Utilization of the $E_{\mu}$-Myc Mouse to Model Heterogeneity of Therapeutic Response

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Abstract

Human aggressive B-cell non-Hodgkin lymphomas (NHL) encompass the continuum between Burkitt lymphoma 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 $E_{\mu}$-Myc transgenic mouse are heterogeneous, mirroring genomic differences between Burkitt lymphoma 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 $E_{\mu}$-Myc model predicts response to therapy. We used genomic analyses to classify $E_{\mu}$-Myc lymphomas, link $E_{\mu}$-Myc lymphomas with NHL subtypes, and identify lymphomas with predicted resistance to conventional and NF-xB–targeted therapies. Experimental evaluation of these predictions links genomic profiles with distinct outcomes to conventional and targeted therapies in the $E_{\mu}$-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.

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Introduction

Aggressive B-cell lymphomas include a spectrum of diagnoses that span Burkitt lymphoma, 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 Burkitt lymphoma” (1). There is considerable clinical and therapy response heterogeneity across and within these diseases. While DLBCL is generally responsive to the R-CHOP chemomunotherapy regimen (containing rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone), Burkitt lymphoma requires more aggressive multiagent 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 (GEMM) 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 $E_{\mu}$-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
Eμ-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 Eμ-Myc background, these studies did not focus on the effects of gene networks and their relevance to the natural genetic heterogeneity seen in the Eμ-Myc model, particularly as it pertains to response to lymphoma therapy.

We previously described that the Eμ-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 Eμ-Myc tumors and methods to use the Eμ-Myc lymphoma model to predict therapy response.

Materials and Methods

Mouse strains and tumor monitoring

Eμ-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. Eμ-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 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 invasibly 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 5 × 10^5 lymphoma cells by the intraperitoneal 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 were 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://filemerge.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 biologic 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^-6. 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; refs. 22, 23) or Prediction Analysis for Microarrays (for greater than two-class models; ref. 24). Datasets used to generate and validate signatures along with accuracy of the signature using leave-one-out cross validation are listed in Supplementary Table S1. Parameters used in generating genomic signatures are listed in Supplementary Table S2. Genomic signatures of cellular pathway activation were applied using the ScoreSignature module (25) on the Duke University GenePattern server.

Immunoblot analysis

Protein was extracted from frozen lymphoma tissue and immunoblots were performed as described...
Southern analysis

Genomic DNA was isolated from frozen lymphoma tissue, digested with EcoRI (10 mg/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 before assessment on a Becton Dickinson 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 with 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 RNaseq spin column (Qiagen). The preparation was treated with DNase I (New England Biolabs) and then further purified using a second RNaseq 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) and then sequenced using the Q5 High Fidelity DNA Polymerase (New England Biolabs). Sequences were analyzed for mismatches and insertions or deletions using BioEdit and the Basic Local Alignment Search Tool (BLAST) hosted by the NCBI.
the \( E\mu\text{-Myc} \) mouse model (7). Our current unsupervised analysis of gene expression profiling data from 112 \( E\mu\text{-Myc} \) lymphomas confirms variation in overall genomic expression (Fig. 1A), and identifies a natural division of these lymphomas into two \( E\mu\text{-Myc} \) subgroups, or “clusters” (Supplementary Fig. 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 (Fig. 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 \( E\mu\text{-Myc} \) 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; Fig. 1C).

These results confirm heterogeneity in the transgenic \( E\mu\text{-Myc} \) model and establish a predictive framework that can prospectively evaluate new and independent \( E\mu\text{-Myc} \) lymphomas.

**Genetic, biologic, and clinical differences between the \( E\mu\text{-Myc} \) subtypes**

Moving beyond simply classifying \( E\mu\text{-Myc} \) 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 biologic 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 biologic function terms that include immune response, protein localization, and regulation of apoptosis (\( P \) values all \( < 10^{-8} \); Bonferroni FWER all \( < 10^{-5} \); Supplementary Table S3).

A further evaluation of gene expression data identified significant differences between the \( E\mu\text{-Myc} \) 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 \( E\mu\text{-Myc} \) 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 (\( P = 5 \times 10^{-7} \), \( \chi^2 \) test; Supplementary Table S4).

