R-CHOP resistance in diffuse large B-cell lymphoma: biological and molecular mechanisms

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Abstract
Although the first-line rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone regimen (R-CHOP) substantially improved outcomes for patients with diffuse large B-cell lymphoma (DLBCL), 40% of the patients suffered from relapsed/refractory disease and had poor survival outcomes. The detailed mechanism underlying R-CHOP resistance has not been well defined. For this review, we conducted a thorough search for literature and clinical trials involving DLBCL resistance. We discussed DLBCL biology, epigenetics, and aberrant signaling of the B-cell receptor (BCR), phosphatidylinositol 3-kinase (PI3K)/Akt, nuclear factor kappa light chain enhancer of activated B-cells (NF-κB), and the Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) pathways as defining mechanisms of DLBCL heterogeneity and R-CHOP resistance. The cell of origin, double- or triple-hit lymphoma and double-protein-expression, clonal evolution, tumor microenvironment, and multi-drug resistance help to contextualize DLBCL resistance in an (epi)genetically and biologically comparative manner. With better understanding of the biological and molecular landscape of DLBCL, a more detailed classification system and tailored treatments will ideally become available to further improve the prognosis of DLBCL patients.

Keywords: Diffuse large B-cell lymphoma; Tumor microenvironment; Multi-drug resistance; Genetic heterogeneity

Introduction
Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin lymphoma (NHL), comprising approximately 25% of the total mature NHL cases in the United States and 45.8% in China.¹,² Rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) has been considered as a standard first-line therapy following improvements in the prognosis of patients with DLBCL, resulting in a 10-year overall survival (OS) of 43.5%.[³] However, 20% of the patients treated with R-CHOP had refractory disease, and approximately 20% of the patients who initially benefited from R-CHOP reported relapsed cases.[⁴] The 30% to 50% of patients with primary or secondary resistance to R-CHOP had significantly poorer survival outcomes and a median OS for about 6.3 months.[⁵] The mechanisms of R-CHOP resistance have not been adequately elucidated. For this review, we conducted a thorough search for literature and clinical trials involving DLBCL resistance and recapitulated the mechanisms of R-CHOP resistance regarding tumor biology and molecular pathways. Moreover, we addressed practical agents targeting each pathway, thus paving the road for future hierarchical classification and tailored treatments.

Tumor biology of R-CHOP resistance

Cell of origin
Gene expression profiling (GEP) analysis has defined activated B-cell-like (ABC) and germinal center B-cell-like (GCB) as the two major subtypes of DLBCL (approximately 50% and 30%, respectively), according to cell of origin (COO). GEP studies have shown that genes defining the ABC subgroup, such as interferon regulatory factor 4 (IRF4), FLICE-like inhibitory protein (FLIP), and B-cell lymphoma 2 (BCL-2), are normally induced during B cell proliferation and activation as characteristics of plasma cells.[⁶] In contrast, the GCB subgroup exhibits genes that are preferentially expressed in germinal center B-cells, such as cluster of differentiation 10 (CD10), LIM domain only 2 (LMO2), and BCL-6.[⁷] Intriguingly, recurrent enhancer of zeste 2 (EZH2) mutation, phosphatase and tensin homolog (PTEN) deletion, BCL-2 translocation/mutation, and c-REL amplification, which are common in the GCB subtype, are seldom described in the ABC subtype.[⁸] The
GCB subgroup had a significantly better 3-year OS than the non-GCB subgroup when treated with R-CHOP (85% vs. 69%), thereby indicating distinct mechanisms of R-CHOP resistance in the ABC subgroup.[8,9] At the same time, the GCB and ABC subtypes of DLBCL are also associated with an umbrella entity, encompassing biologically and genetically distinct aberrations. Thus, a more precise intra-subgroup classification is recommended.

