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Molecular Classification, Pathway Addiction, and Therapeutic Targeting in Diffuse Large B-cell Lymphoma

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Abstract

The rapid emergence of molecularly-based techniques to detect changes in the genetic landscape of diffuse large B-cell lymphoma (DLBCL) including gene expression, DNA and RNA sequencing, and epigenetic profiling, has significantly impacted the understanding and therapeutic targeting of DLBCL. In this review, we will briefly discuss the new methods used in the study of DLBCL. We will describe the influence of the generated data on DLBCL classification and the identification of new entities and altered cell survival strategies with a focus on the renewed interest in some classic oncogenic pathways that are currently targeted for new therapy. Lastly, we will examine the molecular genomic studies that revealed the importance of the tumor microenvironment in the pathogenesis of DLBCL.

Keywords

Diffuse Large B-cell Lymphoma; Gene Expression Profiling; Oncogenes; B-cell Receptor Signaling; Tumor Microenvironment

Introduction

The clinical heterogeneity of diffuse large B-cell lymphoma (DLBCL), the most common aggressive non-Hodgkin's lymphoma (NHL), prompted investigations to molecularly define and determine the “drivers” behind the more aggressive and refractory forms of the disease. In the last decade, there has been an explosion of new methodologies for investigating the molecular genomics of lymphoma, which have broad applications to study other tumor types (methods summarized in Table 1). Traditional methods of tumor evaluation focus on analysis of one to a few DNA or RNA alterations at a time. Cytogenetics is the gold standard for assessment of chromosomal structure, while the integrity of individual DNA sequences are evaluated using Southern blotting or polymerase chain reaction (PCR), or fluorescent in situ hybridization (FISH) techniques. With more recent technological advancements, methods to examine the entire genome are now available including array comparative genomic hybridization (aCGH) for simultaneous assessment of genetic gains and losses and automated sequencing for mutational analysis at the nucleotide level. A

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similar revolution in RNA technologies has expanded the evaluation of individual mRNA expression by singleplex reverse transcriptase-PCR (RT-PCR) to include expression profiling of all gene transcripts within the whole genome by using gene microarrays. In addition, epigenetic alterations are also studied with new tools such as chromatin immunoprecipitation-sequencing (ChIP-seq) that allow for detection of post-translational chromatin modifications (e.g. histone acetylation and methylation). The recently developed molecular genomic techniques are not limited to the aforementioned methods and this rapidly expanding field has led to an increased knowledge of lymphoma biology. Here, we will discuss the molecular genomic analyses, primarily gene expression profiling (GEP), which contributed to the most recent classification of DLBCL. This review will also focus on the important molecular pathways identified by such studies that are currently under investigation as potential targets for new DLBCL therapy.

DLBCL classification

DLBCL is by far the most common NHL and accounts for 30,000 new cases per year in the United States (1). In the World Health Organization's (WHO) "Classification of Tumors of the Hematopoietic and Lymphoid Tissues" published in 2008, several morphologic and immunophenotypic variations are noted in the category of DLBCL, as well as immunohistochemical and molecular subgroups defined by protein expression and GEP differences (2). It is important to note that within morphologically similar DLBCL cases, GEP analyses reported primary mediastinal B-cell lymphoma (PMBL) as a distinct molecular entity. However, we will discuss DLBCL subtypes based on the 2008 WHO classification, which describes PMBL as a separate non-DLBCL entity (3). Other categories of DLBCL based on the composition of the background cell population, anatomic location, and presence of EBV were pulled out as new subtypes and not included in the not otherwise specified (NOS) category. An additional 8 types of large B-cell neoplasms and 2 borderline categories were also described. These are detailed in Table 2.

