**Title:** Utilization of the $E\mu$-Myc Mouse to Model Heterogeneity of Therapeutic Response

**Running Title:** Mouse model of human lymphoma therapy heterogeneity

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Abstract

Human aggressive B-cell non-Hodgkin lymphomas (NHL) encompass the continuum between Burkitt lymphoma (BL) and diffuse large B-cell lymphoma (DLBCL), and display considerable clinical and biologic heterogeneity, most notably related to therapy response. We previously showed that lymphomas arising in the $E\mu\text{-Myc}$ transgenic mouse are heterogeneous, mirroring genomic differences between BL and DLBCL. Given clinical heterogeneity in NHL and the need to develop strategies to match therapeutics with discrete forms of disease, we investigated the extent to which genomic variation in the $E\mu\text{-Myc}$ model predicts response to therapy. We used genomic analyses to classify $E\mu\text{-Myc}$ lymphomas, link $E\mu\text{-Myc}$ lymphomas with NHL subtypes, and identify lymphomas with predicted resistance to conventional and NF-κB targeted therapies. Experimental evaluation of these predictions links genomic profiles with distinct outcomes to conventional and targeted therapies in the $E\mu\text{-Myc}$ model, and establishes a framework to test novel targeted therapies or combination therapies in specific genomically-defined lymphoma subgroups. In turn, this will rationally inform the design of new treatment options for aggressive human NHL.
Introduction

Aggressive B-cell lymphomas include a spectrum of diagnoses that span Burkitt lymphoma (BL), diffuse large B-cell lymphoma (DLBCL), and lymphomas that lie between these diagnoses, termed by the World Health Organization 2008 classification as “B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and BL” (1). There is considerable clinical and therapy response heterogeneity across and within these diseases. While DLBCL is generally responsive to the R-CHOP chemo-immunotherapy regimen (containing rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone), BL requires more aggressive multi-agent regimens that are accompanied by higher toxicities. Although these lymphoma subtypes are generally treated differently, patients are not always cured and responses are not always complete. Prior studies evaluating the heterogeneity of aggressive B-cell lymphomas using primary patient samples have begun to highlight that biologic and genomic complexity underlies clinical variation (2-6). At the current time, these studies have not resulted in clinically used biomarkers to aid in therapy selection. The inherent limitations in the availability and quality of patient-derived samples suggest that experimental models could greatly facilitate efforts to understand heterogeneity in aggressive lymphomas and in therapy response and to develop appropriate therapeutic options.

Genetically engineered mouse models (GEMMs) have provided significant insight into human cancer biology. These models result from the activation or loss of a single gene, and are generally considered to represent a distinct and homogenous phenotype. However, our previous work has provided evidence of heterogeneity in GEMMs, specifically the MMTV-Myc model of breast cancer and the Eμ-Myc model of B-cell lymphoma (7, 8). In both cases, we evaluated large numbers of tumors from these transgenic mice and found that natural heterogeneity in
histologic characteristics and genome-scale expression data exists, suggesting secondary genetic hits drive variation in GEMMs.

The $E\mu$-$Myc$ transgenic mouse was developed as a model of $Myc$-driven aggressive B-cell lymphoma (9, 10). Multiple investigations have identified genes that alter the onset of $E\mu$-$Myc$ lymphoma and/or affect response to single agent chemotherapy (11-19). The $E\mu$-$Myc$ model has also been used in a genetic screen to identify genes that modulate response to doxorubicin (20). By focusing largely on perturbing single genes in the $E\mu$-$Myc$ background, these studies did not focus on the effects of gene networks and their relevance to the natural genetic heterogeneity seen in the $E\mu$-$Myc$ model, particularly as it pertains to response to lymphoma therapy.

We previously described that the $E\mu$-$Myc$ transgenic mouse model of lymphoma develops genomically distinct lymphoma subtypes that reflect the spectrum of human aggressive B-cell lymphomas (7). Here, we describe a genomic analysis strategy to reproducibly classify the distinct forms of $E\mu$-$Myc$ tumors and methods to use the $E\mu$-$Myc$ lymphoma model to predict therapy response.

Materials and Methods

**Mouse strains and tumor monitoring.** $E\mu$-$Myc$ strain (JAX stock# 002728) and C57BL/6J strain (JAX stock# 000664) mice were purchased from Jackson Laboratories, and were bred and housed in a Duke University Medical Center Division of Laboratory Animal Resources facility. All experiments were approved by the Duke University Institutional Animal Care and Use Committee. $E\mu$-$Myc$ mice were monitored twice weekly for visible or palpable lumps, a hunched posture, tachypnea, a swollen belly, or ruffled fur. Upon development of such symptoms, mice were sacrificed and dissected. The spleen and enlarged lymph nodes or masses were removed and placed in RPMI media (GIBCO) with 10% heat-inactivated fetal bovine serum (FBS, Sigma).
Spleens were weighed and lymph node specimens were frozen in liquid nitrogen or fixed in 10% formalin. Single cell suspensions were generated from remaining tissue by squeezing between ground glass slides and filtering the suspension through a sterile 100 μm cell strainer (BD Falcon). Thereafter, we lysed red cells in the cell suspensions and washed the cells twice. Lymphoma cells were either stored inviably at -80°C as cell pellets or resuspended in freezing media (10% DMSO (Sigma) in heat-inactivated FBS), aliquoted, and stored viably in liquid nitrogen.