According to a novel classification system proposed by Schmitz et al[9] based on the co-occurrence of gene aberrations, 23.1% of the patients with ABC-DLBCL exhibited an MCD subtype (MYD88 plus CD79B mutations), 13.6% exhibited BN2 (BCL-6 fusions plus NOTCH2 mutations), and 6.1% exhibited N1 (NOTCH1 mutations). Meanwhile, 37.2% of the patients with GCB-DLBCL exhibited the EZB subtype (EZH2 mutations and BCL-2 translocations) and 11.6% exhibited BN2 while the remaining 51.1% were unclassified. After R-CHOP treatment, patients with the N1 or MCD subtype had significantly inferior outcomes compared to those with the EZB or BN2 subtype. The predicted 5-year OS rates for the MCD, N1, BN2, and EZB subtypes were 26%, 36%, 65%, and 68%, respectively.[9] Drugs that target B-cell receptor-dependent nuclear factor kappa light chain enhancer of activated B-cells (NF-kB) activation (such as Bruton tyrosine kinase [BTK] inhibitors) may be investigated in the BN2 and MCD subtypes while immune-checkpoint inhibitors appear promising in the N1 subtype due to their prominent T-cell gene-expression signature.[9]

Additionally, Chapuy et al[10] identified the following five robust DLBCL clusters with discrete outcomes and coordinated genetic signatures: C1: a low-risk ABC-DLBCL subset of extrafollicular origin with frequent mutations in the NF-xB pathway members (eg, BCL-10 and tumor necrosis factor alpha-induced protein 3 [TNFAIP3]), NOTCH2, and BCL-6 activation; C2: an ABC/GCB-independent subset with 17p loss and TP53 inactivation; C3: a GCB-DLBCL subset with frequent mutations in epigenetic enzymes (eg, lysine methyltransferase 2D [KMT2D], CREB-binding protein [CREBBP], and EZH2), BCL-2, and phosphatase and tensin homolog (PTEN); C4: a GCB-DLBCL subset with mutations in NF-xB modifiers (eg, caspase recruitment domain-containing protein 11 [CARD11], NFKB inhibitor epsilon [NFKBIE], and NFKB inhibitor alpha [NFKBIA]), CD83, and STAT3; C5: an ABC-DLBCL subset with concordant CD79B (48%)/MYD88(26.6%) (50%) mutations and frequent gain of BCL-2, which is associated with extranodal tropism. Accordingly, patients with the C3 or C5 subtype had significantly inferior survival outcomes compared to that of C1, C2, or C4 when treated with R-CHOP, and BTK inhibitors might improve the prognosis of the C5 subtype while epigenetics-modifying agents might be active in the C3 subtype.[10]

**Clonal evolution**

A gain in R-CHOP resistance in DLBCL is increasingly suspected to correlate with the process of clonal evolution. Melchardt et al[11] described the following three major patterns of clonal evolution using high-throughput sequencing (HTS) of 104 genes in 28 DLBCL patients: large global change, subclonal selection, and minimal or no change. Fluctuations of subclones harboring the gene mutations related to R-CHOP resistance (eg, BCL-2 and proto-oncogene serine/threonine-protein kinase 1) were observed as a result of chemoinmunotherapy. More non-synonymous gene mutations in DLBCL patients were associated with a shorter median OS. It was suggested that R-CHOP selected the subclones that expressed gene mutations (eg, tumor protein 53 [TP53]) that favored tumor progression.[11] Likewise, Rizzo et al[12] described the following two patterns of clonal evolution in relapsed DLBCL employing the HTS of variability, diversity, and joining (VDJ) rearrangements on the immunoglobulin heavy chain: an early divergent mode and a late-divergent mode with divergence at the phylogenetic degree. No significant antigen selection in the complementary determining region of the VDJ locus has been observed under immunochemotherapy pressure, suggesting that oncogenic selection rather than antigen selection is a major impetus in R-CHOP resistance.[13]

Under the stress of rituximab treatment, resistant subclones may evolve to lose the surface CD20 antigen (at the RNA or protein level) or to develop genetic mutations of the membrane-spanning four-domains, subfamily A, member I gene.[14] Moreover, regarding the action mechanisms of rituximab, resistant lymphoma subclones could be selected by FcR single nucleotide polymorphisms to diminish the rituximab-induced antibody-dependent cellular cytotoxicity.[14]

