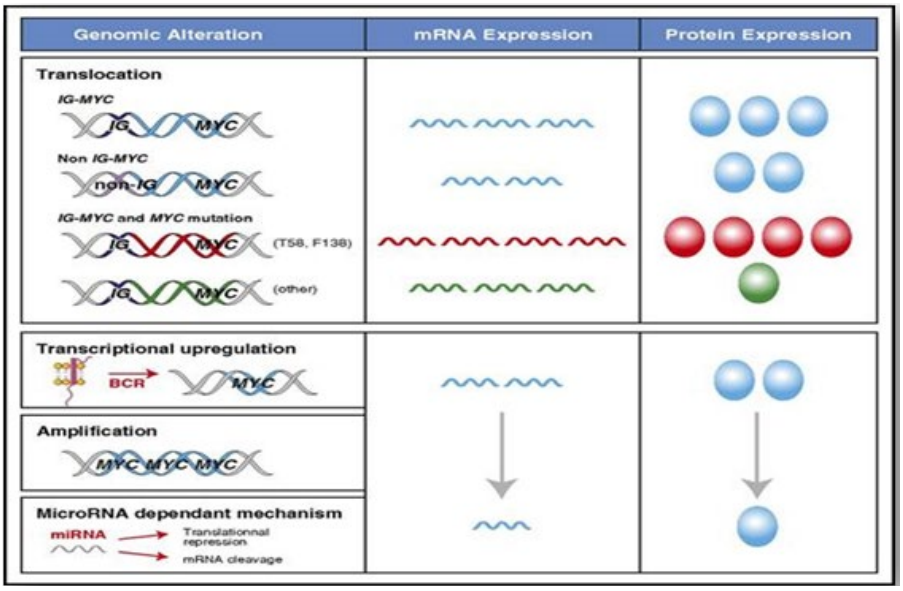
Discussion

In 2016, the World Health Organization (WHO) revised their classification of lymphoid neoplasms to account for the major advances in lymphoma biology since 2008 [1, 2]. This revision emphasizes molecular features of clinical importance, such as genomic alterations in *MYC, BCL2*, and/or *BCL6* oncogenes. An important change is the addition of HGBL with *MYC* and *BCL2* and/or *BCL6* rearrangements, so-called "double-hit" lymphoma (HGBL-DH), as a separate provisional entity.1 This aggressive lymphoma was originally described in 1988 and was shown to be associated with a very poor outcome [3-5]. More than 80% of patients with HGBL-DH harbor concurrent translocations in *MYC* and *BCL2* [6]. The remaining 20% harbor *MYC* and *BCL6* translocations and usually express *BCL2* despite not having a BCL2 translocation [7]. Two large independent studies have demonstrated that concurrent translocations of MYC and BCL6 are not associated with an inferior outcome in diffuse large B-cell lymphoma (DLBCL) [8, 9]. Thus, MYC and BCL2 oncogenes will be the focus of this review. The co-expression of these proteins in DLBCL, so-called dual expressor DLBCL (DE-DLBCL), has also been associated with an inferior survival, although the outcome is superior to that of HGBL-DH.10-15 DE-DLBCL

remains in the DLBCL not otherwise specified (NOS; DLBCL-NOS) category in the revised WHO classification.

The diagnostic work-up of HGBL-DH is controversial. Although some advocate for extensive cytogenetic testing in all patients with DLBCL to identify the ~4% of patients who have HGBL-DH, the costs are prohibitive in many centers. A rational approach to fluorescence in situ hybridization (FISH) testing would be useful, especially in institutions with limited resources or access to FISH.

There is also no standard approach to the treatment of HGBL-DH. There is evidence for a superior progression-free survival (PFS) with intensive induction regimens in selected patients. However, many HGBL-DH patients are elderly and/or frail, which makes them poor candidates for high-dose chemotherapy. Newer targeted therapies are becoming more readily available, but their role in the treatment of HGBL-DH is not yet established. Herein, we review key concepts regarding *MYC* and *BCL2* deregulation in aggressive lymphomas that may guide diagnostic testing and clinical management.