Discovery of cell of origin (COO) subtypes

In 2000, the results of GEP on snap frozen tissue samples using a customized competitive microarray chip known as the Lymphochip first demonstrated the heterogeneity of 60 cases of DLBCL NOS. These cases were morphologically indistinguishable at the histologic level, but clearly had distinct gene expression profiles (4,5). The tumors were grouped into two categories that represent different stages of B-cell differentiation according to the genes predominately expressed, those associated with germinal B-cells or activated peripheral B-cells. The germinal center B-cell (GCB) subtype largely express genes of normal germinal center B-cells, such as *BCL6* and *LMO2*, while activated B-cell (ABC) lymphomas express genes that are upregulated in B-cells with activated B-cell receptor (BCR) signaling including *NF- κ B* and *IRF4* (4,6). However the precise COO that gives rise to the ABC subgroup of DLBCL remains unknown. In 2002, an additional 240 snap frozen cases were profiled using the Affymetrix U133A and U133B oligonucleotide array chip and the GCB and ABC subgroups were confirmed (6). In both papers, the ABC group had a worse outcome as compared to GCB group patients with similar clinical risk factors. In 2008 following the addition of rituximab immunotherapy to the previous standard CHOP

(cyclophosphamide, hydroxydaunorubicin, oncovin, and prednisone) regimen (now known as the current standard, R-CHOP) and the release of an improved Affymetrix oligonucleotide array (U133 plus 2.0, all the transcripts included on the U133A and B set with an additional 6,500 genes), an additional 233 DLBCL cases were profiled for gene expression (7). Similar to the previous study by Alizadeh (2), these data also detected the marked gene expression pattern difference between the 2 DLBCL subgroups and demonstrated the molecularly defined COO classification was prognostically significant in the modern treatment era. This prognostically significant classification of DLBCL was also evident using a novel method for amplification and labeling of RNA (Ribo-SPIA from Nugen Technology) extracted from FFPE tissues. The Ribo-SPIA method proved more amendable to the degraded nature of FFPE extracted RNA and required less input RNA compared to the traditional Eberwine technique (8). Several other groups used GEP to demonstrate the clinical significance of the GCB versus ABC distinction in R-CHOP treated patients using other platforms and profiling of formalin-fixed, paraffin-embedded tissues (FFPE) (9,10). Alternatively, microRNA (11) and DNA methylation (12) profiling have also confirmed the distinct subtypes within DLBCL and the differences in patient outcomes. Although GEP provides an in-depth, overall picture of relative gene expression and distinguishes between molecular subtypes of DLBCL, its use as a diagnostic tool is limited due to the expense and time required for analysis as well as the inability to simultaneously assess morphological features of the tumor.

Immunohistochemistry (IHC) for DLBCL subtype classification

For practical purposes in the clinic, several groups of investigators evaluated panels of immunohistochemical antibodies for the distinction between the different subgroups of DLBCL in a more cost-effective, timely manner. Based on the genes highly associated with the GCB or ABC subtype reported in the GEP studies, algorithms were designed with 3 to 6 antibodies already available for IHC to dichotomize cases into germinal center and non-germinal center subgroups. Due to the heterogeneous gene expression within the GEP identified unclassifiable subtype and the clinical similarity with ABC lymphomas, these cases were not identified separately and therefore the non-GCB classification presumably consists of both ABC and unclassifiable subtypes (13). The first algorithm (Hans' algorithm) used CD10, BCL6, and MUM1 to define GCB and non-GCB subtypes with 83% concordance compared to GEP and the same or better division of patients into prognostic categories (13). A follow-up paper by the same group incorporated additional antibodies, GCET1 and FOXP1 (Choi algorithm) into the Hans' algorithm in order to improve accuracy as compared to GEP and also to resolve conflicting evidence of the clinical relevance for patients treated with R-CHOP using the IHC method (14). More recently, the Tally method modified the Hans' and Choi algorithms by addition of a germinal center associated molecule, LMO2, previously demonstrated to correlate with a better patient outcome (15, 16). Similarly, modifications to the Choi algorithm that evaluate only CD10, FOXP1, and BCL6 show a high concordance to GEP and independent prognostic value for progression-free and overall survival (17). However, discrepancies between the GEP and IHC methods for the prognostic and clinical significance of DLBCL subtype distinction still remain (9).

