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

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 100 cases of a broad range of B-cell lymphomas (BCL) studied via 5hmC immunohistochemistry showed pervasive loss of 5hmC expression. Whole exome sequencing (WES) performed on a subset of diffuse large B-cell lymphoma (DLBCL) 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. 5hmC is increasingly gaining recognition as an important epigenetic mediator, indicative of active demethylation and corresponding gene activation.[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.[3]

TET2 mutations in lymphoid cells exert pleiotropic effects depending on the cellular compartment. Conditional knockout of *TET2* in immature B cells leads to development of lymphoblastic leukemias,[4] whereas TET2-deficiency in hematopoietic stem cells predisposes to myeloid leukemias.[5] Mutations in epigenetic modifying genes, such as *DNMT3A*, *TET2*, or *IDH2*, are associated with lack of 5hmC expression and recurrent in lymphomas of follicular helper T-cell origin.[6] Mutations in epigenetic modifying genes (*viz.*, *EZH2*) are also common in BCL, including follicular lymphoma (FL) and germinal center-derived DLBCL, supporting the rationale for 5hmC evaluation in BCL. An earlier study of *TET1*-deficient mice associated loss of 5hmC immunostaining with development of BCL, suggesting a relationship between *TET1* expression, 5hmC content, and lymphomagenesis.[7] With this background, we evaluated a spectrum of BCL for 5hmC expression and the association of expression with mutations in epigenetic pathway-related genes.

After review of histology, 92 cases of BCL (5 whole section and 87 on tissue microarray [TMA]) were stained for 5hmC (polyclonal 1:1,000, 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.