To truly rule out HGBL-DH in DLBCL, FISH would be required in all patients, even though its prevalence is very low.

The most reliable technique is interphase FISH with break-apart probes that would also detect non-*IG MYC* translocations. To further define the translocation partner, additional tests using fusion probes spanning *MYC* and *IGH, IGκ, or IGλ* could be performed but are not required for the diagnosis.

Immunohistochemistry can be useful for screening patients who require FISH because it is inexpensive, accessible, and routinely used in clinical laboratories [4]. The proliferation marker Ki-67 is always increased in BL but is more variable in HGBL-DH and is not a reliable marker for screening patients that require FISH. Factors affecting poor concordance between hematopathologists include crush artifact and variation in staining intensity and tumor content. Furthermore, thresholds that categorize patients as being positive vary among studies and still require prospective validation. Although BCL2 expression is routinely assessed in pathology, MYC expression is not assessed in all laboratories. The MYC antibody clone Y69 has good interobserver reproducibility. The 40% threshold for MYC and 50% threshold for BCL2 were initially selected based on their prognostic value, not as thresholds for screening patients that require FISH. It is reasonable to continue using these thresholds until a consensus is reached but reporting the percentage of positive cells in the biopsy would be ideal because extremes in protein expression are more reproducible and are helpful in assessing the likelihood of detecting an MYC translocation.

In centers where FISH analysis is not feasible for all patients with DLBCL, using cell of origin (COO) with MYC and BCL2 co-expression would be a useful screening strategy to test patients who are at highest risk of treatment failure. Restricting FISH testing to patients with a GCB phenotype would reduce the number of patients to test by ~50%. However, based on data from more than 1000 patients included in 2 independent studies, only 6% of GCB patients will have HGBL-DH (Table 1). Selecting GCB DLBCLs that also express MYC and BCL2 proteins would reduce testing by >90% because only 15% of patients with GCB-DLBCL will also be dual expressors. By using this strategy, one-third of DLBCLs probed by FISH would be a bona fide HGBL-DH. The HGBL-DH missed would be enriched in cases harboring *MYC* and *BCL6* translocations and those that do not have dual expression, whose clinical significance is unclear.

Table 1.

MYC and BCL2 deregulation in DLBCL and HGBL

Morphology and immunophenotype	DLBCL (%)		HGBL (%)
	GCB	ABC	
MYC protein by IHC	27	36	60
Mechanisms	Translocation	BCR and NF-κB signaling	Translocation
Incidence of translocation	21	5	60
BCL2 protein by IHC	43	63	70
Mechanisms	Translocation	BCR and NF-κB signaling; gene amplification	Translocation
Incidence of translocation	25	5	40
DE-DLBCL	15	23	NA
MYC and *BCL2* translocations	6 (if de novo DLBCL) 21 (if prior FL*)	<1	30
MYC and *BCL6* translocations	2†	2	

Some proponents of IHC as a screening tool recommend screening both for dual MYC and BCL2 expression and for GCB subtype prior to testing any samples by FISH. Scott *et al.* found that this would limit the population tested by FISH to only 11–14% of the total DLBCL population, however, it would result in missing up to a quarter of the cases of HGBL-DH/TH [5].

An alternate strategy to identify patients with HGBL-DH/TH is to screen every pathology sample of a newly diagnosed high grade B-cell lymphoma with FISH probes for dual *IGH-MYC* fusion. If positive, a sequential FISH study could be performed on the sample for *BCL2* and *BCL6* translocations.

A typical FISH dual fusion probe targets the *IGH-MYC* fusion and can detect the classic translocation partner but can also detect the less common translocations between immunoglobulin (IG) and *MYC, (IGK-MYC)* and (*IGL-MYC*). The less common mutations comprise <5% of *MYC* translocations but could be missed if a FISH break-apart probe was used in lieu of the dual fusion probe. The sensitivity of this method should approach 100% in those cases where IG is the translocation partner for *MYC*.

