DLBCL Cell of Origin Typing and Whole Transcriptome Analysis using Single Slides with HTG EdgeSeq

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Introduction

- Diffuse Large B-cell Lymphoma (DLBCL), is the most common form of Non-Hodgkin's Lymphoma (NHL) and has a highly heterogenous molecular pathology.
- In newly diagnosed patients, validated prognostics include the International Prognostic Index (IPI) and Cell of Origin (COO) classification. Gene microarrays were initially used to classify DLBCL into germinal center B-cell-like (GCB) or activated B-cell-like (ABC) COO subtypes.
- Immunohistochemical (IHC) staining of CD10, MUM1 and BCL6 is a proxy used in clinical practice in lieu of transcriptomics due to its expense, complexity and tissue requirements.
- Recent advances in the HTG EdgeSeq platform allow genome-scale profiling with minimal tissue input (1 slide, ≥11mm^2).
- We successfully applied this novel technology to perform simultaneous COO classification, immune cell enrichment and tumor pathway analysis using a single FFPE slide.

Objectives

- Assess the sensitivity and accuracy of EdgeSeq whole-transcriptome panel using single slides of FFPE lymphoma tumor samples.
- Determine if the EdgeSeq platform could replicate Cell of Origin typing from clinically validated assays using limited tissue.

Methods

Samples and COO Typing

- FFPE DLBCL resections and core needle biopsies (n=99) , Follicular Lymphoma (FL; n=19) and normal lymph nodes (LN; n=8) were sourced commercially (Avaden Biosciences, WA, Capital Biosciences, MD and TriMetis, TN).
- Tumor locations included: lymphoid organs, gastrointestinal tract, testes, and the pleural cavity.
- All samples were from newly diagnosed, untreated patients. Informed consent for genomic analysis was obtained by the vendor.
- RNAseq was performed using Illumina Total RNAseq with rRNA depletion on control samples for cross platform validation.
- COO testing was performed using Han's algorithm (provided by tissue vendor) and/or Lymph2Cx diagnostic panel (Nanostring Technologies, Seattle, WA).
- EdgeSeq procedure was as follows: FFPE samples were sectioned into 5µm slides, tissue area measured, and processed into lysates. Lysates were hybridized with HTG's Human Transcriptome Panel (HTP) probes (19,000 genes). Average sample input size was 40mm^2 for resections and 8mm^2 for cores. Note: HTP panel will be referred to as EdgeSeq Whole-Transcriptome (WT) panel.

Bioinformatics

- Differential gene expression analysis was performed using linear models in R. Published DLBCL/FL expression dataset was downloaded from NCBI GEO (GSE132929).
- Pathway gene sets were extracted from mSigDB (Hallmark) and Immune cell signatures were extracted from the literature (xCell) [1].
- The *glmnet* R package was used to select informative gene features using penalized regression and to create a logistic classifier.

EdgeSeq is Optimized for Rapid, Genome-Wide RNA Analysis of Single FFPE Slides

- HTG EdgeSeq employs a nuclease protection assay to quantify RNA expression directly from FFPE tissue lysates (Figure 1). High sensitivity and multiplexing capacity is achieved by using Illumina sequencers for counting RNA molecules.
- Control FFPE samples were processed in parallel by RNAseq and single-slide EdgeSeq; correlation was high with an R^2 of 0.7 (n = 8; Figure 2A).
- 12,000 genes were detected on average in the lymphoma samples; resections and core needle biopsies performed comparably (Figure 2B). Genes important in Cell of Origin classification were reliably detected (Figure 2C).
- Compared to current clinically validated methods for COO typing (Table 1), EdgeSeq offers broader profiling with lower sample input requirements.