**Tumor microenvironment**

The tumor microenvironment is considered to be a defining feature in B-cell differentiation and DLBCL tumorigenesis, including immune cells, stromal cells, and extracellular components [Figure 1]. The response to R-CHOP was affected by the pretreatment of tumor microenvironment concerning fibrosis, angiogenesis, and immune cells.[15] The tumor microenvironment was differentially constructed at the gene and protein levels when comparing R-CHOP-sensitive DLBCL with resistant DLBCL.[16] CD37 deficiency, programmed cell death-ligand 1 (PD-L1), and CD47 upregulation on malignant B-cells revealing immune evasion were observed in resistant cases.[17,19] However, it was found that CD47 upregulation correlated with poorer prognosis in patients with non-GCB DLBCL compared to the GCB subtype, thereby indicating that a distinct microenvironment existed in different COOs.[20] A CD47 blockade by Hu5F9-G4 can synergize with rituximab to overcome R-CHOP resistance in DLBCL.[19] Janus kinase-signal transducer and activator of transcription 3 (JAK-STAT3) signaling propagates several levels of intimate interaction between tumor cells and the immunological microenvironment to regulate angiogenesis, inflammation, immuno-suppression, and oncogenesis.[21]

The apoptosis-protective adhesion to stromal cells, termed cell adhesion-mediated drug resistance (CAM-DR), is another underlying mechanism of R-CHOP resistance in DLBCL.[22] CAM-DR was revealed to correlate with
rituximab resistance through ADAM metalloproteinase domain 12 (ADAM-12) upregulation and phosphatidylinositol 3-kinase (PI3K)-Akt signaling modulation. However, as per findings reported by Lenz et al., genetically identified non-malignant signatures of stromal-1 (fibrosis and myeloid infiltration) and stromal-2 (vessel formation) in pretreatment biopsies were found to be prognostically favorable and unfavorable for patients with DLBCL who received R-CHOP, respectively. This finding was in concordance with a report by Cardesa-Salzmann et al. which stated that increased microvessel densities independently signified an inferior OS for patients with de novo DLBCL treated with R-CHOP.

**Multi-drug resistance**

Multi-drug resistance (MDR) describes acquired cross-resistance to a wide variety of structurally and functionally unrelated agents. The integrated membrane protein P-glycoprotein (Pgp) ATP binding cassette-1 serves as an ATP-dependent efflux pump for the active removal of its substrates through the lipid bilayer and for the reduction in intracellular cytotoxic concentration, thereby resulting in MDR. Pgp is encoded by MDR-1, one of the most investigated MDR genes located on chromosome 7q21.12, where significant polymorphisms exist. In addition to Pgp, other transporters in the ATP-binding cassette transporter family have been identified to confer drug resistance, such as multidrug-resistance-related protein-1 (MRP-1) and ATP-binding cassette subfamily G member 2 (ABCG2). Doxorubicin, vincristine, and prednisone are substrates for Pgp and are known to induce MDR-1 expression. DLBCL patients with high expression of Pgp, MRP-1, or ABCG2 demonstrated a significantly poor outcomes.

**Targeting molecular mechanisms in R-CHOP resistance**

The findings reported by Schmitz et al and Chapuy et al. suggested a different targeted therapies for genetically distinct DLBCL subsets. For example, the perturbation of proximal B-cell receptor (BCR) signaling is suggested for the C5 and MCD subtypes, BCL-2 inhibitors for C5, NF-κB signaling blockade for C1 and BN2, and inhibition of JAK-STAT3 signaling for C4. Herein, we summarized the most investigated gene and protein aberrations in DLBCL as well as the cross-linked signaling pathway intricacies at the intersection of DLBCL biology and the clinic. These abnormalities are further elucidated by the perturbation of epigenetics in this study. Existing resistance to R-CHOP necessitates the use of alternative
strategies and individualized therapies. In this context, rational drugs under investigation targeting R-CHOP resistance are further enumerated and discussed on a molecular basis [Figure 2].