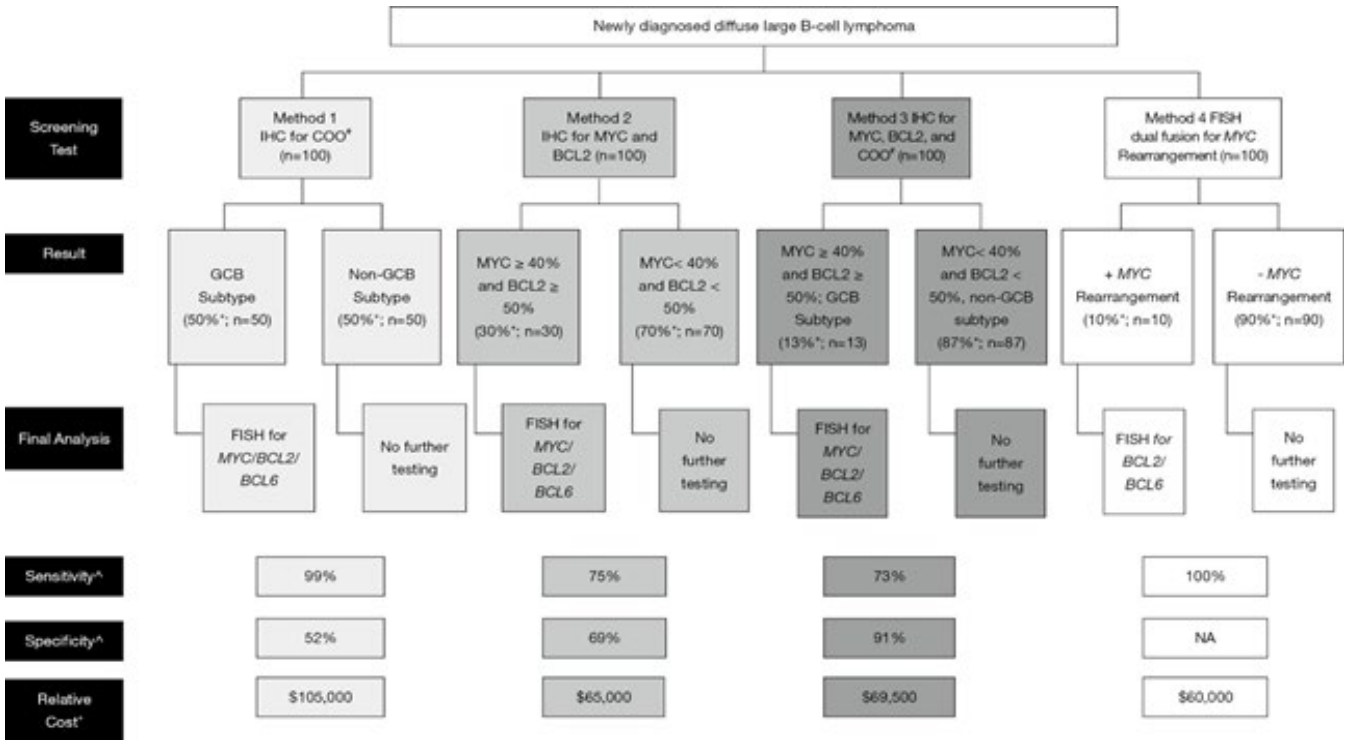
MYC can have other translocation partners with non-*IG* genes. In a study that identified 54 patients with *MYC* translocations 24 were translocated with non-*IG* gene partners, most commonly (q24; p13) in 13 of 24 patient samples [6]. In multivariable analysis a non-IG gene translocation partner was associated with more favorable survival compared with an *IG* gene partner [7]. Similarly, another retrospective study identified 28/225 DLBCL patient samples that harbored a *MYC* translocation. They were able to identify an *IG* translocation partner in 12 of the 24 available samples.

MYC translocation with *IG* translocation partner gene was associated with worse OS compared with *MYC* translocation with non-IG translocation partner gene [8]. Therefore, although performing FISH with a dual fusion *IGH-MYC* probe would not detect non-*IG* partner *MYC* translocations, but this may not be as clinically relevant.