**Generation of Eµ-Myc lymphoma in C57BL/6 mice and treatment.** To transplant Eµ-Myc lymphomas to C57BL/6 mice, we thawed, washed, and counted viably frozen Eµ-Myc lymphoma cells. We suspended the lymphoma cells in RPMI media (GIBCO) and injected 5x10⁵ lymphoma cells by the i.p. route into C57BL/6J mice. We monitored recipient mice daily, and rated illness on a scale from 1 to 5, where 1 was moribund, 3 was obvious lymphoma, and 5 was perfect health. Mice with a rating of 3 were either sacrificed or treated with chemotherapy – cyclophosphamide (300 mg/kg i.p. once), doxorubicin (10 mg/kg i.p. once), or bortezomib (1 mg/kg i.p. twice weekly for four doses) – and were thereafter monitored daily using the rating scale until progression (score less than 3), at which time they were sacrificed and lymphoma tissue was collected. Cyclophosphamide and doxorubicin were purchased from the Duke University Medical Center inpatient pharmacy, and bortezomib was purchased from LC laboratories.

**Gene expression profiling data preparation and normalization.** Lymphoma tissue samples were homogenized using the Lysing Matrix A tube for tissue (MP Biomedicals). Lymphoma cell pellet samples were homogenized by passing through a tuberculin syringe and centrifuged through a QIAshredder homogenizer column (Qiagen). RNA was extracted from the
lysates using Qiagen RNeasy kits (Qiagen). RNA integrity was verified with an Agilent 2100 Bioanalyzer. Microarray processing and RNA hybridization to Affymetrix Mouse 430 2.0 or Mouse 430A 2.0 GeneChip arrays were performed according to the manufacturer’s instructions in the Duke University DNA microarray core facility.

From microarray CEL files, data was normalized with either MAS5 or RMA algorithms for appropriate downstream analyses. Gene expression data can be accessed in the NCBI Gene Expression Omnibus (GSE40760). For analyses comparing data obtained from microarrays processed at different times (separate batches), the ComBat algorithm (21) was used to reduce batch effect. For analyses comparing human and mouse microarray data, we used ChipComparer and FileMerger (http://chipcomparer.genome.duke.edu/ and http://filemerger.genome.duke.edu/), as described previously (8).

**Microarray analysis.** We performed unsupervised analyses of unfiltered genomic data from Eμ-Myc lymphomas using the k-means clustering algorithm. For supervised analyses, we determined which genes were differentially expressed between the two clusters using the Wilcoxon rank sum test and Bonferroni correction (q value < 0.2). Gene ontology and biological function of differentially expressed genes was assessed using DAVID (http://david.abcc.ncifcrf.gov/home.jsp), and significant gene ontology terms were selected from Bonferroni values less than 10^{-5}. We also used genomic data to develop new genomic models (“signatures”) from existing microarray datasets using the binary regression (Binreg) algorithm (for two-class models) (22, 23) or Prediction Analysis for Microarrays (PAM; for greater than two-class models (24)). Datasets used to generate and validate signatures along with accuracy of the signature using leave-one-out cross validation are listed in Table S1. Parameters used in generating genomic signatures are listed in Table S2. Genomic signatures of cellular pathway
activation were applied using the ScoreSignature module (25) on the Duke University GenePattern server. Gene expression microarray data can be found on Gene Expression Omnibus at NCBI, accession number GSE40760.

**Immunoblot analysis.** Protein was extracted from frozen lymphoma tissue and immunoblots were performed as described previously (15). 100 μg protein samples were fractionated by gel electrophoresis and transferred to Immobilon membrane to detect c-MYC, p19ARF, MDM2, p53, p65/RELA, phosphorylated p65/RELA, β-Actin, and GAPDH. Antibodies used were as follows: c-MYC (Santa Cruz N-262 sc 764, 1:500), p19ARF (Calbiochem Ab-1 PC435, 1:1000), MDM2 (Santa Cruz c-18 sc812, 1:1000), p53 (Calbiochem Ab-1 OP03, 1:1000), p65/RELA (Santa Cruz C-20 sc-372, 1:1000), phosphorylated p65/RELA (Cell Signaling 93HI 3033S, 1:1000), β-Actin (Cell Signaling 13E5 4970S, 1:5000), and GAPDH (Santa Cruz FL-335 sc-25778, 1:500). Equal protein loading was verified by staining blots with Ponceau Red and probing for GAPDH or β-Actin.

**Southern analysis.** Genomic DNA was isolated from frozen lymphoma tissue, digested with EcoR1 (10 mg per sample) and fractionated by agarose gel electrophoresis. Thereafter, DNA was transferred to membranes and probed with a radiolabeled heavy chain J3-J4 joining region genomic fragment, as described before (15).

**Flow cytometry.** Eμ-Myc lymphoma surface expression of B220, IgM, IgD, CD43, and CD138 were assessed with flow cytometry, using the following reagents: Mouse BD Fc block (BD Pharmingen 553142) and antibodies (B220-APC BD Pharmingen 553092, IgM-FITC BD Pharmingen 553437, IgD-PE BD Pharmingen 558597, CD43-FITC BD Pharmingen 553270, and CD138-PE BD Pharmingen 553714). Cells were washed, incubated with block then antibody, washed again, and fixed prior to assessment on a Becton Dickenson FACSCanto II flow
cytometer. Data were analyzed with FlowJo (Tree Star, Inc.). We also stained and performed flow cytometry on pooled bone marrow, mesenteric lymph node and spleen cells from healthy C57BL/6 mice, which were used as a normalization control between experiments.

