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Letter to the Editor

Loss of 5-hydroxymethylcytosine expression is near-universal in B-cell lymphomas with variable mutations in epigenetic regulators.

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Epigenetic alterations are increasingly recognized in human malignancies, with loss of 5-hydroxymethylcytosine (5hmC) expression by immunohistochemistry as a potential correlational proxy for malignant epigenetic change. In this study, nearly one hundred cases of a broad range of B-cell lymphomas studied via 5hmC immunohistochemistry showed pervasive loss of 5hmC expression. Whole exome sequencing performed on a subset of DLBCLs showed a significant fraction of cases (58.8%) with missense mutations in such genes CREBBP, IDH1, IDH2, TET1, TET2, and WT1.

Epigenetic pathways related to gene expression, including DNA methylation, are often dysregulated in human cancers. 5-hydroxymethylcytosine is increasingly gaining recognition as an important epigenetic mediator, indicative of active demethylation and corresponding gene activation.\textsuperscript{1,2} While patterns of gene expression resulting from epigenetic modifications are often variable, 5hmC loss (as a surrogate for TET loss-of-function and 5mC persistence) is associated with some human cancers, including melanoma, mesothelioma, acute myeloid leukemias, and myelodysplastic syndrome.\textsuperscript{3}

\textit{TET2} mutations in lymphoid cells exert pleiotropic effects depending on the cellular compartment. Conditional knockout of \textit{TET2} in immature B-cells leads to development of lymphoblastic leukemias,\textsuperscript{4} whereas TET2-deficiency in hematopoietic stems cells predisposes to myeloid leukemias.\textsuperscript{5} Mutations in epigenetic modifying genes, such as \textit{DNMT3A}, \textit{TET2}, or \textit{IDH2}, are associated with lack of 5hmC expression and recurrent in lymphomas of follicular helper T-cell origin.\textsuperscript{6} Mutations in epigenetic modifying genes (viz. \textit{EZH2}) are also common in B-cell lymphomas, including follicular lymphoma and germinal center-derived DLBCLs, supporting the rationale for 5hmC evaluation in B-cell lymphomas. An earlier study of \textit{TET1}-deficient mice associated loss of 5hmC immunostaining with development of B-cell lymphomas, suggesting a relationship between \textit{TET1} expression, 5hmC content, and lymphomagenesis.\textsuperscript{7}

With this background, we evaluated a spectrum of B-cell lymphomas for 5hmC expression and the association of expression with mutations in epigenetic pathway-related genes.
After review of histology, 92 cases of B-cell lymphomas (5 whole section and 87 on TMA) were stained for 5hmC (Polyclonal 1:1000, Active Motif), see figure 1). Five normal lymph nodes were also examined in tandem. A subset of the whole section cases were subject to double stain performed for CD3 (red)/5hmC(brown) to assess 5hmC in the T-cell compartment. All staining was performed on the Leica Bond III™ autostainer. Staining was scored dichotomously as absent or present.

Whole exome sequencing of 17 DLBCL cases from whole sections was also performed. Tumor DNA from 17 large-cell lymphoma samples within the above cohort was isolated using the AllPrep DNA/RNA FFPE kit (Qiagen) and matched germline DNA was obtained using peripheral blood with the DNeasy Blood/Tissue kit (Qiagen). One case showing slightly increased large cells, morphologically closer to nodular lymphocyte predominant Hodgkin lymphoma (NLPHL), was excluded from WES analysis to maintain homogeneity.

The Agilent SureSelect XT Human All Exon v6 Kit captured whole-exome and untranslated region (UTR), with reads generated using Illumina HiSeq2500 and NovaSeq6000 at Theragen Bio Co., Ltd, due to performance of sequencing over two separate experiments. See table S1 for additional methods on read alignment. Somatic mutations were then called using Mutect2 through GATK4 either paired germline DNA or best practices provided panel of normals. Identified mutations were then post-processed by filtering according to best practices and annotated using the vcf2maf tool, which integrates the annotation tool Variant Effect Predictor (VEP) from Ensembl and a format conversion step. The final annotated mutations from WES were then analyzed in R (version 4.0.3) using tidyverse (version 1.3.0) best practices and the package maftools (version 2.4.12). See Table S1 and Figure S1 for results pertaining to cell of origin status and predicted significance of observed mutations and their locations.

The study was approved by the UOC Institutional review board (IRB13-1297).
Normal lymph nodes, retained 5hmC in the mantle, marginal, and paracortical areas with isolated loss only in the germinal centers B-cells with scattered follicular dendritic cells showing retained 5hmC. Notably, a significant majority of intrafollicular (likely CD4 follicular helper T-cells) and extrafollicular T-cells showed loss of 5hmC on the CD3/5hmC double stain (figure 2A).