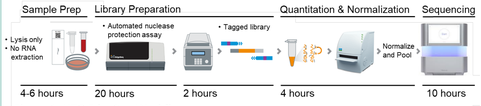


Figure 1: HTG EdgeSeq Workflow

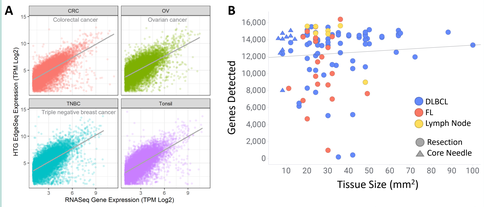


Figure 2: Accuracy and Gene Detection of EdgeSeq Whole-Transcriptome Panel

(A) Scatterplot of RNAseq and EdgeSeq gene expression in control samples with best fit line in grey. (B) Scatterplot of number of genes detected above negative controls versus input tissue size. (C) Histogram of percentage of genes detected in COO panels from landmark publications.

Table 1: Cell of Origin Classification Methods

COO typing method	Recommended FFPE sample input (5µm)	Targets	Readout
IHC (Han's algorithm)	4 slides	CD10, MUM1, BCL6	GCB or Non-GCB
Lmyph2Cx	2-8 slides (>16-2mm^2)	20 genes	GCB or ABC
HTG COO	1 slide (≥2mm^2)	92 genes	GCB or ABC
RNAseq	8-10 slides or curls	~20,000 genes	GCB or ABC

Cell of Origin Typing from Single Slides can Recapitulate Gold Standard Test Results

- The Nanostring Lymph2Cx panel is widely used for RNA COO typing. Lymph2Cx assessed gene expression was highly correlated to EdgeSeq, both resections and core needle biopsies showed similar performance (R^2 = 0.73, n = 25 and R^2 = 0.75, n = 8).
- This suggests that the EdgeSeq WT panel achieved comparable sensitivity with less sample input and a larger gene set (Figure 3).

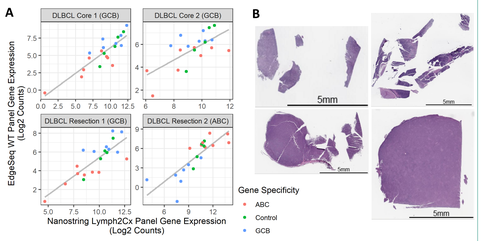


Figure 3: Gene Expression Comparison of Lymph2Cx and EdgeSeq WT Panel

(A) Scatterplot of gene expression in log2 counts using EdgeSeq Whole Transcriptome panel (y-axis) and Lymph2Cx (x-axis). Points represent genes in the panel, colored by their COO specificity (red ABC, blue GCB). Line indicates least squares fit. (B) H&E images for tissue samples used in (A).

- A machine learning approach was used to create a COO classifier using logistic regression. The training set comprised 43 samples (26 GCB, 17 Non-GCB) and the test set comprised 8 samples (6 GCB, 2 Non-GCB).
- Genes from validated COO panels [2,3] were used for classifier training; 14 genes were selected as highly informative by the algorithm.
- The classifier achieved 100% accuracy on the training set and 88% accuracy on the test set with 1 misclassification (Figure 4).

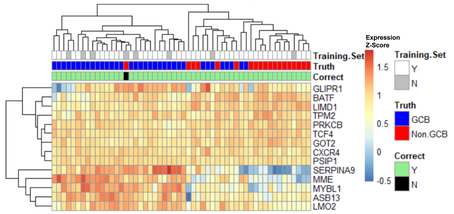


Figure 4: Classification Performance for COO Typing

Heatmap of gene expression for informative genes in COO typing. Cell colors reflect z-score of expression. Classifier labels, true COO status and training set membership are indicated by the top rows.