**Double- or triple-hit lymphoma and double-expressor lymphoma**

Double-hit lymphoma (DHL) and triple-hit lymphoma (THL) were embraced as a new category of high-grade B-cell lymphoma by the 2016 World Health Organization classification of tumors with MYC/8q24 translocation accompanied by rearrangement of BCL-2/18q21, BCL-6/3q27, or both.\(^{[89]}\) Approximately 80% to 90% of the DHLs or 19% to 34% of the DLBCLs are also double-expressor lymphomas (DELs) with dual overexpression of c-MYC and BCL-2 detected by immunohistochemistry.\(^{[151]}\) Patients with DHL, THL, or DEL were generally considered to have a poor outcomes in the era of R-CHOP; however, the prognostic value of BCL-2, BCL-6, or MYC remains controversial.\(^{[12,33]}\) MYC plays a dominant role in proliferation, cell growth, apoptosis, and oncogenic transcription\(^{[34]}\) while BCL-2 maintains cellular viability via apoptosis inhibition.\(^{[15]}\) The dose-adjusted etoposide, prednisone, vincristine, cyclophosphamide, and doxorubicin regimen was shown to be a better choice for DLBCL patients with MYC translocation, although no randomized controlled trials have confirmed this so far.\(^{[16]}\) Landsburg et al.\(^{[37]}\) reported that ibrutinib monotherapy was highly effective in relapsed/refractory (rr) DEL. Regarding the overexpression of BCL-2 in DHL or DEL, BCL-2 inhibitor ABT-199 was demonstrated to result in an overall response rate (ORR) of 38% in rrDLBCL in a phase I trial.\(^{[38]}\) In a HOVON phase II trial,\(^{[39]}\) R-CHOP plus lenalidomide appeared to provide benefits to patients with MYC

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**Figure 2:** Targeting molecular pathways in R-CHOP-resistant DLBCL. This is the schematic representation of the BCR, PI3K-Akt, NF-κB, and JAK-STAT3 molecular signaling pathways in DLBCL. Aberrant expression of these pathways have emerged as top candidate targets implicated in R-CHOP resistance. These abnormalities are partially elucidated by epigenetic abnormalities. BCR: B cell receptor; BTK: Bruton’s tyrosine kinase; CARD11: Caspase-associated recruitment domain 11; DLBCL: Diffuse large B cell lymphoma; EZH2: Enhancer of zeste 2; FOXO: Forkhead box O; HDAC: Histone deacetylase; JAK-STAT3: Janus kinase/signal transducer and activator of transcription 3; MALT-1: Mucosa-associated lymphoid tissue lymphoma translocation-1; MAPK: Mitogen-activated protein kinase; NEMO: NF-κB-kappa B essential modulator; NF-κB: Nuclear factor kappa light chain enhancer of activated B cells; PI3K-Akt: Phosphatidylinositol 3-kinase/Akt; PKC: Protein kinase C; PTEN: Phosphatase and tensin homolog; PDK-1: Phosphatidylinositol-dependent kinase 1; PRDM1: Positive regulatory domain containing 1; R-CHOP: Rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone; SYK: Spleen tyrosine kinase; SPK: Src family of protein tyrosine kinases; IRAK: Interleukin 1 receptor associated kinase.
translocation with a 2-year OS and disease-free survival (DFS) of 73% and 75%, respectively. A previous study showed that the XPO1 inhibitor could decrease c-MYC expression, and combined use of the BCL-2 and XPO1 inhibitors was highly effective in eradicating DHL cells in vitro and prolonging host survival in vivo in a mouse DHL model.\[60\]