Among all lymphoma cases (n = 92), the majority of high-grade, low-grade B-cell and Hodgkin lymphomas showed loss of 5hmC in neoplastic cells (94%, 94%, and 88.5% of cases, respectively, Figure 1B, Figure 2 B-D). 5hmC loss occurred in over 90% of lymphoma cells in any given case. Partial/variable loss only occurred in four cHLs (two were considered 5hmC retained, while two with extensive loss in over 90% of cells were considered 5hmC lost), demonstrating occasional weak staining in a subset of Hodgkin cells. When the DLBCLs-not otherwise specified (NOS) with available cell of origin (COO) data were stratified by COO, there were 11 germinal center B-cell (GCB), 11 non-GCB, and one undetermined COO, with all cases showing 5hmC loss. Cases with weak staining and partial loss was not observed, except in one MCL.

Rare cases of high-grade B-cell lymphoma with retained 5hmC expression consisted of one primary mediastinal B-cell lymphoma and one DLBCL-Richter transformation (RT) from chronic lymphocytic leukemia and small lymphocytic lymphoma (CLL/SLL). The only two low-grade B-cell lymphoma with retained 5hmC expression were both CLL/SLL. Hodgkin lymphomas with retained 5hmC expression were split between two cHL (9% of cHL) and one NLPHL (25% of NLPHL).

Only two DLBCL-RT from CLL/SLL were included within the DLBCL-NOS set. One case showed retained 5hmC expression and the other showed concordant 5hmC loss in both the high-grade DLBCL and residual low-grade CLL/SLL components. In most lymphomas, reactive background small lymphoid cells with strong retained 5hmC expression served as internal controls. However, in some Hodgkin and
DLBCL cases (including transformed follicular lymphoma), a significant fraction of non-neoplastic background milieu also demonstrated loss of 5hmC expression (Figures 2B, 2C and Figure S1).

WES analysis interrogating for mutations in epigenetic regulators (IDH1, IDH2, TET1, TET2, KMT2D, EZH2 and CREBBP) showed missense mutations in 10 of 17 DLBCLs tested (58.9% of cases) with CREBBP seen most frequently (figure 3). Details on the nature of these mutations and effect on protein are detailed in supplementary Figure S2.

This study demonstrated near-universal loss of 5hmC expression by IHC across a wide range of high- and low-grade B-cell lymphomas (89%) and Hodgkin lymphomas (88%). The observations support results from prior studies in B-cell lymphoma by Matsuda et al and Siref et al, and extends the observation to additional B-cell lymphomas including MCL (typical and blastoid variants) as well as DLBCL-NOS.10,11

The Matsuda study evaluated four subtypes of B-cell lymphomas (FL, CLL, MCL, and BL) and noted uniform loss of 5hmC expression in follicular & Burkitt lymphomas.10 Lack of staining in follicular lymphoma is congruent with our findings. We included two cases of high-grade B-cell lymphoma (one double hit, another without), and both showed uniform loss of 5hmC expression. Their study also found that all CLL and most MCL cases retained 5hmC expression, with only two of 11 MCLs demonstrating loss. In contrast, our study noted loss of expression in the majority of CLL cases (six of eight) and all four MCL cases (Figure 1a). Their study included one DLBCL-RT, but staining pattern details in the transformed component were not reported. We expected an increased likelihood of 5hmC loss in the transformed component, but noted an inverse pattern with retained 5hmC in one DLBCL-RT and loss in one CLL component, suggesting that loss is not correlated with progression in B-cell lymphomas. Our data in HL cases is aligned with the observations of Siref et al which reported near-universal loss of expression in cHL.11
Additionally, we assessed 17 DLBCL cases via WES for missense mutations explanatory of 5hmC expression loss in the aforementioned epigenome-related genes (DNMT3A, TET1, TET2, IDH1, IDH2 and WT1). While about half of these cases demonstrated mutations in one or more of these genes, most without alterations still showed 5hmC loss. This aligns with observations by Lemonnier et al in T-cell lymphoma where a significant proportion of cases with 5hmC loss did not show mutations in TET2 or DNMT3A. From a mechanistic perspective, B-cell lymphomas carry mutations in these epigenetic regulator genes less frequently. Rather, a subset of FL and DLBCLs (enriched for germinal center COO) harbor mutations in the epigenetic modulator EZH2 that promotes increased suppressive trimethylation via H3K27me, affecting 5mC hydroxylation. The minimal number of mutated cases impedes speculation since we focused on just DLBCL to ensure homogeneity. However, non-GCB predominance in mutation-positive DLBCLs in our study suggests that 5hmC loss is likely independent of EZH2 mutation status, although the exact mechanism remains poorly understood. From a translational perspective, it was recently demonstrated that 5hmC in circulating cell-free DNA assessed by chemical labeling-based sequencing technology correlated with prognosis in newly-diagnosed DLBCL and hence examining TET1, TET2 in conjunction with 5hmC and 5mC may have prognostic utility in the setting of B-cell lymphomas.