Comparison of Cell of Origin (COO) classification methods

The use of IHC for subgrouping DLBCL has the advantages of being inexpensive, widely available, and possibly more accurate for cases with infrequent tumor cells. However, IHC is currently limited by the lack of additional antibodies specific for the ABC subtype, qualitative and subjective nature of scoring, and tissue quality issues that lower reproducibility. In contrast, GEP and other molecular assays are more quantitative and objective, can analyze significantly more genes, and allows for the unclassifiable category to remain distinct from the ABC subgroup. As a disadvantage most of the discovery work for GEP was performed on snap-frozen tissues that are typically only collected in academic centers. In order to be widely available and have clinical application molecular-based methods need to be easily useable on (FFPE) tissues, which are often the only biopsy material available. However, several research groups demonstrated the feasibility of using expression profiling and other techniques on FFPE tissues with new extraction and labeling methods, and novel platforms that require shorter probes and less nucleic acid input (8, 10, 11, 13, 18). The identification of molecularly defined subtypes of DLBCL with distinct and significant differences in patient outcome suggest GCB and ABC lymphomas depend upon different pathways for growth and survival that may serve as targets for novel therapeutic strategies.

Molecular pathways and oncogenes for targeted therapy in DLBCL

B-cell receptor signaling and downstream pathways

Chronic active BCR signaling was recently identified as a critical pathway in NHL, especially in the ABC DLBCL subtype. Approximately 10% ABC DLBCL have mutations in caspase recruitment domain-containing protein 11 (CARD11) that constitutively activate NF- κ B (19). However, the mechanism that engages wild-type (wt)-CARD11 was not delineated. A RNA interference (RNAi) screen revealed that a component of the BCR signaling pathway, Bruton's tyrosine kinase (BTK) was essential for survival of ABC DLBCL with wt-CARD11 (18). In ABC DLBCL cell lines, BCR formed prominent clusters in the plasma membrane with low diffusion similar to BCRs in antigen stimulated normal B-cells (19). It was further shown that while antigen specificity of the BCR is provided by surface immune globulin, signaling is mediated by CD79A and CD79B. The CD79A/B heterodimer serves as a framework for the assembly and membrane expression of the BCR. Engagement of the BCR by antigen induces SRC-family kinases to phosphorylate tyrosines in the immune receptor tyrosine-based activation (ITAM) motifs of CD79A and CD79B. Spleen tyrosine kinase (SYK) is activated by binding to the phosphorylated ITAMs, triggering downstream signaling that involves BTK, phospholipase C- γ (PLC γ), and protein kinase C- β (PKC β) (19). BTK forms a complex with PLC γ that activates PKC β . This leads to the phosphorylation of CARD11 leading to recruitment of BCL10 and mucosa associated lymphoid tissue lymphoma translocation gene 1 (MALT1) into a multi protein CBM complex; this ultimately activates IKK β thereby engaging the canonical NF- κ B pathway. In addition to NF- κ B, phosphatidylinositol 3-kinase (PI3Kinase), extracellular signal regulated kinase (ERK) and nuclear factor of activated T cells (NFAT) pathways are activated as well (Figure 1) (20). This is in contrast to "tonic" BCR signaling

that is required to maintain resting mature B-cells. It was shown that ablating the immunoglobulin heavy (IgH) chain leads to loss of mature B-cells over a period of 1–2 weeks (21). Furthermore, ablation of the CD79A/Igα in mouse models decreased expression of BCR of both IgM and IgD class (22).

BTK and BTK inhibitor PCI32765—The significant discovery of constitutive BCR signaling and development of targeted inhibitors to BTK was subsequently translated in early phase clinical trials. Interim results of a Phase 2 study of PCI32765 (BTK Inhibitor) in ABC-DLBCL were recently presented, and notably there was a 40% overall response rate in relapsed refractory DLBCL (23).

PKCβ and PKCβ inhibitor enzastaurin—B-cells express multiple protein kinase C (PKC) isoforms (24). Of these, PKCβ mediates BCR dependent NF-κB activation by recruiting IKK to interact with the CBM complex and TAK1 (25,26). Enzastaurin is an adenosine triphosphate-competitive, selective inhibitor of PKCβ. In preclinical studies, enzastaurin induced apoptosis and inhibited the proliferation of DLBCL cell lines and xenografts at low micro-molar doses. In *in vitro* kinase assays, comparable concentrations of enzastaurin inhibited PKCβ and other PKC isoforms by approximately 90% with little effect on multiple other serine/threonine and tyrosine kinases (27).