**Statistical analyses.** Statistical analyses were performed with the statistical environment R using standard packages and the pamr and survival packages. Binary regression modeling was performed using MATLAB (26). Pathway signature scores were generated using the ScoreSignature module in GenePattern (25). Progression free survival was calculated as days from therapy to when the mouse had consistent progression of illness compared to the day of therapy, was moribund and sacrificed, or died.

**Trp53 Sequence analysis.** RNA was isolated from frozen lymphoma cell pellets: cells were lysed in Qiagen RLT buffer, the lysate homogenized by passage through a 20 gauge needle followed by a QIAshredder spin column, and the RNA purified using an RNeasy spin column (Qiagen). The preparation was treated with DNase I (New England Biolabs) and then further purified using a second RNeasy spin column. The RNA was analyzed for quality using the RNA 6000 Pico Kit and the Agilent 2100 Bioanalyzer (Agilent Technologies). RNA (1 µg) was reverse-transcribed using the qScript cDNA SuperMix (Quanta BioSciences). The coding region of the *Trp53* cDNA was PCR amplified in three overlapping segments using the Phusion High Fidelity PCR Master Mix with HF Buffer (New England Biolabs). The size and quantity of the PCR products were verified by agarose electrophoresis. The PCR products were treated with ExoSAP-IT (Affimetrix) and Sanger sequenced. FinchTV was used for the chromatogram analysis. Sequences were analyzed for mismatches and insertions or deletions using BioEdit and the Basic Local Alignment Search Tool (BLAST) hosted by the NCBI.

**Results**
A method to predict Eµ-Myc lymphoma subtypes. We previously described the intrinsic heterogeneity of global gene expression in lymphomas that develop from the Eµ-Myc mouse model (7). Our current unsupervised analysis of gene expression profiling data from 112 Eµ-Myc lymphomas confirms variation in overall genomic expression (Figure 1A), and identifies a natural division of these lymphomas into two Eµ-Myc subgroups, or “clusters” (Figure S1). Iterative unsupervised k-means clustering analyses demonstrate reproducibility (99.9% of runs), whereas attempts to subdivide the dataset into more than two clusters resulted in instability in the cluster assignments for the samples.

Moving beyond descriptive analyses, we developed a prediction model (or “signature”) that can be used to classify a new lymphoma sample into one of the two clusters (Figure 1B). This 500-probe cluster classification signature has 97% accuracy of correctly classifying training data on leave-one-out cross validation. We evaluated a test set of 76 new Eµ-Myc lymphomas, originating in C57BL/6 congenic mice, in which the genomic signature identified 56 Cluster 1 lymphomas and 20 Cluster 2 lymphomas, using a cut-off score of 0.5. Concordant with the prior description (7), there is a significant difference in time to onset of these two types of lymphoma, with Cluster 1 lymphomas more likely to occur at an earlier age than Cluster 2 lymphomas (median time to onset of 121 vs. 326 days, respectively, p = 0.0003, log-rank test, Figure 1C).

These results confirm heterogeneity in the transgenic Eµ-Myc model and establish a predictive framework that can prospectively evaluate new and independent Eµ-Myc lymphomas.

Genetic, biologic, and clinical differences between the Eµ-Myc subtypes. Moving beyond simply classifying Eµ-Myc lymphomas, we evaluated differences between the identified genomic clusters. For example, in supervised analyses of differentially expressed genes between the two
clusters, we found that genes upregulated in Cluster 1 lymphomas are significantly enriched for gene ontology biological function terms related to RNA processing, regulation of transcription and translation, and cell cycle, whereas genes upregulated in Cluster 2 lymphomas are significantly enriched for gene ontology biological function terms that include immune response, protein localization, and regulation of apoptosis (p values all < $10^{-8}$; Bonferroni FWER all < $10^{-5}$, Table S3).

A further evaluation of gene expression data identified significant differences between the $E_{\mu}$-Myc lymphoma clusters in terms of gene sets that represent B-cell maturation. Using a stage-specific genomic signature developed from sorted murine B-cells of different maturation stages (27), we classified $E_{\mu}$-Myc lymphomas as pro/pre B-cell stage (n = 53, 70%), follicular/marginal zone stage (n = 14, 18%) and germinal center B-cell stage (n = 9, 12%). Lymphomas classified as deriving from pro/pre B-cells were more common in the Cluster 1 subgroup, whereas lymphomas classified as from the follicular/marginal zone stage were more common in Cluster 2 subgroup (p = 5x$10^{-7}$, Chi-squared test, Table S4).

Since stage of differentiation and activation has prognostic value in human DLBCL (6), we evaluated the $E_{\mu}$-Myc lymphoma genomic data within the context of germinal center B-cell (GCB) versus activated B-cell (ABC) lymphoma subtypes. While there is variation in the stage of differentiation of DLBCL, BL arise uniformly from B-cells at the GCB stage (5, 28). Using genomic data from human lymphoma samples (29), we evaluated a GCB/ABC genomic signature in human and $E_{\mu}$-Myc lymphomas (Figure 2A). In an independent dataset of human DLBCL samples (4), the signature correctly distinguished lymphomas annotated as GCB or ABC type (Figure 2B, p < $2x10^{-16}$, Wilcoxon rank-sum test), and when a cut-off score of 0.5 was used, the accuracy was 94% (n=329/350). Applying this signature to $E_{\mu}$-Myc lymphoma data
identified significant differences between the two cluster subtypes (Figure 2C, respective median scores 0.18 vs. 0.99, where 0 represents GCB and 1 represents ABC, p < 0.0001, Wilcoxon rank-sum test). Using a cut-off score of 0.5, Cluster 1 lymphomas were more likely to have a GCB score, while Cluster 2 lymphomas were more likely to have an ABC score (p = 0.0001, Pearson’s Chi-squared test). These different supervised analyses of genomic data underscore the distinct differences between \( E\mu-Myc \) lymphomas and suggest there is variation in cell of origin maturation and differentiation.