**BCR signaling pathway**

The BCR signaling pathway exhibits a functionally determinant role in B-cell activation, proliferation, and differentiation. The constitutive activation of PI3K and phosphatidylinositol-dependent kinase 1 (PDK-1) is essential for the survival of ABC-DLBCL cells with chronic active BCR signaling.\[44,42\] Signaling adapters caspase-associated recruitment domain 11 (CARD11), BIMP3, CARD-containing MAGUK protein 1 (CARMA1), BCL-10, and mucosa-associated lymphoid tissue lymphoma translocation-1 (MALT-1) comprise the CARMA1–BCL-10–MALT-1 (CBM) complex, but it is an integral whole, contributes to the upregulation of constitutive NF-κB activation in ABC-DLBCL.\[43\] Additionally, the MALT-1 subunit of the CBM complex features indirect NF-κB-promoting activity via proteolysis of the NF-κB inhibitors CYLD, RELB, A20, and BCL-10.\[44\] Indeed, chronic-active BCR signaling plays a pivotal in the survival of almost all ABC-DLBCLs and driven by frequent activating mutations of the immunoreceptor tyrosine-based activation motifs in CD79B and CD79A or of the coiled-coil domain in CARD11.\[44\] In contrast, GCB-DLBCLs are prone to survive with a BCR-negative immunophenotype.\[45\]

Previous studies have provided a framework for targeted inhibition of the BCR signaling pathway in B-cell receptor-dependent ABC-DLBCL.\[44,46-52\] Apart from COO algorithms, genetic assessments to confirm the exact lesion of the molecular pathways are recommended. For example, BTK inhibition is lethal for the upstream CD79B mutant cells but dispensable for the downstream CARD11-mutant DLBCL, which would be killed by downstream blockade of the NF-κB pathway.\[53\]

**PI3K-Akt signaling pathway**

PI3K-Akt signaling activation is enabled by sequential phosphorylation of PI3K, phosphatidylinositol-4,5-bisphosphate (PIP2), and Akt protein kinase B.\[54\] Akt directly activates NF-κB signaling via phosphorylated IkBs at Thr23\[55\] and indirectly activates NF-κB signaling by stimulating the mitogen-activated protein kinase (MAPK) family.\[56\] PTEN serves as a major negative regulator of PI3K-Akt signaling by dephosphorylating PIP3, and PI3K/Akt dysregulation was indicated in 55% of the GCB-DLBCLs and 14% of the non-GCB DLBCLs with PTEN deficiency.\[57\]

The PI3K-Akt signaling pathway is involved in rituximab action, chronic activation of BCR signaling, and CAM-DR. High levels of phosphorylated Akt had an adverse prognostic impact on patients with DLBCL treated with R-CHOP.\[58\] Conceivably, components of the PI3K-Akt signaling pathway exhibit validated targets of DLBCL. Everolimus and temsirolimus are rapamycin analogs that target the raptor mammalian target of rapamycin. In a phase II trial of everolimus for rrDLBCL, an ORR of 30% was reported.\[59\] Furthermore, single-agent ibrutinib or copanlisib for inhibition of BTK or PI3K triggered simultaneous activation of the other pathway, thereby highlighting the significance of combined therapy.\[42\]

**NF-κB signaling pathway**

NF-κB signaling is activated by canonical (activates p50/rel and p50/relA) and non-canonical (activates p52/relB) pathways with both anti-apoptotic and apoptotic functions.\[60\] As downstream effectors of chronic active BCR signaling, the sustained activity of NF-κB signaling exerts a prominent tumor survival feature in ABC-DLBCL but not in GCB-DLBCL.\[61\] Both p50 and p65 activation proved to be unique mechanisms of R-CHOP resistance in ABC-DLBCL.\[62,63\] ABC-DLBCLs facilitate the canonical NF-κB signaling pathway by aberration of MYD88, BCL-10, CARD11, CD79A, CD79B, cyclin D2, CCR7, IRF4, FLIP, NFKB1A, TRAF2, and TNFAIP3.\[64,65\] Cells with MYD88 mutations exhibit constitutive activation of canonical NF-κB signaling.\[60\] Therefore, the target components of NF-κB signaling are of therapeutic importance. Lenalidomide and its analog thalidomide, which have antiangiogenic and pleiotropic immunomodulatory functions, exert direct tumor toxicity by binding to cereblon to inhibit downstream NF-κB signaling.\[66\] The regimen of lenalidomide plus R-CHOP appeared to mitigate the inferior survival of non-GCB DLBCLs in two phase II trials of 112 patients with newly diagnosed DLBCL.\[66\]