SYK and SYK inhibitors R406 and Fostamatinib—In DLBCL cell lines that express tonic BCR dependent survival signals targeted inhibition of SYK abrogates BCR signaling and induces apoptosis. Tumor cells deficient in SYK did not show *in vivo* proliferation and inhibition of SYK by R406 and R788 (Fostamatinib, prodrug of R406) led to tumor regression *in vivo* (28).

PI3K and PI3K inhibitor CAL-101—The PI3K/AKT/mTOR (mammalian target of rapamycin) pathway has emerged as an important pro-survival pathway in lymphoma. Utilizing chronic lymphocytic leukemia (CLL) cells from untreated patients, pro-survival effects from the involvement of BCR signaling pathway were noted following activation with surface IgM. However, when the cells were treated with a PI3K inhibitor there was decreased myeloid cell leukemia sequence-1 (MCL1) expression and increased apoptosis (29,30). In mouse models, p110δ was necessary for B-cells to respond to BCR clustering or toll like receptor ligands, and it mediated survival and proliferation (31). Activation of the PI3K pathway by cell surface receptors is directly mediated by class I isoforms of which the p110δ isoform is highly expressed in hematopoietic cells. In a kinome wide-screen, CAL-101 was identified as a potent and selective inhibitor of p110δ. CAL-101 blocked constitutive PI3K signaling and decreased phosphorylation of AKT. This in turn led to an increase in poly (ADP-ribose) polymerase and caspase cleavage and induction of apoptosis (32). This potent compound is currently in clinical development for a variety of NHL.

mTOR and mTOR inhibitor rapamycin—The mTOR kinase is a 289-kDa serine–threonine kinase that exists in mutually exclusive complexes referred to as mTORC1 and mTORC2. Each complex has several components, one of which is mTOR, the catalytic subunit (33,34). Rapamycin (Sirolimus, the international nonproprietary name) is the parent drug of the class of mTOR inhibitors that target mTORC1. The rapamycin analogs

Temsirolimus and Everolimus are FDA approved for renal cell carcinoma. These agents have demonstrated activity against lymphoma cells both *in vitro* and *in vivo*. Everolimus inhibited cell cycle progression in DLBCL cells by inducing a G1 arrest. Cell cycle arrest was also accompanied by reduced phosphorylation of retinoblastoma protein (RB) as well as reduced expression of cyclin D3, and it also augmented rituximab induced cytotoxicity in rituximab sensitive cell lines (35).

Antigen presentation pathway: MHC class I and II

MHC1-biallelic gene mutations resulting in inactivation of beta-2-microglobulin were recently identified in sequencing studies of DLBCL as being one of the key pathways affected by mutations (36). Earlier IHC studies demonstrated that loss of the MHC I or MHC II antigens was associated with fewer infiltrating T cells and poorer outcome in DLBCL (37,38). More recently, decreased gene expression levels of MHC Class II were associated with poor prognosis in CHOP, CHOP-like, and R-CHOP- treated patients (6, 37–41). The mechanism of decreased MHC II expression in DLBCL is not known, but appears to be at the transcriptional level and under the control of the master transactivator of MHC II expression, known as CIITA (42). Point mutations in CIITA are known to result in a group of inherited immunodeficiency syndromes known as the Bare Lymphocyte Syndromes. However, these mutations are not found in DLBCL patient samples (43). Interestingly, CIITA is recurrently translocated in PMBL leading to decreased expression of MHC II and increased expression of ligands of programmed cell death 1 and 2 (PDL1 and PDL2) on T-cells (44). Thus, there are likely multiple mechanisms for immune escape involving antigen presenting pathways, which serve as new therapeutic targets.