Our prior work linked \( E\mu-Myc \) lymphoma subgroups with human aggressive lymphoma subtypes. We evaluated genomic data from aggressive human lymphomas (the BL to DLBCL spectrum) and confirmed that the genomic signature we developed to classify \( E\mu-Myc \) lymphomas significantly differentiates molecularly defined human aggressive B-cell lymphoma subtypes (where a score of zero represents Cluster 1 and a score of one represents Cluster 2). BL is most similar to Cluster 1 \( E\mu-Myc \) lymphomas, while DLBCL (non-molecular BL) is most similar to Cluster 2 \( E\mu-Myc \) lymphomas (Figure 3). Together with the analysis of genomic data with regards to GCB versus ABC distinction, these results define Cluster 1 \( E\mu-Myc \) lymphoma as a representation of human BL and Cluster 2 \( E\mu-Myc \) lymphoma as a representation of the ABC subtype of human DLBCL.

We also evaluated the mutational status of the \( Trp53 \) gene and the overall status of the p53-p19\(^{ARF}\)-MDM2 tumor suppressor axis in representative clones of the two \( E\mu-Myc \) clusters, as past work has documented mutation of p53 in over a quarter of \( E\mu-Myc \) lymphomas and overall disruption of the p53-p19\(^{ARF}\)-MDM2 tumor suppressor axis in about 80% of lymphomas arising in this mouse model (30). Five out of seven Cluster 1 lymphomas harbored mutation in the \( Trp53 \) cDNA, including extensive deletions and others that would lead to missense amino acid
substitutions or frameshifts, while all seven of the Cluster 2 lymphomas evaluated contained wild type \textit{Trp53} cDNA (p = 0.026, Chi-squared test) (Figure S2B). In addition, one of the Cluster 1 lymphomas with wild type p53 exhibited elevated MDM2 protein levels, whereas the other had moderately elevated p19\textsuperscript{ARF} levels. Five of the Cluster 2 lymphomas exhibited modest elevation of p19\textsuperscript{ARF} protein levels, two had modest increase in MDM2 levels, and one had substantial increase in MDM2 levels (Figure S2A). As such, it appears that \textit{Trp53} gene disruption is an additional distinction between the two lymphoma clusters. However, given the observed heterogeneity within and between the lymphoma clusters with regards to mutation of \textit{Trp53} and expression of p19\textsuperscript{ARF} and MDM2, \textit{Trp53} mutation status and the p53 tumor suppressor axis do not appear to be the sole determinants of the differences between the clusters.

In addition to genomic evaluations of \textit{E\mu-Myc} lymphomas, we assessed surface expression of B-cell markers by flow cytometry. While individual lymphomas varied in expression of IgM, IgD, CD43, and CD138, we found no significant difference between the \textit{E\mu-Myc} lymphoma clusters in terms of surface marker expression (data not shown).

From a clinical perspective, \textit{E\mu-Myc} lymphomas are classically described as presenting with diffuse lymphadenopathy (10). We observed this presentation in the majority of \textit{E\mu-Myc} lymphomas, but also found sick mice with a single enlarged lymph node, mediastinal disease, or gut-centered disease. Cluster 1 lymphomas were significantly more likely to present with lymphadenopathy that was diffuse or involving more than one lymph node region, whereas Cluster 2 lymphomas were significantly more likely to present with lymphadenopathy isolated to one lymph node group, particularly gut-centered (p = 0.0005, Pearson’s Chi-squared test, Table S5).
Taken together, our evaluations of $E\mu$-Myc lymphomas categorized into two clusters based on gene expression patterns not only describe biological and clinical differences between the clusters but also link them to distinct human lymphoma entities. Thus, genomic heterogeneity within the transgenic $E\mu$-Myc mouse model appears to mirror genomic heterogeneity in human lymphomas.

$E\mu$-Myc lymphomas exhibit variation in chemotherapy response that reflects variation in chemotherapy response of human aggressive lymphomas. Human aggressive B-cell lymphomas display heterogeneity with regards to response to therapy. Within DLBCL, clinical markers (IPI score (31, 32)) or molecular markers (ABC vs. GCB type (6)) distinguish patients with variable responses to multi-agent chemotherapy or chemo-immunotherapy. BL is uniformly treated with high-dose multi-agent regimens (33), and retrospective analyses revealed that DLBCLs that are genomically similar to BL have improved outcomes when treated intensively like BL (5).

Given the links between human aggressive B-cell lymphomas and the $E\mu$-Myc model, we were interested in determining the extent to which the $E\mu$-Myc model could be used to model heterogeneity in response to lymphoma therapy. We used gene expression signatures as a method by which to generate hypotheses regarding therapy response, and followed these analyses with experimental testing using $E\mu$-Myc tumors.