Since stage of differentiation and activation has prognostic value in human DLBCL (6), we evaluated the \( E\mu\text{-Myc} \) 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, Burkitt lymphoma 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 \( E\mu\text{-Myc} \) lymphomas (Fig. 2A). In an independent dataset of human DLBCL samples (4), the signature correctly distinguished lymphomas annotated as GCB or ABC type (Fig. 2B; \( P < 2 \times 10^{-16} \), Wilcoxon rank sum test), and when a cut-off score of 0.5 was used, the accuracy was 94% (\( n = 329/350 \), 2 test; Supplementary Table S4). Applying this signature to \( E\mu\text{-Myc} \) lymphoma data identified significant differences between the two cluster subtypes (Fig. 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, whereas cluster 2 lymphomas were more likely to have an ABC score ($P = 0.0001$, Pearson $\chi^2$ 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 Burkitt lymphoma 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). Burkitt lymphoma is most similar to cluster 1 $E\mu$-Myc lymphomas, whereas DLBCL (nonmolecular Burkitt lymphoma) is most similar to cluster 2 $E\mu$-Myc lymphomas (Fig. 3). Together with the analysis of genomic data with regard to GCB versus ABC distinction, these results define cluster 1 $E\mu$-Myc lymphoma as a representation of human Burkitt lymphoma 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-p19ARF-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-p19ARF-MDM2 tumor suppressor axis in about 80% of lymphomas arising in this mouse model (30). Five 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, whereas all seven of the cluster 2 lymphomas evaluated contained wild-type $Trp53$ cDNA ($P = 0.026; \chi^2$ test; Supplementary Fig. S2B). In addition, one of the cluster 1 lymphomas with wild-type p53 exhibited elevated MDM2 protein levels, whereas the other had moderately elevated p19ARF levels. Five of the cluster 2 lymphomas exhibited modest elevation of p19ARF protein levels, two had modest increase in MDM2 levels, and one had substantial increase in MDM2 levels (Supplementary Fig. S2A). As such, it appears that $Trp53$ gene disruption is an additional distinction between the two lymphoma clusters. However, given the observed heterogeneity within and between the lymphoma clusters with regard to mutation of $Trp53$ and expression of p19ARF and MDM2, $Trp53$ 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 $E\mu$-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 $E\mu$-Myc lymphoma clusters in terms of surface marker expression (data not shown).

From a clinical perspective, $E\mu$-Myc lymphomas are classically described as presenting with diffuse lymphadenopathy (10). We observed this presentation in the majority of $E\mu$-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 $\chi^2$ test, Supplementary Table S5).

Taken together, our evaluations of $E\mu$-Myc lymphomas categorized into two clusters based on gene expression patterns not only describe biologic 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.
uniformly treated with high-dose multiagent regimens (33), and retrospective analyses revealed that DLBCLs that are genomically similar to Burkitt lymphoma have improved outcomes when treated intensively like Burkitt lymphoma (5).

Given the links between human aggressive B-cell lymphomas and the \( E\text{\textmu}-\text{Myc} \) model, we were interested in determining the extent to which the \( E\text{\textmu}-\text{Myc} \) 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 \( E\text{\textmu}-\text{Myc} \) tumors.

We began by utilizing a genomic signature developed to distinguish lymphomas that are sensitive or resistant to CHOP-like chemotherapy regimens (Fig. 4A; ref. 29). Interestingly, \( \text{MYC}^+ \) DLBCLs were 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 \( \text{MYC}^+ \) DLBCLs have inferior outcomes to standard chemotherapy compared with other DLBCLs (34). Single genes that are known to modulate chemotherapy sensitivity in the \( E\text{\textmu}-\text{Myc} \) model (which include \( \text{Top2a}, \text{Akt}, \text{Bcl-2}, \text{p53}, \text{Pten}, \) and \( p19^{ARF} \); refs. 11–13, 16, 20, 35) were not present in the CHOP response signature, but two probes for \( \text{AKT3} \) and three probes for \( \text{PTEN} \) 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 patients with DLBCL treated with either CHOP or R-CHOP (ref. 4), the signature significantly separated groups of patients with distinct responses to these two regimens (Figs. 4B and C). We next evaluated the genomic signature of CHOP resistance in the \( E\text{\textmu}-\text{Myc} \) lymphoma samples. As shown in Fig. 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 \( E\text{\textmu}-\text{Myc} \) lymphoma, we tested selected \( E\text{\textmu}-\text{Myc} \) lymphoma clones for response to single-agent doxorubicin and cyclophosphamide in vivo. These studies rely on the transplantability of \( E\text{\textmu}-\text{Myc} \) 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 (Supplementary Fig. S3A), expression of B-cell surface markers (Supplementary Fig. S3B), clonality as defined by single immunoglobulin heavy chain
Our prior work and the work of others defined gene expression signatures of cellular pathway activity and context of chemotherapy response in aggressive human lymphomas, and for this reason, Eμ-Myc mice might be able to identify novel targets to rationalize and design treatment for human aggressive B-cell lymphomas.