Oncogenes

BCL2

The B-cell lymphoma-2 (*BCL2*) gene translocations with the IgH gene are characteristic of low-grade follicular lymphoma. The t(14;18) (q32;q21) puts the *BCL2* gene under the direction of the IgH promoter, which is constitutively active in B-cells. These translocations also occur in approximately 15% of DLBCL with increased frequency (35%) in GCB DLBCL. Amplification of the *BCL2* gene detected by FISH also occurs in 20% of DLBCL with the majority of amplifications occurring in the ABC subtype. Approximately 35% of ABC DLBCL harbor amplifications, which correlated with poor overall survival. Thus, the 2 types of DLBCL have different genetic mechanisms for increasing expression of BCL2 (45–47). Increased mRNA expression is reported for about 33% of DBLCL including cases with and without t(14;18). Higher *BCL2* mRNA levels are seen in cases with amplifications, and therefore in the ABC subtype. In addition to these genetic alterations for deregulation of BCL2 expression, there are other mechanisms for BCL2 upregulation in DLBCL, such as constitutive activation of the NF-κB pathway. There are likely other as of yet identified post-transcriptional/translational mechanisms as well since translocation, amplification, and upstream pathway activation do not account for the high-level of BCL2 protein detected in some cases of DLBCL. BCL2 protein detection using IHC demonstrates 40% of DLBCL cases express BCL2 protein with a positivity rate of 62% in ABC DLBCL and 30% of GCB DLBCL. Interestingly, BCL2 protein expression appears to increase with advancing age as

well as the occurrence of *BCL2* translocation (from age 20 – 50) and ABC DLBCL (48). These factors may contribute to the long recognized negative prognostic impact of age on DLBCL outcome. It is well established that *BCL2* protein expression strongly correlates with worse overall survival and serves as a potential therapeutic target to promote chemotherapy sensitivity (45,48,49). Several molecular therapeutic agents targeting *BCL2* at either the transcriptional or protein level are in development. Oblimersen is a single-stranded DNA molecule with sequence complementary to *BCL2* mRNA. The mechanism of action involves hybridization with the *BCL2* mRNA causing hydrolysis, which subsequently results in decreased *BCL2* protein levels (49). For targeting *BCL2* at the protein level, Obatoclax (GX-15-070), a small molecule *BCL2* family pan-inhibitor, binds to *BCL2*, *BCLXL*, and *MCL1*. Preclinical data suggest several mechanisms of action including some that are independent of BAX/BAK mediated apoptosis (51). Similarly ABT-737 interacts with multiple *BCL2* family members, *BCL2*, *BCLXL* and *BCLW*. However, this small molecule was identified during a screen of compounds with modest affinity to proximal binding sites of the BH3 binding groove of *BCLXL*. Optimization of the ABT-737 small molecule to improve oral bioavailability led to the development of ABT-263, a chemical analogue that has been clinically tested; however, it exhibits similar broad spectrum affinity for *BCL2* family members. The ABT compounds have approximately 100× greater affinity for the *BCL2*-family proteins (52). While ABT 263 favors binding to *BCL2*, *BCLXL*, *BCLW*, it still binds *MCL1* with greater affinity compared to other inhibitors of multiple *BCL2* family members, like obatoclax (51). The newest *BCL2* inhibitor by Abbott, ABT-199, binds to *BCLXL* with significantly less affinity than ABT-263 and, therefore, is associated with less thrombocytopenia. This compound is currently in Phase I/II clinical trials (53). An alternative strategy to lower *BCL2* expression and potentially improve DLBCL and other lymphoma patient response is to target *BCL2* at the transcriptional level.

One such strategy for therapeutic modification of transcription is through small molecule interaction with DNA secondary structures. Drug targeting of DNA secondary structures is a fast-developing and novel area of research for various oncogenes, including *BCL2*, and different tumor types. DNA secondary structures, the G-quadruplex and the i-motif, are formed with either the guanine or cytosine rich strand, respectively, through negative supercoiling induced during transcription. Due to their proximity to oncogene promoter regions and evidence in *in vitro* models the formation of these structures are proposed to play an important role in transcriptional regulation *in vivo* (54). There are both G-quadruplex and i-motif DNA secondary structures that form within the *BCL2* promoter region and regulate transcription (55,56). Therefore the potential for developing small molecules to target these DNA structures is ongoing and in early, preclinical studies (55). Similarly, G-quadruplex structures regulate transcription of the *MYC* oncogene and can be targeted with small molecules to lower expression of the oncogene and induce cytotoxicity in NHL cells (57).