We began by utilizing a genomic signature developed to distinguish lymphomas that are sensitive or resistant to CHOP-like chemotherapy regimens (Figure 4A)(29). Interestingly, MYC was differentially expressed in the 200-probe signature, being more highly expressed in CHOP-resistant lymphomas (10.1 vs. 9.1 median RMA expression value, $p = 0.0001$, Wilcoxon rank
sum test). This is notable because MYC+ DLBCLs have inferior outcomes to standard chemotherapy compared to other DLBCLs (34). Single genes which are known to modulate chemotherapy sensitivity in the Eµ-Myc model (which include Top2a, Akt, Bcl-2, p53, Pten, and p19ARF (11-13, 16, 20, 35)) were not present in the CHOP response signature, but two probes for AKT3 and three probes for PTEN were differentially expressed between CHOP sensitive and CHOP resistant lymphomas at nominal p-values between 0.01 to 0.05 (Wilcoxon rank sum test). When this genomic signature is applied to an independent dataset of DLBCL patients treated with either CHOP or CHOP with rituximab (R-CHOP) (4), the signature significantly separated groups of patients with distinct responses to these two regimens (Figures 4B and 4C). We next evaluated the genomic signature of CHOP resistance in the Eµ-Myc lymphoma samples. As shown in Figure 4D, Cluster 2 lymphomas were predicted to be more chemotherapy sensitive than Cluster 1 lymphomas (p < 0.0001, Wilcoxon rank-sum test).

To validate the genomic predictions of differential sensitivity to chemotherapy between Cluster 1 and Cluster 2 Eµ-Myc lymphoma, we tested selected Eµ-Myc lymphoma clones for response to single agent doxorubicin and cyclophosphamide in vivo. These studies rely on the transplantability of Eµ-Myc lymphomas into immunocompetent C57BL/6 background strain mice. We selected lymphomas (C1-1, C1-3, C1-4, C1-5, C2-1, and C2-2) that were representative of the two clusters and that retained their characteristics after transplantation into recipient mice. These characteristics include genomic signature scores (Figure S3A), expression of B cell surface markers (Figure S3B), clonality as defined by single immunoglobulin heavy chain rearrangement (Figure S3C), and MYC, p53, p19ARF, and MDM2 protein expression (Figures S3D and S3E). As such, the transplanted tumors appear to represent valid models of the two forms of Eµ-Myc lymphoma.
\(E\mu-Myc\) lymphoma recipients were treated with single agent doxorubicin or cyclophosphamide with one-time dosing, as previously described (13). As shown in Figure 5, therapeutic responses correlated with genomic signature predictions of CHOP resistance: mice bearing the Cluster 2-type \(E\mu-Myc\) lymphoma had significantly longer responses with either of the single agent conventional chemotherapy agents than mice bearing the Cluster 1-type lymphoma. After doxorubicin treatment, mice bearing the Cluster 1 lymphomas C1-1 (n = 7), C1-3 (n = 5), and C1-4 (n = 5) continued to have ill appearance and rapid progression of lymphoma, while mice bearing Cluster 1 lymphoma C1-5 (n = 6) responded to therapy for about two weeks prior to progressing. On the other hand, almost all the mice bearing the Cluster 2 lymphomas C2-1 (n = 4) or C2-2 (n = 4) improved to normal appearance for approximately three to four weeks prior to progression. After cyclophosphamide therapy, mice bearing the Cluster 1 lymphomas C1-1 (n = 9), C1-3 (n = 7), C1-4 (n = 6), or C1-5 (n = 6) returned to a healthy appearance for approximately four weeks prior to progression. However, mice bearing the Cluster 2 lymphomas C2-1 (n = 5) or C2-2 (n = 4) returned to a fully healthy appearance for approximately six weeks prior to progression. As a negative control, mice bearing either Cluster 1 or Cluster 2 lymphomas that were not treated had progressive disease and were sacrificed one to two days from when treatment would have been administered. Upon dissection, there was a severe burden of lymphoma in all mice (data not shown).

The selected \(E\mu-Myc\) lymphoma clones are representative of the lymphoma clusters in terms of predictions of chemotherapy response, based on the CHOP resistance genomic signature. The Cluster 1-type lymphomas C1-1, C1-3, C1-4, and C1-5 had a CHOP resistance signature score of 0.8, 0.97, 0.94, and 0.98 respectively, while the Cluster 2-type lymphoma C2-1 and C2-2 had a
CHOP resistance signature score of 0.35 and 0.02. These scores correlate with the chemotherapy responses shown in Figure 5.

Since perturbations of the p53 tumor suppressor axis are associated with inferior response to conventional chemotherapy in aggressive human lymphomas (36, 37) and in Eμ-Myc lymphomas (35), we also evaluated the response to chemotherapy based on the status of p53. As noted above, each of the Cluster 2 lymphomas exhibited wild type Trp53 whereas the majority of the Cluster 1 lymphomas contained an aberrant Trp53 gene. Nevertheless, there were examples of Cluster 1 lymphomas that did contain a wild type Trp53 gene, and as seen in Figures S4A and S4B, the response to chemotherapy for a Cluster 1 Trp53 wild type was more similar to the Cluster 1 lymphomas with mutant Trp53 rather than the Cluster 2 lymphomas. This suggests that p53 status is likely not the sole determinant of response to chemotherapy in this model.

Together, these findings support the concept that variation in therapy response exists in transgenic Eμ-Myc mice. Additionally, transcriptional programs shared between human aggressive B-cell lymphomas and Eμ-Myc mice appear to serve as predictive biomarkers of response to conventional chemotherapy. As such, Eμ-Myc mice have utility as a model for variation in therapeutic response in human lymphomas, and for this reason, Eμ-Myc mice might be able to identify novel targets to rationally select and design treatment for human aggressive B-cell lymphomas.