The positive predictive value of this method is not 100% as there are about 5% of all patients with DLBCL that have a single *MYC* translocation that is not paired with a *BCL2/BCL6* translocation. As multiple publications have demonstrated worse prognosis and increased risk of CNS relapse with single *MYC* translocation in DLBCL, one could argue that it is clinically relevant to identify even patients with a single translocation.

Ultimately, decisions regarding the diagnostic workflow of tumors with DLBCL morphology will depend on laboratory resources, test prioritization when tissue is limited, and pathologist/physician preferences. The degree to which classification into HGBL-DH/TH alters clinical management will be a major factor [8]. The results of this project, in concert with these factors, can inform decisions about whether and how to adopt screening tests, allow rational design of screening algorithms, and provide estimation of the impact of the screening implementation.

In summary, with the high sensitivity of screening all newly diagnosed high grade B-cell lymphoma tumor samples for *MYC* rearrangements with a dual fusion FISH probe seems to be the most efficient way not to miss the diagnosis of HGBL-DH/TH. A summary of this screening method can be found in Figure 1: Method 4.



The next logical question is that should clinicians wait for final FISH results prior to initiation of chemotherapy or not. As the processing times for FISH studies are highly variable between labs, a practical concern physician often face is whether to wait for final FISH results prior to initiation of chemotherapy. This problem is of relevance if sequential testing of the pathology is performed, potentially leading to further delays. As high-grade B-cell lymphomas such as DLBCL are clinically very aggressive, these patients require urgent therapy. As such, we do not recommend delaying the initiation of therapy to wait for FISH results. Once FISH results are available, the treatment plan can be tailored to match the patient's molecular risk. For example, if a patient is diagnosed with DLBCL and FISH results are unavailable, we proceed with RCHOP therapy for cycle 1. Subsequently, if the FISH studies confirm HGBCL-DH/TH, the remainder of the cycles can be switched to REPOCH and include CNS prophylaxis.

Sensitivity of the screening methods should certainly be considered when selecting an institutional standard for screening DLBCL patient samples for HGBL-DH/TH. However, another factor that should be considered is the cost of molecular analysis of these patient samples. Unfortunately, it is a bit difficult to ascertain the exact cost of these studies as the charges can be variable from one insurance company to another.as per a rough estimation, each IHC stain costs approximately $100 and each FISH study is approximately $400–$500 USD (https://www.cms.gov/medicare-coverage-database) Using these factors, relative cost of each screening method which is pictured in Figure 1 is calculated. Notably, Method 1 (screening for GCB by IHC) is the most expensive and Methods 2–4 are similar in relative cost. Therefore, it is again recommended to use Method 4 (screening with FISH for *MYC* rearrangements) as the most cost-effective screening method.

The poor prognosis and adverse outcomes following standard chemoimmunotherapy for patients with aggressive B-cell lymphomas harboring dual rearrangements of *MYC* and *BCL2* and/or *BCL6* is now well-established. Due to the need for more intensive induction chemotherapy than RCHOP and the potential need to implement CNS prophylaxis, it is crucial for treating physicians to know whether a patient with newly diagnosed DLBCL fits into the HGBL-DH/TH category. In a cost-conscious era, routine, and widespread testing for biologic determinants of outcome may not be appropriate, and a critical appraisal of predictors is warranted.

Since it is hard to distinguish clinical phenotype or pathologic morphology to accurately predict for underlying HGBL-DH/TH, the molecular features of the underlying lymphoma are a more objective means to screen for HGBL-DH/TH. The paper has summarized the data to support various methods of screening by molecular features including protein expression and sequential FISH.

Diagnosing D/THL is challenging without further FISH analysis. However, further FISH analysis in all patients with DLBCL or other aggressive B-cell lymphomas is hindered due to practical limitations

Based on the research, it is recommended to screen all patient samples with newly diagnosed high-grade B-cell lymphoma with a dual fusion FISH probe for *MYC-IG* translocation. If the FISH study is positive, the sample can then be tested for *BCL2/BCL6* translocations. This appears to be most sensitive and cost-effective method to diagnose patients with HGBL-DH/TH and to best assist treating physicians in the clinical management of these patients.

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