**JAK-STAT3 signaling pathway**

JAKs belong to the cytosolic tyrosine kinase family. JAKs are phosphorylated and activated upon cytokine binding. Subsequently, STAT proteins are activated and transition to the nucleus to facilitate transcription of downstream genes (eg, IL-6, IL-10, cyclin D1, cyclin D2, MYC, BCL-xl, and BCL-2), thereby influencing cell viability, immunosuppression, angiogenesis, and oncogenesis.\[67\] The STAT3-BCL-2-IL-10 loop is involved in R-CHOP resistance.\[68\] STAT3 expression was detected in 37% of the DLBCLs and in 54% of the ABC-DLBCLs treated with R-CHOP, thereby indicating poor survival, especially for the ABC subtype.\[69\] Conceivably, activation of the JAK-STAT3 signaling pathway of ABC-DLBCL suggests promising therapeutic targets, including JAK, STAT3, and IL-10 receptors.\[70\] According to results from a phase I b trial, STAT3 antisense oligonucleotide AZD9150 showed efficacy in patients with rrNHL.\[71\]

**Epigenetics**

Epigenetic modifications confer crucial information to the transcription phenotypes and biological behaviors of tumorigenesis and drug resistance. Mutations of epigenetic modifiers (EP300, KMT2D, and SETDB1) were observed at both diagnosis and relapse, suggesting their roles as driver mutations of tumorigenesis in DLBCL.\[13\] Methyl-
atation and acetylation are the most investigated epigenetic patterns underlying R-CHOP resistance in DLBCL. Distinct epigenetic profiles contributing to the ABC and GCB phenotypes were detected and helped to redefine the molecular subtypes of DLBCL.24 The prominent feature of ABC-DLBCLs and JAK activation was found to correlate with phosphorylation of histone H3 tyrosine 41 rather than STAT regulation.23 MicroRNAs are also involved in drug resistance in DLBCL,74 and the abnormal epigenetic profiling of microRNAs represents a putative mechanism of the positive regulatory domain-containing 1 (PRDM1) repression in DLBCL with wild-type PRDM1.75

Notably, unlike the aberrant genes and pathways mentioned above, epigenetic abnormalities are reversible. CD20 repression and rituximab resistance detected in the RRBL1 cell line were successfully reversed by the histone-deacetylase inhibitor trichostatin A.76 The class-1 histone deacetylase inhibitor panobinostat increased DLBCL cell death.77 Subsequent investigation revealed that DNA methyltransferase azacitidine followed by R-CHOP proved feasible in a phase I trial of 12 high-risk patients with newly diagnosed DLBCL.78 The efficacy of tazemetostat, a single-agent inhibitor of histone methyltransferase EZH2 for the treatment of rrDLBCL, was also evaluated in trials.79

Conclusions

At present, we are well equipped to predict outcomes for DLBCL patients treated with R-CHOP using genetic, immunohistochemical, serum, and imaging markers. However, proper classification of DLBCL patients and identification of R-CHOP non-responders at diagnosis are critical. The BCR, PI3K-Akt, NF-kB, and JAK-STAT3 molecular signaling pathways are established mechanisms underlying R-CHOP resistance. The COO, clonal evolution, tumor microenvironment, MDR, and epigenetics are important for biological considerations. Novel target agents based on these mechanisms are currently being used in preclinical and clinical trials of DLBCL. Although there is only modest evidence to support their roles as first-line alternatives for R-CHOP, they must not be underestimated in the management of resistant DLBCL. Ideally, with better understanding of the biological and molecular landscape of DLBCL, a more detailed classification system and tailored treatments will be available in the near future.

Conflicts of interest

None.

References