**Evaluating pathway specific targeted therapies in the context of Eμ-Myc lymphoma heterogeneity.** Our prior work and the work of others defined gene expression signatures of cellular pathway activity and demonstrated that these signatures can serve as predictive biomarkers of response to pathway-specific targeted therapies (23, 26, 38). As shown in Figure
6A, we found significant distinctions in cellular pathway activity between types of human aggressive B-cell lymphomas, with RAS, MYC, PI3K, and E2F1 pathways significantly upregulated in BL, while TGFβ, STAT3, TNFα, EGFR, and interferon pathways are significantly upregulated in DLBCL (non-molecular BL). Visually, a similar pattern of pathway activity is seen between Cluster 1 and Cluster 2 Eµ-Myc lymphoma samples as is seen between BL and DLBCL (Figure 6B). To quantitate the similarity, we calculated binary logistic regression coefficients of the genomic signatures with respect to the human and Eµ-Myc lymphoma, and found a significant correlation between the coefficients for the lymphomas (cor = 0.961, Pearson’s correlation test, Figure 6C).

Our goal in this research is to use these genomic signatures of pathway activity to identify novel therapeutic targets for cancers. To initiate these studies in lymphoma, we began by evaluating the NF-κB pathway, a known oncogenic pathway in DLBCL. We evaluated a genomic signature of the NF-κB pathway in human and Eµ-Myc lymphoma, and thereafter tested the extent to which therapeutic inhibition of the NF-κB pathway is beneficial in specific subgroups of lymphoma.

In human aggressive B-cell lymphomas, the NF-κB pathway appears to have divergent functions. For example, in DLBCL, NF-κB pathway is a well-recognized pro-survival and oncogenic mechanism (39), while in BL, the NF-κB pathway appears to act in a pro-apoptotic fashion within the context of MYC overexpression (40). The central role of the NF-κB pathway in DLBCL has led to the clinical evaluation of bortezomib, a proteosome inhibitor that suppresses the NF-κB pathway, where there appears to be specific efficacy in the ABC subtype of DLBCL (41, 42). Because of the variable role and activity of the NF-κB pathway in the
spectrum of human aggressive B-cell lymphomas, we hypothesized that the same might be seen in $E{\mu}$-$Myc$ lymphomas.

To assess NF-$\kappa$B pathway activity in $E{\mu}$-$Myc$ lymphomas, we utilized a genomic signature of the “TNF$\alpha$ pathway,” which was developed from gene expression data obtained from endothelial cells treated with TNF$\alpha$ (GSE9055 (43)) and is known to reflect activation of the NF-$\kappa$B pathway. Using this genomic signature, we found significantly higher predictions of NF-$\kappa$B pathway activity in the Cluster 2 $E{\mu}$-$Myc$ lymphomas compared to the Cluster 1 lymphomas (Figure 7A, $p < 0.0001$). The predictions of differential NF-$\kappa$B pathway activity between the two lymphoma clusters is supported by finding elevated protein expression of phosphorylated p65/RELA in Cluster 2 lymphomas compared to Cluster 1 (Figure S5) and by differential mRNA expression of NF-$\kappa$B target genes between the two lymphoma clusters (Table S6).

Because of the differential NF-$\kappa$B pathway activity in the two $E{\mu}$-$Myc$ lymphoma clusters, we hypothesized that therapeutic inhibition of the NF-$\kappa$B pathway would be more beneficial in mice bearing Cluster 2 $E{\mu}$-$Myc$ lymphomas than in mice bearing Cluster 1 $E{\mu}$-$Myc$ lymphomas. As bortezomib is known to inhibit the NF-$\kappa$B pathway (44) and has been evaluated in human aggressive lymphomas, we tested $E{\mu}$-$Myc$ lymphoma Cluster 1 and Cluster 2 clones for their response to bortezomib.

As seen in Figure 7B, single agent bortezomib therapy was significantly more effective in mice bearing the Cluster 2 $E{\mu}$-$Myc$ lymphomas than Cluster 1 $E{\mu}$-$Myc$ lymphomas. The recipients of Cluster 2 lymphomas C2-1 ($n = 6$) and C2-2 ($n = 8$) treated with bortezomib had stabilization of disease for approximately one to two weeks, while recipients of Cluster 1 lymphomas C1-1 ($n = 6$), C1-3 ($n = 6$), C1-4 ($n = 6$), and C1-5 ($n = 6$) treated with bortezomib almost uniformly progressed within several days of the first injection. As with
cyclophosphamide and doxorubicin, Cluster 2 lymphomas (all Trp53 wild type) had longer progression free survival than Cluster 1 lymphomas (either Trp53 wild type or mutant/deleted) after bortezomib treatment (Figure S4C). The NF-κB pathway signature scores for the Cluster 2 lymphomas (0.84 and 0.8 respectively) were higher than the NF-κB pathway signature scores for the Cluster 1 lymphomas (0.35, 0.25, 0.18, and 0.29 respectively), and the scores correlated with the clinical responses seen.

The results of applying and testing genomic signatures of pathway activity to the Eμ-Myc model demonstrate that they can be used as biomarkers of therapeutic response. Since Eμ-Myc lymphoma is a model for heterogeneity seen in human aggressive B-cell lymphomas, and since mouse and human lymphomas have similar patterns of pathway activity as measured by genomic signatures, we believe that Eμ-Myc mice can serve as a model to identify and test novel targeted therapies for human aggressive B-cell lymphoma subtypes.

Discussion

Perhaps the greatest challenge facing the development of new drugs and effective treatment for human cancers is the heterogeneity of the disease. Cancer in general is not one disease, and the same holds true for B-cell lymphomas. BL, DLBCL, and B-cell lymphomas with features intermediate between DLBCL and BL represent a spectrum that exhibits clinical heterogeneity and diversity of underlying genetic alterations. An ability to define and understand this heterogeneity is clearly critical to adapting and applying effective treatment strategies to individual patients.