WES 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

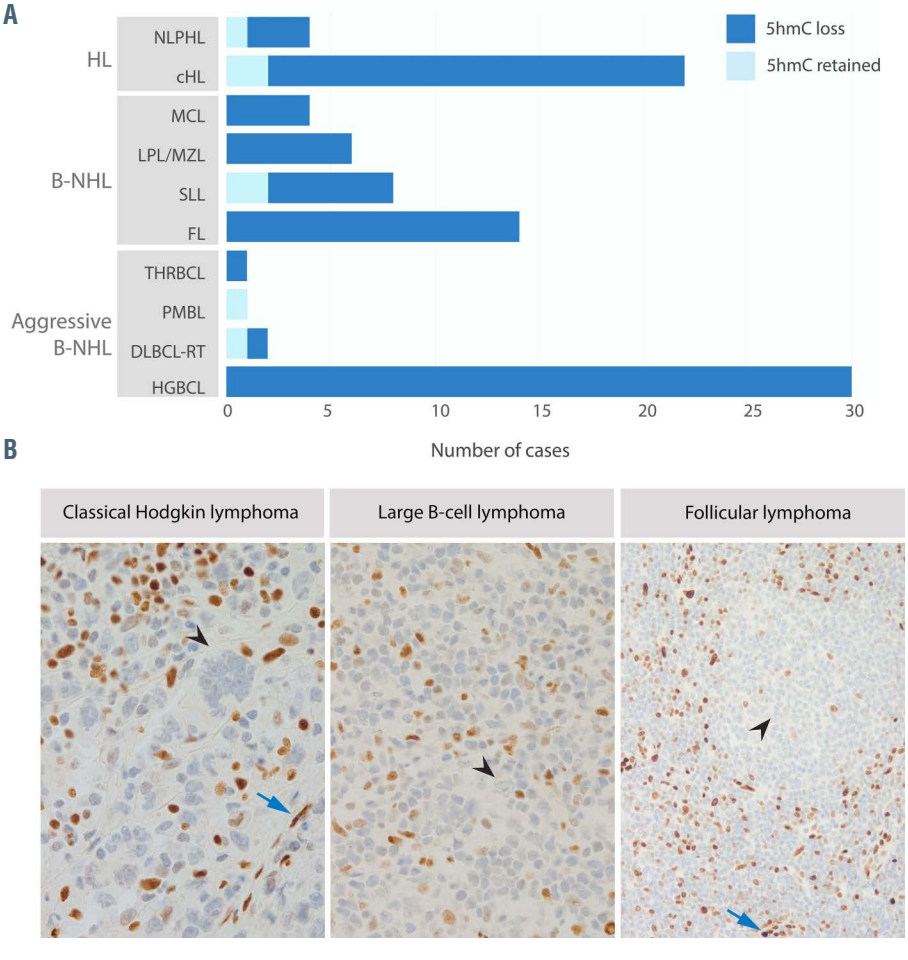

Figure 1. 5-hydroxymethylcytosine expression by lymphoma subtypes. (A) Lymphomas were organized into three groups: high-grade B-cell lymphoma (HGBCL) /diffuse large B-cell lymphoma (DLDCL) low-grade (including mantle cell lymphoma [MCL]), and Hodgkin lymphoma [HL]; with constituent subtypes specified, and average percent 5-hydroxymethylcytosine (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 HL showed loss of 5hmC expression. One very weakly stained MCL case was considered within the negative group. The HGBCL cases included 1 double-hit lymphoma and one HGBCL-not otherwise specified (NOS) as per World Health Organization 2017 schema. Graphs were generated within Plotly and Visual Studio code.[14] (B) Three sample cases of lymphomas demonstrating loss of expression in the neoplastic cells (black arrowhead) depicting Hodgkin/Reed-Sternberg cell, diffuse DLBCL as well as neoplastic follicles of follicular lymphoma (FL). Background endothelial cells and reactive lymphoid cells demonstrate strong retained expression of 5hmC (blue arrows). NLPHL: nodular lymphocyte predominant Hodgkin lymphoma; cHL: classical Hodgkin lymphoma; B-NHL: B-cell non-Hodgkin lymphoma; LPL: lymphoplasmacytic lymphoma; MZL: marginal zone lymphoma; SLL: small lymphocytic lymphoma; THRBCL: T-cell/histiocyte-rich B-cell lymphoma; PMBL: primary mediastinal (thymic) large B-cell lymphoma; RT: Richter transformation.

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 regions (UTR), with reads generated using Illumina HiSeq2500 and NovaSeq6000 at Theragen Bio Co., Ltd, due to performance of sequencing over two separate experiments. See the *Online Supplementary 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,[8] 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).[9] See the *Online Supplementary Table S1* and *Online Supplementary 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 center B (GCB) 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 (HL) showed loss of 5hmC in neoplastic cells (94%, 94%, and 88.5% of cases, respectively, Figure 1B, Figure 2B and D). 5hmC loss occurred in over 90% of lymphoma cells in any given case. Partial/variable loss only occurred in four classical HL (cHL) (2 were considered 5hmC retained, while 2 with extensive loss in over 90% of cells were considered 5hmC lost), demonstrating occasional weak staining in a subset of Hodgkin cells. When the DLBC-not otherwise specified (NOS) with available cell of origin (COO) data were stratified by COO, there were 11 GCB cell, 11 non-GCB cell, and one undetermined COO, with all cases showing 5hmC loss. Cases with weak staining and partial loss was not observed, except in one mantle cell lymphoma (MCL).

Rare cases of high-grade BCL (HGBCL) with retained 5hmC expression consisted of one primary mediastinal BCL and one DLBCL-Richter transformation (RT) from chronic lymphocytic leukemia and small lymphocytic lymphoma (CLL/SLL). The only two low-grade BCL with retained 5hmC expression were both CLL/SLL. HL 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