MYC

Translocations of the cellular-myelocytomatosis gene (*MYC*) are a defining feature of Burkitt's lymphoma (BL). The most common translocation is the t(8;14)(q24;q32) involving *MYC* and the IgH promoter region, which places the *MYC* gene under the direction of the

IgH promoter like *BCL2-IgH* translocations as previously described. Alternatively, non-Ig translocation partners involve the kappa (t(2;8)(p12;q24)) or lambda t(8;22)(q24;q11) light chain promoters and taken together these 3 different *MYC* translocations are detected in >90% of BL. In contrast, translocations involving *MYC* are found in only 10% of DLBCL and usually involve non-Ig translocation partners (58–60). *MYC* translocations are also detected in the entity known as B-cell lymphoma, unclassifiable with features intermediate between DLBCL and BL. Many of these lymphomas are referred to as "double-hit" and contain not only *MYC* translocations (usually with non-Ig partner genes), but also translocations or other genetic abnormalities of *BCL2* and/or *BCL6* (58,59). *MYC* translocations are also detected in low-grade follicular lymphomas that have transformed into more aggressive neoplasms. Most recently, 49% of plasmablastic lymphomas demonstrated *MYC* translocations with Ig partner genes (61). Prognostically, as a sole abnormality or in low-complex karyotypes, *MYC* translocations *per se* do not confer a worse patient outcome in lymphoma. However, in conjunction with *BCL2*, *BCL6*, *CCND1* or as part of a complex karyotype, patient outcomes are usually much worse (62). In addition to *MYC* translocations, *MYC* amplifications were also detected in DLBCL. Using FISH, 7% of all DLBCL had *MYC* amplification, including 22% of GCB DLBCL (63). Recently, a novel technique of colorimetric *in situ* hybridization (CISH), detected more frequent amplification, in up to 70% of DLBCL as well as frequent abnormal signal clusters reminiscent of homogeneously staining regions (64). By aCGH, *MYC* gains were prognostically unfavorable, especially in the setting of concurrent chromosomal deletion of 8p (65). Elevated mRNA levels of *MYC* are also associated with poor patient outcome (10). Until recently there was no available antibody for adequate IHC detection of *MYC* in FFPE tissues. Now with the development of a rabbit monoclonal *MYC* antibody by Epitomics, *MYC* protein was evaluated in large series of R-CHOP treated DLBCL with concurrent genetic, GEP, prognostic, and outcome data (48,66). *MYC* protein was associated with poor outcome irrespective of mRNA or genetic changes indicating that it is the net effect on *MYC* protein expression including all genetic, post-transcriptional, and post-translational regulation, which impacts DLBCL biology and patient outcome. Thus, more than one method may be necessary to assess *MYC* deregulation. This same study demonstrated that *MYC* abnormalities including translocations or increased mRNA levels, and protein levels, were all prognostic in DLBCL, however only in the context of concurrent *BCL2* protein expression (but not *BCL2* translocations) (48). Thus, a particularly aggressive form of DLBCL has both the proliferative and other metabolic advantages of *MYC* expression while being able to evade chemotherapy-induced apoptosis by over-expression of *BCL2* protein. Despite being a well-characterized oncogene, efforts at targeting *MYC* have been fraught with challenges.

Previous approaches have included using triple helix forming oligonucleotides that affect gene transcription or antisense nucleotides that interfere with translation of *MYC* mRNA (67). An interesting strategy has included targeting MAX, the obligate bHLH-LZ (basic-helix-loop-helix leucine zipper) partner which heterodimerizes with *MYC*. These small molecule inhibitors affect *MYC*-MAX binding to E (enhancer)-box DNA sequences that lie within promoters, and serve as binding sites for downstream transcription factors. Several of these compounds are hindered by their relative low potencies with cytotoxicity obtained at

50–100µmol/L levels, which are not physiologically achievable (68). Another approach for *MYC* targeting at the transcriptional level is to interfere with bromodomain containing proteins that mediate acetylation to lysine residues on histones and are required for chromatin remodeling during transcription. JQ1 is a novel small molecule inhibitor that selectively binds to the acetyl lysine binding site of the bromodomain and extra terminal domain (BET) protein family consisting of BRD2, BRD3, BRD4, and BRDT. JQ1 is currently in preclinical development and is reported to lower *MYC* expression and in-turn arrest tumor growth in a multiple myeloma murine model (69). As previously mentioned, G-quadruplex DNA secondary structures formed within the *MYC* promoter region are also another novel therapeutic target in hematological malignancies that have applications for treatment of DLBCL (57,70).