In summary, we corroborate previously published data and extend current insights by demonstrating loss of 5hmC expression in most B-cell lymphomas. This loss may be diagnostically useful in establishing a malignant B-cell phenotype in limited samples without flow cytometry/molecular data. However, the loss of 5hmC in reactive background T-cells (in normal and malignant nodes) indicates that 5hmC loss in T-cells is not a surrogate of aberrant/neoplastic phenotype.
References


Table and Figure Legends

**Figure 1**: 5hmC expression by lymphoma subtypes. **A.** Lymphomas were organized into three groups: high-grade/large cell, low-grade (including MCL), and Hodgkin; with constituent subtypes specified, and average percent 5hmC loss per group tabulated by total numbers of cases. Overall, 94% of large B-cell lymphomas, 94% low-grade B-cell lymphomas (including MCL) and 88.5% of Hodgkin lymphomas showed loss of 5hmC expression. One very weakly stained MCL case was considered within the negative group. The high-grade B-cell lymphoma (HGBCL) cases included one double-hit lymphoma and one HGBCL-NOS as per WHO 2017 schema. Graphs were generated within Plotly and Visual Studio code. **B.** Three sample cases of lymphomas demonstrating loss of expression in the neoplastic cells (black arrowhead) depicting Hodgkin/Reed-Sternberg cell, large B-cell (DLBCL) as well as neoplastic follicles and follicular lymphoma. Background endothelial cells and reactive lymphoid cells demonstrate strong retained expression of 5hmC (blue arrows).

**Figure 2.** T-cell panel: CD3(brown)/5hMC(red) double stain on reactive node and B-cell lymphoma. Panel A shows low power of germinal center-mantle interface. Noted normal mantle cells positive for 5hmC while perifollicular T-cells are in red. High power of the paracortical areas (panel on right top) show several T-cells with only CD3(red) while 5hMC(brown) is negative in these cells. Germinal center areas at high power with scattered T-cells (likely CD4+ follicular helper T-cells) with loss of 5hmC. Normal follicular dendritic cells (large doublets) express strong 5hmC while germinat center B-cells are also negative. Panel B, C and D correspond to one case each of CHL, DLBCL, and THRBCL with neoplastic cells negative for 5hMC (black arrowheads) with a significant component of microenvironment T-cells also negative for nuclear 5hmC.
Figure 3: Whole exome sequencing data looking at epigenetic regulators \((IDH1, IDH2, TET1, TET2)\) mutations in 17 DLBCLs. A. Distribution of cases stratified by mutations and Cell of origin status showing that most cases with mutations were enriched in the non-GCB cell of origin. For more information, see Supplementary figure S2.
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Supplementary
Supplementary Tables & Figures

Table S1. Cases by COO status and predicted significance of observed mutations. Predicted significance of observed mutations with SIFT, Polyphen 2 and COSMIC. Read alignment, mutation calling, and annotation were performed using a pipeline modified from the Genomic Data Commons (GDC) data harmonization pipelines. Reads were aligned to the GRCh38.d1.vd1 reference sequence using bwa-mem (version 0.7.17). According to GenomeAnalysisToolkit (GATK) best practice protocol, duplicates were marked and removed, followed by base quality score recalibration (BQSR) using GATK4 (version 4.1.3.0).

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Figure S1: Large B-cell lymphoma lacking 5hmC in lymphoma cells and microenvironment lymphoid cells:  

A. Low-power H&E depicting diffuse lymphoid infiltrate of a non-GCB DLBCL rich in histiocytes with scattered small clusters of large cells (inset). Focus rich in small lymphocytes (*) corresponding to reactive T-cells is noted at the periphery. B. The marked area is devoid of small B-cells while background malignant large B-cells are positive for CD20. C. The marked area comprises predominantly CD3+ T-cells with a prominent T-cell rich background elsewhere. D. The marked area (rich in T-cells) is largely negative for 5hmC. Most of the lymphoma cells are also negative with staining restricted to scattered histiocytes and endothelial cells. Only occasional tumor cells were positive in this case. Tumor cells marked with black arrow in inset. Specifically endothelial cells showed the strongest staining while most T-cells showed weak to negative staining in this case.
Figure S2. Predicted functional significance of mutations and their locations.