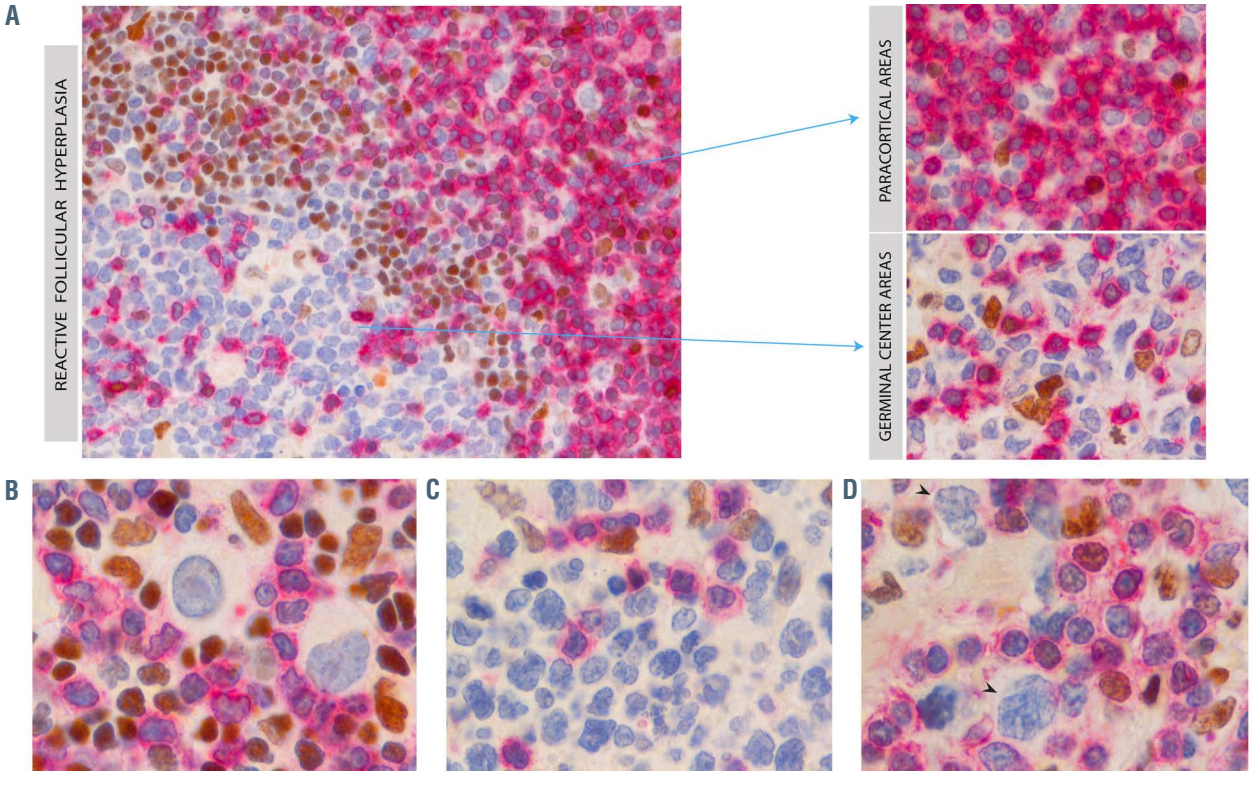

Figure 2. T-cell panel: CD3 (red)/5-hydroxymethylcytosine (brown) double stain on reactive node and B-cell lymphoma. (A) Low power magnification of germinal center-mantle interface. Noted normal mantle cells positive for 5-hydroxymethylcytosine (5hmC) while perifollicular T cells are in red. High power magnification 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 germinal center B cells are also negative. (B, C and D) Correspond to 1 case each of classical Hodgkin Lymphoma (cHL), diffuse large B-cell lymphoma (DLBCL), and T-cell/histiocyte-rich B-cell lymphoma (THRBCL) with neoplastic cells negative for 5hMC (black arrowheads) with a significant component of microenvironment T cells also negative for nuclear 5hmC.

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 FL), a significant fraction of non-neoplastic background milieu also demonstrated loss of 5hmC expression (Figures 2B and C; *Online Supplementary Figure S1*).

WES analysis interrogating for mutations in epigenetic regulators (*IDH1*, *IDH2*, *TET1*, *TET2*, *KMT2D*, *EZH2* and *CREBBP*) showed missense mutations in ten of 17 DLBCL tested (58.8% of cases) with *CREBBP* seen most frequently (Figure 3). Details on the nature of these mutations and effect on protein are detailed in the *Online Supplementary Figure S2*.

This study demonstrated near-universal loss of 5hmC expression by immunohistochemistry across a wide range of HGBCL and LGBCL (>90%) and HL (88%). The observations support results from prior studies in BCL by Matsuda *et al.* and Siref *et al.*, and extends the observation to additional BCL including MCL (typical and blastoid variants) as well as DLBCL-NOS.[10,11]

The Matsuda study evaluated four subtypes of BCL (follicular lymphoma [FL], chronic lymphocytic leukemia [CLL], MCL, and Burkitt lymphoma [BL]) and noted uniform loss of 5hmC expression in FL and BL.[10] Lack of staining in FL is congruent with our findings. We included two cases of HGBCL (1 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 MCL demonstrating loss. In contrast, our study noted loss of expression in the majority of CLL cases (6/8) 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 BCL. 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 explanative 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*.[6] From a mechanistic perspective, BCL carry mutations in these epigenetic regulator genes less frequently. Rather, a subset of FL and DLBCL (enriched for germinal center COO) harbor mutations in the epigenetic modulator *EZH2* that promotes increased suppressive trimethylation via *H3K27me*, affecting 5mC