Evaluating pathway-specific targeted therapies in the context of Eμ-Myc lymphoma heterogeneity

Together, these findings support the concept that variation in therapy response exists in transgenic Eμ-Myc mice. In addition, 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 remotely select and design treatment for human aggressive B-cell lymphomas.

Evaluating pathway-specific targeted therapies in the context of Eμ-Myc lymphoma heterogeneity
demonstrated that these signatures can serve as predictive biomarkers of response to pathway-specific targeted therapies (23, 26, 38). As shown in Fig. 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 Burkitt lymphoma, whereas TGFβ, STAT3, TNFα, EGFR, and IFN pathways are significantly upregulated in DLBCL (nonmolecular Burkitt lymphoma). Visually, a similar pattern of pathway activity is seen between cluster 1 and cluster 2 Eμ-Myc lymphoma samples as is seen between Burkitt lymphoma and DLBCL (Fig. 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 ($r = 0.961$, Pearson correlation test, Fig. 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 prosurvival and oncogenic mechanism (39), whereas in Burkitt lymphoma, the NF-κB pathway appears to act in a proapoptotic 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 Eμ-Myc lymphomas.

To assess NF-κB pathway activity in Eμ-Myc lymphomas, we utilized a genomic signature of the “TNFα pathway,” which was developed from gene expression data obtained from endothelial cells treated with TNFα (GSE9055; ref. 43) and is known to reflect activation of the NF-κB pathway. Using this genomic signature, we found significantly higher predictions of NF-κB pathway activity in the cluster 2 Eμ-Myc lymphomas compared with the cluster 1 lymphomas (Fig. 7A; $P < 0.0001$). The predictions of differential NF-κB pathway activity between the two lymphoma clusters is supported by finding elevated protein expression of phosphorylated p65/RELA in cluster 2 lymphomas compared with cluster 1 (Supplementary Fig. S5) and by differential mRNA expression of NF-κB target genes between the two lymphoma clusters (Supplementary Table S6).

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

As seen in Fig. 7B, single-agent bortezomib therapy was significantly more effective in mice bearing the cluster 2 Eμ-Myc lymphomas than cluster 1 Eμ-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 1 to 2 weeks, whereas 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
initiated by the \textit{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 \textit{E\textmu-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 \textit{E\textmu-Myc} lymphomas derive from \textit{Myc}-overexpressing B cells and human B-cell lymphomas do not necessarily have \textit{MYC} aberrations, our approach shows that additional genomic programs connect human and murine lymphomas.

Although the \textit{E\textmu-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 \textit{E\textmu-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 \textit{E\textmu-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

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. Burkitt lymphoma, DLBCL, and B-cell lymphomas with features intermediate between DLBCL and Burkitt lymphoma 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 \textit{E\textmu-Myc} transgenic mouse was developed as an example of B-cell lymphoma

Figure 7. Prediction of NF-\textit{kB} pathway activity and response of the two \textit{E\textmu-Myc} lymphoma clusters to bortezomib therapy. A, box and whisker plot of the NF-\textit{kB} signature scores comparing cluster 1 and cluster 2 \textit{E\textmu-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 with cluster 2 lymphomas (blue) after treatment with bortezomib. \( P \) values calculated using the log-rank test.

injection. As with cyclophosphamide and doxorubicin, cluster 2 lymphomas (all \textit{Trp53} wild-type) had longer progression-free survival than cluster 1 lymphomas (either \textit{Trp53} wild-type or mutant/deleted) after bortezomib treatment (Supplementary Fig. S4C). The NF-\textit{kB} pathway signature scores for the cluster 2 lymphomas (0.84 and 0.8, respectively) were higher than the NF-\textit{kB} 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 \textit{E\textmu-Myc} model demonstrate that they can be used as biomarkers of therapeutic response. As \textit{E\textmu-Myc} lymphoma is a model for heterogeneity seen in human aggressive B-cell lymphomas, and as mouse and human lymphomas have similar patterns of pathway activity as measured by genomic signatures, we believe that \textit{E\textmu-Myc} mice can serve as a model to identify and test novel targeted therapies for human aggressive B-cell lymphoma subtypes.

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Although the \textit{E\textmu-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 \textit{E\textmu-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 \textit{E\textmu-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 Eμ-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 Eμ-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 Eμ-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 Eμ-Myc mice confirms these findings as 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 Eμ-Myc mouse model as 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 chemotherapeutic 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 Eμ-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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: R.E. Rempel, J.R. Nevins, D.R. Friedman
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Analysis and interpretation of data (e.g., statistical analysis, biosistics, computational analysis): R.E. Rempel, X. Jiang, P. Fullerton, T.Z. Tan, J.A. Lau, J.R. Nevins, D.R. Friedman
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Ye, J.-A. Lau, J.-T. Chi

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