Lack of activation-induced cytidine deaminase expression in in situ follicular neoplasia

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Title:
Lack of activation-induced cytidine deaminase expression in *in situ* follicular neoplasia

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The immunoglobulin heavy chain (IGH) genes undergo class switch recombination (CSR) and somatic hypermutation (SHM) to produce a diverse repertoire of high affinity antibodies. CSR and SHM occur in mature B lymphocytes in the highly specialized microenvironment of germinal centers (GC) and depend on activation-induced cytidine deaminase (AID), an enzyme present in germinal center B-cells. AID mediates deamination of cytosine (C) to uracil (U) at cytosine:guanine (C:G) base pairs to generate uracil:guanine (U:G) mismatch, and double-strand DNA breaks (DSBs) into switch (S) region sequences of IGH. This process introduces point mutations at a high frequency in IGHV and IGLV genes allowing for affinity maturation of the antibody response and replaces the heavy chain constant region of the antibody from the default IgM to IgG, IgA or IgE isotypes.1

In addition to IGH, endogenous AID affects numerous genes across the B cell genome but at much lower levels than the immunoglobulin loci. Off target AID activity during the germinal center reaction contributes to lymphomagenesis.2 In malignancies of mature B-cell origin, recurrent translocations often arise as a result of DSBs within the IGH switch region and a partner gene. These translocations arise as a result of aberrant AID mediated CSR.2 Mutations in various oncogenes implicated in the pathogenesis of B cell lymphomas like BCL6, PAX5, MYC and BCL2 share features similar to immunoglobulin gene mutations suggesting a role for aberrant SHM.1

Based on RNA expression data, AID appears to be selectively expressed in GC B cells and GC-derived malignancies.3 Follicular lymphoma (FL) originates from GC B cells and shows heterogeneity in the SHM pattern, suggesting the possible heterogeneous expression of AID in this entity. The reported expression of AID in FL by immunohistochemistry (IHC) varies from 25% to 100%, acknowledging that some of this variation may be due to technical and definitional differences.3,4,5 More recently, Scherer and colleagues demonstrated that AID mRNA and protein expression were highly correlated in FL by quantitative RT-PCR and IHC, respectively. However, there was great variability with 38% of cases showing less than 10 IHC-positive cells/high power field (hpf).6 This translates to 1% or less of cells in a hpf of small lymphocytes. Interestingly, duodenal-type FL (DFL) lacks AID expression, which has been postulated
to be related to its extremely indolent clinical behavior. Immunophenotypically, DFL cells typically strongly co-express CD10 and BCL2 proteins. In situ follicular neoplasia (ISFN) is characterized by partial or total colonization of reactive germinal centers by BCL2+/CD10+ co-expressing B cells harboring *IGH-BCL2* fusions in an otherwise architecturally preserved lymph node. BCL2 and CD10 IHC staining is characteristically intense and is higher than that seen in systemic FL cells, much like DFL. Given all the overlapping characteristics of these entities, we explored AID expression in ISFN.

Institutional pathology archives (2002-2017) were searched for cases of ISFN and reviewed to confirm diagnosis. We identified 16 patients with ISFN (6 male, 10 female) with adequate tissue for study. The median age was 66 years (range 50–83 years) at the time of diagnosis. Additional clinical information was obtained by review of the medical records and was available for 13 patients. The ISFN was discovered incidentally in all the cases, during pathologic review of biopsies performed for a variety of clinical indications. Ten patients had other hematolymphoid or non-hematolymphoid neoplasm at some point in time. Two patients had a history of preceding FL, and 1 patient had concurrent FL at another anatomic site. Of the non-FL patients, none developed subsequent FL on median follow up of 43.5 months (range 13-97 months). 11 patients were alive at last follow up, one was lost to follow up and one patient died from complications of chronic myelomonocytic leukemia and related treatment. The clinicopathologic features are summarized in supplementary table 1.

Morphologically, the lymphoid and immune architecture was preserved in all ISFN cases, with a few follicles demonstrating BCL2 highly overexpressing GC B-cells. Initially, six ISFN cases were subjected to immunohistochemical staining with AID which suggested lack of staining in ISFN cells as compared to the expression in reactive GC cells (Figure 1). In order to confirm this impression, we then performed AID/BCL2 double Immunofluorescent (IF) staining on 16 ISFN cases. Double IF staining showed lack of AID expression in strongly BCL2 expressing cells in the ISFN follicles in all the cases (Figure 2). Thus, all cases were considered AID-negative. Internal positive controls (nearby reactive follicles and cells within the same follicle) stained appropriately (BCL2-, AID+).
typical systemic nodal FL, we also evaluated 15 cases of low grade FL for AID expression using double IF from a tissue microarray constructed during the same time period. Seven of 15 (47%) low grade FL cases demonstrated co-expression of BCL2 and AID in 10% or more of neoplastic cells within follicles on double IF staining (Figure 2). This difference between systemic nodal FL and ISFN was statistically significant (P < 0.001). We could not evaluate AID expression in three cases with manifest FL at other time points/sites due to the lack of availability of these specimens. Given the known correlation between AID mRNA and protein levels by IHC, we further verified the lack of AID expression at mRNA level on 2 cases each of ISFN and FL by performing RNAscope in situ hybridization. The findings in ISFN were similar to both the IHC and double IF results, confirming the absence (or extremely low levels) of AID in ISFN follicles, compared to manifest FL (Figure 3). Methodological details are presented in accompanying supplemental information.