Equally important to attaining the goal of personalized lymphoma therapy is the development of disease models that can be used for the evaluation of new therapeutic strategies. The Eμ-Myc
transgenic mouse was developed as an example of B-cell lymphoma initiated by the Myc oncogene, and various studies have used this model to investigate how the manipulation of cooperating genes can alter time to lymphoma onset or modulate response to chemotherapy (11-13, 16-18, 35). However, the naturally inherent heterogeneity of the model is not well appreciated.

Our prior studies highlighted the fact that the $E\mu$-Myc model develops a variety of genomically diverse lymphomas that occur naturally and have distinct differences in time to lymphoma onset (7). Here, we confirm these findings and develop methods that can be used to classify new lymphomas and link them to distinct human lymphoma subtypes. A key and critical component of our approach is the capacity to use gene expression profiling as a common currency to allow for cross-species comparisons (8). Genomic signatures developed in the human context and applied to mouse data, and vice versa, demonstrate similarities in biology between human and murine lymphomas. Even though all $E\mu$-Myc lymphomas derive from Myc-overexpressing B-cells and human B-cell lymphomas do not necessarily have MYC aberrations, our approach shows that additional genomic programs connect human and murine lymphomas.

Although the $E\mu$-Myc model shares genomic programs with aggressive human B-cell lymphomas, it does have certain limitations. Recent next generation sequencing efforts have identified numerous mutations that are found with varying frequency in human aggressive lymphomas (45-47). Although the $E\mu$-Myc model may not recapitulate the full extent of these genetic alterations, the resulting gene expression patterns demonstrate the downstream similarities between human and murine lymphomas. In addition, we note differences comparing the subtypes of $E\mu$-Myc and human aggressive lymphoma, such as presentation (localized vs. extensive disease), cell of origin determination (GCB vs. ABC subtypes), and response to
chemotherapy. These differences highlight that the mouse model is not an exact representation of aggressive human lymphomas. Despite these limitations, we believe our approach provides the rationale for using $E\mu$-Myc mice and genomic signatures to model heterogeneity of therapy response in aggressive human B-cell lymphomas.

Most importantly, we now shift the focus of these studies from purely descriptive to one where $E\mu$-Myc mice can serve as a model to test therapies that may have future utility in human aggressive lymphomas. Our evaluation of conventional chemotherapy drugs used in the treatment of lymphoma validates that $E\mu$-Myc mice can serve to model heterogeneity in response to therapy in the human setting. Aberration in the TP53 gene, resulting in abnormal protein expression, is known to modulate the response of human lymphomas to conventional chemotherapy (36, 37). Our evaluation of the p53 axis in $E\mu$-Myc mice confirms these findings since all of the $Trp53$ mutations were found in Cluster 1 lymphomas, which displayed the greatest resistance to chemotherapy. Nevertheless, our work suggests that additional genetic programs may affect chemotherapy response in the $E\mu$-Myc mouse model since one Cluster 1 lymphoma with wild type $Trp53$ also exhibited chemotherapy resistance. However, this limited analysis with only one such clone precludes conclusions regarding the relative roles of p53 status versus expression-based clusters in predicting therapeutic response. Further experiments with additional lymphoma clones as well as with other conventional chemotherapy agents will be necessary to fully assess this.

Our previous work and that of others have established a link between pathway activation and sensitivity to pathway-specific therapeutic agents (23, 38). Using the $E\mu$-Myc model to evaluate targeted therapeutics in different subtypes of lymphoma holds great promise. In experiments beginning to explore these possibilities, we observed that treatment with bortezomib, a
proteosome inhibitor that suppresses NF-κB pathway activity, had preferential benefit in treating lymphomas derived from the Cluster 2 subgroup, which we demonstrate models the ABC-subtype of DLBCL. These data confirm results emerging from human clinical trials regarding the use of bortezomib-containing regimens in the ABC subgroup of DLBCL (41, 42). Moving forward, our results suggest that predictions of pathway activity could be used to select and test new therapeutic options and combinations in an in vivo experimental model that reflects characteristics of subtypes of human lymphoma. Ultimately, such findings could inform patient stratification and design of future clinical trials.

References Cited


Figure Legends

**Figure 1.** Gene expression profiling identifies two types of \( E\mu-\text{Myc} \) lymphoma. A) Heatmap of the \( E\mu-\text{Myc} \) lymphoma training set, with lymphomas clustered based on similarities in expression of the 2% of probes with the highest standard deviation across all samples. Data was centered and scaled; red color represents upregulated gene probes and blue color represents downregulated gene probes. B) Heatmap of the 500-probe genomic signature that discriminates Cluster 1 and Cluster 2 \( E\mu-\text{Myc} \) lymphomas, with samples in columns and probes in rows. Color scheme as in Figure 1A. C) Kaplan-Meier curves evaluating time from birth to onset of lymphoma for \( E\mu-\text{Myc} \) mice, grouped by genomic lymphoma cluster (Cluster 1: \( n=56 \), Cluster 2: \( n=20 \)). Significance determined by the log-rank test.