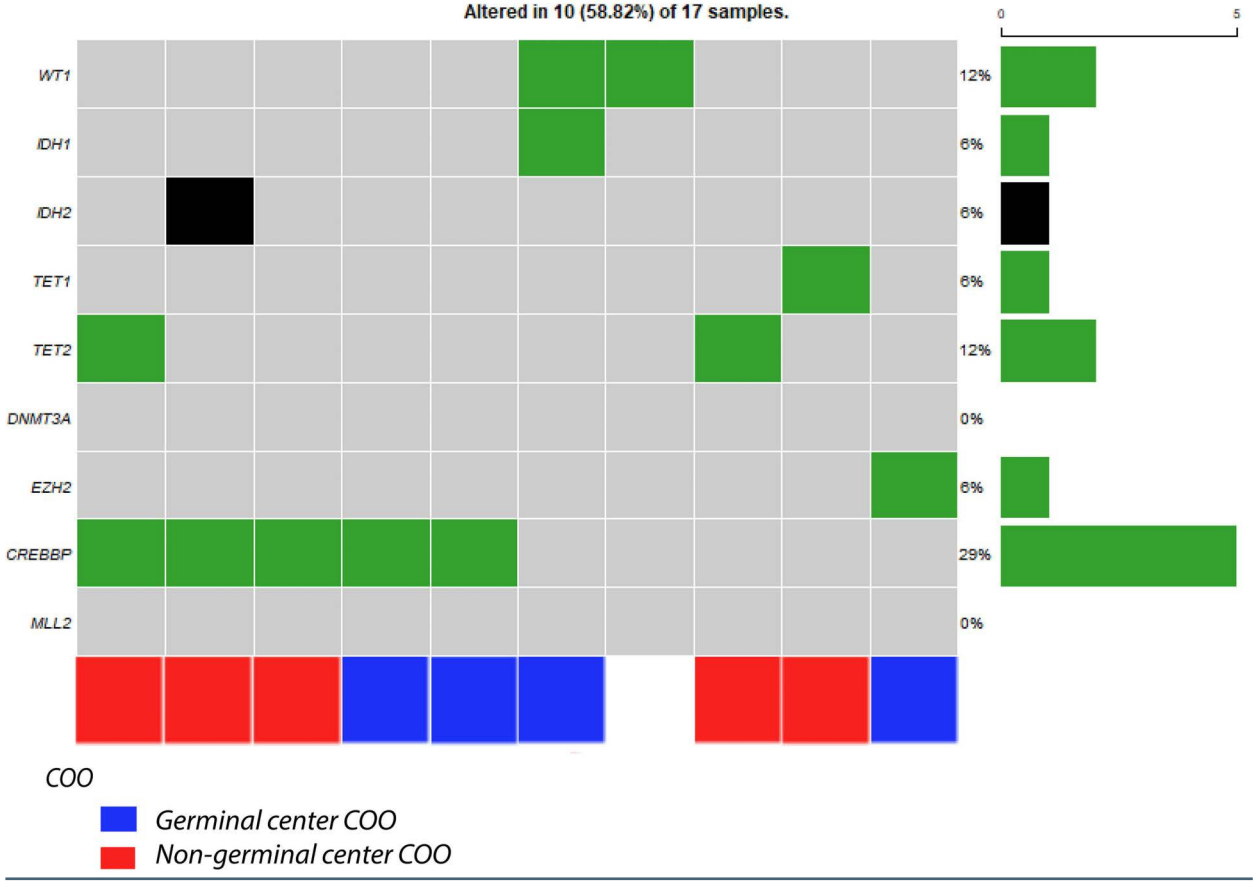

Figure 3. Whole exome sequencing data looking at epigenetic regulators (*IDH1*, *IDH2*, *TET1*, *TET2*) mutations in 17 diffuse large B-cell lymphomas. (A) Distribution of cases stratified by mutations and cell of origin (COO) status showing that most cases with mutations were enriched in the non-germinal center COO. For more information, see the *Online Supplementary Figure S2*.

hydroxylation.[12,13] The minimal number of mutated cases impedes speculation since we focused on just DLBCL to ensure homogeneity. However, non-GCB predominance in mutation-positive DLBCL 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 BCL.[2]

In summary, we corroborate previously published data and extend current insights by demonstrating loss of 5hmC expression in most BCL. 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.

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