ISFN is an indolent condition with a very low risk (<5%) of progression to FL. The ISFN cells carry a t(14;18)(q32;q21), similar to usual-type FL. This abnormality leads to constitutive overexpression of BCL2, inhibition of apoptosis and accumulation of inappropriately rescued B cells with a prolonged life span. This event is believed to be the first genetic hit in the natural history of FL pathogenesis, but additional genetic hits are required for progression to malignant follicular lymphoma. ISFN carries few secondary genetic changes. In contrast, secondary genetic alterations are found in 70–90% of FL at initial diagnosis in addition to the t(14;18)/\textit{IGH-BCL2} fusion. AID expression is a marker of the germinal center reaction. The additional genetic hits in follicular lymphoma B cells are postulated to be facilitated by AID, contributing to genomic instability. A subset of FL cases (25-100%) has been shown to express AID. We confirm this in our study by showing approximately half of the low grade FL cases expressed high level AID in neoplastic follicles by double IF. In our study, none of the ISFN cases expressed detectable AID in BCL2 intensely positive neoplastic cells in germinal centers. The absence of detectable AID in ISFN may be related to greater genetic stability and its generally benign behavior. We cannot exclude very low levels of AID expression, below the technical limits of our assays. Indeed, it has been shown that levels of AID in non-isotype switched IgM-expressing FL cells correlate with \textit{IGHV...
mutations and aberrant SHM in BCL6.\textsuperscript{6} Interestingly, circulating follicular lymphoma-like and ISFN cells, by virtue of the “allelic paradox” phenomenon, are IgM-expressing and show much lower levels of genomic alterations compared to overt FL, consistent with the theory of very low or near absent AID activity at this “early lesion” stage.\textsuperscript{9}

The AID expression results in our study are similar to the findings described by Katsuyoshi et al in duodenal-type FL.\textsuperscript{7} All 17 studied cases of duodenal-type FL lacked expression of AID. Interestingly, a clonal relationship has been proven in a recent case report of ISFN and duodenal-type FL in the same patient.\textsuperscript{12} Our study further demonstrates commonalities between these two entities and reinforce the hypothesis that these two entities might represent different tissue manifestations of a single precursor lesion.\textsuperscript{12} Despite the lack of AID expression, duodenal-type FL B cells have been shown to be at the memory B-cell stage with somatic and ongoing mutation, which later was suggested to be a consequence of BACH2 expression in tumor cells.\textsuperscript{7,13} This may be worth exploring given copy number gains of the BACH2 locus in ISFN cells.\textsuperscript{9} Interestingly, there is emerging evidence of ongoing IGH SHM in ISFN as recently shown by Kosmidis et al.\textsuperscript{14} Ongoing SHM in ISFN despite the absence of AID expression is not without precedent in typical FL. Ongoing mutations and intraclonal heterogeneity was detected in AID-negative FL samples, albeit at significantly lower levels than AID-positive cases.\textsuperscript{4} The regulation of AID expression is complex, involving transcription, posttranscription, and posttranslational mechanisms.\textsuperscript{15} This provides for many avenues to explore the mechanism behind ongoing mutations, mechanism for downregulated AID, and the global genetic profile of ISFN that will enhance our understanding of this entity.
References:


**Figure 1:** BCL2 and AID Immunohistochemistry:

A - Intense BCL2 staining in follicle involved by ISFN (Blue arrow) and negative BCL2 staining in reactive follicle (Black arrow).
B. In contrast, ISFN follicle (Blue arrow) lacks AID, and reactive follicle (black arrow) is positive for AID.

**Figure 2:** Immunofluorescent AID/BCL2 double staining:

A-C- Follicle (arrowhead) involved by ISFN with BCL2 staining red cells(A) and few AID staining green cells(B) shows lack of AID/BCL2 double staining cells(C). A nearby reactive follicle (arrow) is negative for BCL2 (A), positive for AID (B) and shows lack of AID/BCL2 double staining cells (C).

D-F: High magnification of the ISFN follicle showing BCL2 (D) positive cells (red), fewer AID (E) staining cells (green), and absence of AID/BCL2 double staining cells (F).

G-I- High magnification of a follicle from lymph node with FL: BCL2 (red) is diffusely positive in lymphoma cells(G) and AID(green) is positive in scattered lymphoma cells(H). AID/BCL2 double staining highlights many dual positive (arrows) lymphoma cells (I).