Abnormal Microenvironment & Stromal Signatures

Historically the study of lymphoma involved isolation and analyses of the malignant lymphocytes. However, recent data showcase the importance of also considering the tumor microenvironment in the pathogenesis of lymphoma. GEP data identified predictive gene signatures that correlated outcome of molecular DLBCL subtypes to anthracycline based chemotherapy. Of interest, expression of the genes in the “lymph node” signature, which encode for components of the extracellular matrix that mediate fibrosis, predicted a favorable patient outcome (6). A whole genome array study with tissue from 176 newly diagnosed DLBCL patients using, multiple clustering methods grouped the DLBCL into 3 different categories according to gene expression of tumor microenvironment and host response, (1) oxidative phosphorylation, (2) B-cell receptor/proliferation, and (3) host response (71). The “host response” (HR) cluster was identified by a microenvironment gene signature that had unique characteristics including fewer genetic abnormalities, increased expression of multiple components of the T-cell receptor, CD2, and additional molecules associated with T/NK-cell activation and the complement cascade. These tumors occurred in younger patients, and had unique clinical presentations involving splenomegaly and bone marrow involvement (71). In a different study using GEP and clinical data from 787 DLBCL patients stratified into 2 cohorts (CHOP or R-CHOP treated), a 2 gene model was developed reflecting the contributions of the tumor and the microenvironment to the pathogenesis of DLBCL. The first gene, *LMO2*, was validated as an independent predictor of survival in the GCB DLBCL subtype and the second gene was associated with the DLBCL microenvironment, tumor necrosis factor receptor superfamily member 9 (*TNFRSF9*) (72). Tertiles of the 2-gene score stratified patients with distinct outcomes with corresponding 2-year overall survivals. When assessed within DLBCL subtypes, high 2-gene scores identified patients with relatively adverse outcomes in the GCB DLBCL subtype. Consistent with this correlation to poor prognosis, the low scores for *LMO2* and *TNFRSF9* expression in the ABC-like tumors identified patients with superior outcomes (10). In further support that signaling from the tumor microenvironment contributes to the progression of DLBCL and ultimately patient outcome, a GEP study with R-CHOP treated patient demonstrated two “stromal” signatures: “stromal-1” and “stromal-2” (7).

The stromal-1 signature was associated with increased patient survival and included expression of genes related to the extracellular matrix and histiocytes, such as, secreted

protein, acidic and rich in cysteine (SPARC) and connective tissue growth factor (CTGF). SPARC, also known as osteonectin, is a glycoprotein frequently overexpressed in multiple tumor types including different solid tumors, and associated with increased tumor invasion and metastasis (73). CTGF is a cysteine rich matricellular protein that interacts with integrin receptors and with LRP-5 to inhibit WNT signaling. Elevated levels of CTGF have been noted in number of solid tumors, and hematological malignancies, such as acute lymphoblastic leukemia, and may promote tumor cell proliferation and migration (74). Although the precise role of SPARC and CTGF expression within the tumor microenvironment in DLBCL pathogenesis is unclear, both of these proteins are currently targeted for therapy in solid tumors where they exhibit pro-tumor effects. A novel albumin bound drug, Abraxane (nanoparticle bound albumin-Paclitaxel), can effectively target SPARC (75). A CTGF-specific monoclonal antibody, FG-3019, inhibits tumor growth and metastases in a pancreatic xenograft mouse model (74). Overall, lymphoma-associated histiocyte expression of SPARC and CTGF correlates with an increased survival following R-CHOP in DLBCL patients (7). However, as future research resolves the specific function of stromal-1 genes in the interaction between malignant DLBCL cells and cells within the microenvironment there may be a potential use for these therapies.