**Figure 2.** Gene expression connects \( E\mu-\text{Myc} \) lymphoma clusters with different lymphoma cell of origin subtypes. A) Heatmap of the genomic signature that discriminates germinal center B-cell (GCB) from activated B-cell (ABC) lymphomas. Red color represents upregulated gene probes and blue color represents downregulated gene probes. B) Box and whisker plot of GCB-ABC signature scores in an independent dataset of human B-cell lymphomas, grouped by GCB or ABC lymphoma type. Signature score of 0 represents GCB, while 1 represents ABC. Bold line represents median, box represents the interquartile range (IQR), whiskers represent 1.5 times the IQR, and dots represent outliers. P value calculated using the Wilcoxon rank sum test. C) Box and whisker plot of GCB-ABC signature scores in Cluster 1 and Cluster 2 \( E\mu-\text{Myc} \) lymphomas. Graphing parameters as described in Figure 2B. P value calculated by Wilcoxon rank sum test.

**Figure 3.** Cross-species comparison of gene expression connects \( E\mu-\text{Myc} \) lymphoma clusters with subtypes of human non-Hodgkin lymphomas. Box and whisker plot of \( E\mu-\text{Myc} \)
signature scores in three types of aggressive human B-cell lymphomas (data from GSE4475 dataset). Bold line represents median, box represents the interquartile range (IQR), whiskers represent 1.5 times the IQR, and dots represent outliers. P value calculated using the Kruskal-Wallis test.

**Figure 4. Gene expression signature of chemotherapy sensitivity predicts the two \( E\mu\text{-Myc} \) lymphoma clusters have different responses to chemotherapy.** A) Heatmap of the genomic signature that discriminates human lymphomas with the longest remission after CHOP chemotherapy (“sensitive”) to those with the shortest remission (“resistant”). Red color represents upregulated gene probes and blue color represents downregulated gene probes. Overall survival of DLBCL patients after treatment with CHOP (B) or R-CHOP (C), grouped by CHOP signature score. P value calculated by log-rank test. D) Box and whisker plot of CHOP signature scores in Cluster 1 and Cluster 2 \( E\mu\text{-Myc} \) lymphomas. Parameters as described in Figure 2A. P value calculated by Wilcoxon rank-sum test.

**Figure 5. \( E\mu\text{-Myc} \) lymphomas from the two clusters have significantly different progression free survival after chemotherapy treatment.** A) Progression free survival in recipient mice bearing Cluster 1 lymphomas (red) compared to Cluster 2 lymphomas (blue) after treatment with doxorubicin. B) Progression free survival in recipient mice bearing Cluster 1 lymphomas (red) compared to Cluster 2 lymphomas (blue) after treatment with cyclophosphamide. P values calculated using the log-rank test.

**Figure 6. Gene expression signatures of pathway activity in \( E\mu\text{-Myc} \) and human lymphomas.** A) Heatmap of scores from cellular and oncogenic pathway activity signatures in molecularly defined Burkitt lymphoma (BL) compared to diffuse large cell lymphoma (DLBCL). Red represents upregulated pathways and blue represents downregulated pathways. B) Heatmap,
with color scheme as in Figure 6A, comparing Cluster 1 and Cluster 2 $E_\mu$-Myc lymphomas from the training set. C) Binary regression coefficients from fitting each pathway activity signature scores to the phenotype of BL vs. DLBCL are plotted against binary regression coefficients from fitting each pathway activity signature score to the phenotype of Cluster 1 vs. Cluster 2, and were compared using Pearson’s correlation.

**Figure 7. Prediction of NF-\kappaB pathway activity and response of the two $E_\mu$-Myc lymphoma clusters to bortezomib therapy.** A) Box and whisker plot of the NF-\kappaB signature scores comparing Cluster 1 and Cluster 2 $E_\mu$-Myc lymphomas. Bold line represents median, box represents the interquartile range (IQR), whiskers represent 1.5 times the IQR, and dots represent outliers. P value calculated using the Wilcoxon rank sum test. B) Progression free survival in recipient mice bearing Cluster 1 lymphomas (red) compared to Cluster 2 lymphomas (blue) after treatment with bortezomib. P values calculated using the log-rank test.
Figure 1

A.

B.

Cluster 1  Cluster 2

C.

Fraction Free of Lymphoma

Time (days)

Cluster 1

Cluster 2

p = 0.0003
A. B. C.

Figure 2

GCB−ABC Signature Score

GCB−ABC Signature Score

p < 2 x 10^{-16}
p = 8 x 10^{-8}
Figure 3

$p < 2 \times 10^{-16}$

$E_\mu - \text{Myc Cluster Signature Score}$

mBL intermediate non-mBL

Human Lymphoma Type (Molecular Diagnosis)
A. CHOP sensitive | CHOP resistant

B. Overall Survival after CHOP

C. Overall Survival after R-CHOP

D. CHOP Resistance Signature Score

Figure 4
Figure 6

A. BL vs. DLBCL

B. Cluster 1 vs. Cluster 2

C. Human Lymphoma Regression Coefficients

- $E\mu$-Myc Lymphoma Regression Coefficients
  - cor = 0.961
**Figure 7**

**A.**

Box plot showing the distribution of NF-κB Signature Score for different clusters of $E_{\mu}$-Myc Lymphoma. The box plot indicates a significant difference among the clusters with $p < 0.0001$.

**B.**

Survival curve showing the fraction surviving until progression for different clusters after Bortezomib treatment. The survival curves are differentiated by cluster:
- Cluster 1-1 (red, solid line)
- Cluster 1-3 (red, dashed line)
- Cluster 1-4 (red, dotted line)
- Cluster 1-5 (red, dashed-dotted line)
- Cluster 2-1 (blue, solid line)
- Cluster 2-2 (blue, dashed line)

The survival times are compared, with $p = 7 \times 10^{-10}$.
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