Exploring NHL Molecular Pathobiology with Minimal Tissue Requirements

- DLBCL is a more aggressive cancer than FL and the differences in tumor microenvironment have been well studied [4]. We compared DLBCL/FL differences in our cohort of patients with a similar, published expression study and found high concordance (Figure 5A).
- Examples of two relevant genes are depicted in Figure 5B. *AURKA* is an oncogenic Aurora family kinase; the inhibitor alisertib is being tested in a relapsed/refractory DLBCL population [5]. *BACH2* is a transcription factor that plays a role in B-cell maturation, class-switching and enhances B-cell proliferation in germinal centers, a hallmark of FL [6].
- Elevation of MYC, E2F and MTORC1 was observed in DLBCL, these are well known pathways; inhibitors are currently being tested for clinical efficacy (Figure 5C) [7,8].

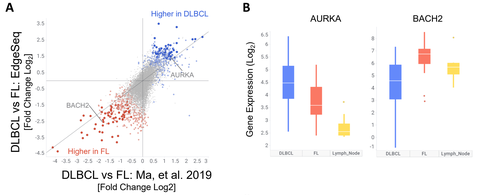
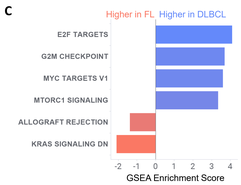


Figure 5: Transcriptional comparison of DLBCL to FL

(A) Scatterplot of DLBCL versus FL fold changes from two cohorts. Each dot represents a gene. Top 200 up/down-regulated genes are highlighted. Dark blue/red indicates genes highly significant in both cohorts. Diagonal line represents a perfect correlation. (B) Boxplots for AURKA and BACH2 expression. (C) Significantly enriched pathways using mSigDB Hallmark database.



- A major advantage of genome-wide over targeted gene panels is freedom to use a variety of gene signature analysis tools. We applied the xCell method for immune cell estimation and identified changes in both lymphoid and myeloid compartments (Figure 6A) [1].
- Macrophage gene signatures were enriched in DLBCL, particularly M2 subtype; a recent paper suggests these may be recruited by tumors [9] and play a role in response to R-CHOP [10]. FL was highly enriched for T regulatory cells, consistent with studies showing aberrant T-reg retention [11].
- We conducted a similar immune cell enrichment analysis comparing GCB and non-GCB DLBCL samples. Class-switched memory B-cell signature was elevated in Non-GCB, this corroborates recent reports suggesting aberrant memory B cells are the source of ABC tumors as opposed to plasmablasts (Figure 6C) [12] .

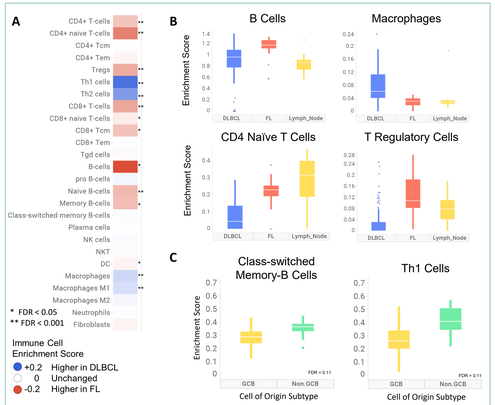


Figure 6: Investigation of the NHL tumor microenvironment with gene signatures

(A) Heatmap of xCell signature enrichment scores for Lymphoid and Myeloid cell subsets comparing DLBCL to FL. (B) Enrichment scores for B-cell, Macrophage, CD4+ naïve T cells and Tregs across DLBCL, FL and LN. (C) Enrichment score for class-switched memory B-cells and Th1 cells comparing GCB vs Non-GCB DLBCL.

Conclusions

- Combined COO typing and whole transcriptome analysis from a single slide efficiently uses patient core needle biopsies and resections.
- HTG EdgeSeq Whole Transcriptome panel data is highly correlated to Lymph2Cx panel, the gold standard for RNA based COO classification.
- A simple classifier achieved high accuracy, thus a more comprehensive validation for COO typing is warranted
- Whole transcriptome profiling enables identification of immune cell signatures and hallmark pathways differences between NHL subtypes.
- Use of EdgeSeq for low-input longitudinal core needle sampling may yield insights into tumor evolution and therapeutic mechanisms of action across the DLBCL treatment landscape.

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