The stromal-2 signature includes endothelial- and angiogenesis-related genes and is targetable with drugs with anti-VEGF, PDGFR, ANG/TIE2 activity, and inhibitory effects on the CXCR4-CXCL12 axis (76). In the angiopoietin (ANG) pathway, ANG1 ligand binds TIE2, the tyrosine kinase receptor for ANG, expressed on endothelial cells, which allows for dimerization and phosphorylation of TIE2 (77). ANG1 is constitutively active in several organs whereas ANG2 functions as an ANG1 antagonist by increasing vascular permeability and neovascularization in conjunction with VEGF in tissues undergoing vascular remodeling. ANG2 overexpression is correlated with a worse prognosis in a variety of cancers including solid tumors and hematological malignancies (acute myeloid leukaemia and chronic lymphocytic leukaemia) (78,79). AMG386 is a first-in-class fusion peptibody that counteracts the interaction of ANG1 and ANG2 ligands with TIE2. This peptibody mediated anti-angiogenic and anti-tumor activity in human colon cancer xenografts (80) and showed promising clinical activity in a recent phase II clinical trial for ovarian cancer (81). Monoclonal antibodies, MEDI-3617 and AMG780, directed against ANG2 are also currently under clinical development (77).

Summary

Molecular diagnostic techniques have been extensively applied to characterize the diagnostic signatures as well as the "therapeutic" signatures of DLBCL. In the future, this type of detailed information will allow placement of patients not only into specific diagnostic categories reflecting natural history, etiology, and prognosis but will also direct selection of new targeted agents. An outstanding example in the field of lymphoma was the recognition of the ABC subtype of DLBCL and the activation of the B-cell receptor signaling pathway through critical mutations. These findings directly led to clinical trials of the promising BTK-inhibitor drugs in DLBCL as well as other types of lymphomas with BCR abnormalities. New information on the prognostic significance of alterations in oncogenes and combinations of oncogenes was also revealed along with the importance of

the tumor microenvironment. These latter areas are also becoming the focus of intense clinical therapeutic investigation. It is anticipated that in the future, the field will develop the same level of detailed information for the other major categories of lymphoma including Hodgkin's lymphoma, and the highly lethal T-cell NHL.

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Fig 1.
Schematic Representation of BCR Signaling Pathway and Small Molecule Inhibitors of BCR Signaling.

Table 1

High Throughput methods for tumor investigation

Field	Target	Method	Information
Epigenetics	Chromatin	Methylation profiling	Methylation patterns of nucleotides
		Chromatin immunoprecipitation, “ChIP on Chip” or “ChIP- Seq”	Transcription factor associations with DNA; DNA sequences involved in histone methylation or acetylation
Genomics	DNA	Automated sequencing	DNA sequence
		SNP array with sequencing	Copy-neutral loss of heterozygosity; association with risk or outcome of malignancy
		Array competitive comparative genomic hybridization	Gains and losses of DNA
Transcriptomics	RNA	Gene expression profiling	mRNA levels
		RNA sequencing	Changes in RNA (and underlying DNA) sequences
		siRNA	Critical mRNAs for cell functioning
Proteomics	Proteins	Mass spectroscopy	Quantity and quality of expressed proteins

Table 2

Classification of aggressive B cell NHL *

DLBCL, Not otherwise specified	
Morphologic variants	Centroblastic
	Immunoblastic
	Anaplastic
	Rare cytologies
Molecular subgroups	Germinal center B cell-like
	Activated B-cell-like
Immunohistochemistry subgroups	CD5+
	Germinal center B cell-like
	Non-Germinal Center B cell-like
DLBCL, Subtypes	
	T cell/histiocyte rich large B cell lymphoma
	Primary DLBCL of the Central Nervous System
	Primary cutaneous DLBCL, leg type
	Epstein Barr Virus positive DLBCL of the elderly
Other lymphomas of large B cells	
DLBCL associated with chronic inflammation	
Lymphomatoid granulomatosis	
Primary Mediastinal (thymic) large B cell lymphoma	
Intravascular large B cell lymphoma	
ALK positive DLBCL	
Plasmablastic lymphoma	
Large B cell lymphoma arising in HHV8-associated multicentric Castleman disease	
Primary effusion lymphoma	
Borderline categories	
B cell lymphoma with features intermediate between DLBCL and Burkitt lymphoma	Often characterized by concurrent translocations of MYC and BCL2 and/or BCL6 (double- and triple-hit lymphomas)
B cell lymphoma with features intermediate between DLBCL and classical Hodgkin lymphoma	

* according to WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, 2008