**Figure 3:** RNAscope, in situ hybridization for AID in ISFN (results in punctate staining):

A, B - Follicle involved by ISFN- Strongly positive for BCL2 mRNA (A) and negative for AID mRNA (B). The few AID positive cells are residual benign germinal center cells which are larger than the small cleaved ISFN cells. Insets are from exactly the same field on serial section.

C, D - Follicular Lymphoma- Positive for both BCL2 (C) and AID mRNA (D).
Supplemental Data

Supplemental Methods:

Immunohistochemistry:

The antibody panel used to assess ISFN cases was as follows: CD3 (2GV6, Ventana), CD20 (L26, Dako), BCL6 (PG-B6p, Dako), CD10 (56C6, Leica), BCL2 (124, Cell Marque, Rocklin, CA), AID (ZA001, ThermoFisher, Waltham, MA). For all markers except AID, formalin-fixed, paraffin-embedded (FFPE) tissue sections were stained using an automated IHC instrument (Benchmark Ultra, Ventana Medical Systems, Tucson, AZ). For AID, the tissue sections were subjected to heat-induced epitope retrieval (pH 9.0, 20 minutes) and incubated with anti-AID mouse monoclonal antibody (ZA001, 1:200) for 15 minutes at room temperature. Visualization was achieved with Bond Polymer Refine detection system in Bond Max instrument (Leica Microsystems, Buffalo Grove, IL).

Immunofluorescent AID/BCL-2 double staining:

Deparaffinization and heat-induced epitope retrieval of FFPE samples was performed using the Bond Max instrument (Leica Biosystems, Buffalo Grove, IL). Avidin and biotin was blocked with block A and block B reagents (ThermoFisher, Waltham, MA), respectively. Before adding antibody against AID (1:100) for 30 minutes, samples were incubated in 10% normal goat serum (Vector; Burlingame, CA), 5% BSA/PBS (Sigma-Aldrich; St. Louis, MO). AID was visualized with biotinylated anti-mouse antibody (Vector) followed by streptavidin conjugated QD605 (ThermoFisher). Subsequent staining with BCL-2 (clone: EP36, 1:200, CellMarque, Rocklin, CA) was preceded by avidin and biotin blocking. BCL-2 was visualized with biotinylated anti-rabbit antibody followed by streptavidin conjugated QD655 (ThermoFisher). Hoechst 33342 (ThermoFisher) was used as a nuclear counterstain.
Quantitation of Double AID/BCL2 staining double staining:

Sections labeled with AID – QD 605 and BCL2 – QD 655 were viewed with emission filters of 605/20 and 655/20 nm. Image files were analyzed using the Image-Pro Plus software (Media Cybernetics; Silver Spring, MD). First, the image of Hoechst 42333 counterstain, together with images of QD staining, was loaded into the composite preview. Using the “count/size” function and “Watershed” split, individual cells were segmented based on Hoechst 42333 counterstain. Subsequently, the composite preview was switched to the AID – QD 605 or the BCL2 – QD 655 image, and the data were analyzed to determine the percentage of double positive cells. Samples with ≥10% AID/BCL2 co-expressing cells within follicles were considered as AID positive regardless of the nuclear or cytoplasmic localization of the molecule.

RNAscope

For mRNA AID detection, FFPE tissue sections were stained using an automated IHC instrument (Discovery Ultra, Ventana Medical Systems, Tucson, AZ). Briefly, tissue sections were subjected to heat-induced epitope retrieval, pH 9.0 for 24 minutes at 97°C, followed by 16 minutes protease treatment at 37°C and 2 hour hybridization with Hs-AICDA probe (Advanced Cell Diagnostics, Newark, CA) at 43°C. Visualization was achieved with mRNA DAB Detection Kit (Roche, Basel, Switzerland). For readers not familiar with RNAscope assays, a positive signal is a single dot or multiple dots within a cell. Per manufacturer, positivity for AID in a sample is defined as at least 1 dot in every ten cells visible at 20-40X magnification. For our purposes of illustrating colocalization of AID with BCL2, we considered individual cells with at least one dot as positive for AID.

Statistics:

Results were compared using Fisher Exact test performed at a significance level of P<0.05, using statistical analysis software (StatSoft, Inc., Tulsa, OK)
Supplementary table 1: Clinicopathological features of patients with in situ follicular neoplasia.

<table>
<thead>
<tr>
<th>n</th>
<th>Site</th>
<th>Indication for LN removal</th>
<th>M/F</th>
<th>Age (years)</th>
<th>Previous lymphoma</th>
<th>Concurrent lymphoma</th>
<th>New lymphoma on follow up</th>
<th>Other malignancy</th>
<th>Other benign change in same specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Left tonsil</td>
<td>n/a</td>
<td>M</td>
<td>80</td>
<td>n/a</td>
<td>DLBCL, GC in same lymph node</td>
<td>n/a</td>
<td>Squamous cell ca (conjunctiva)</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>Right inguinal LN</td>
<td>LAD concerning for lymphoma recurrence</td>
<td>F</td>
<td>83</td>
<td>-</td>
<td>FL-G2 (diagnosed 20 years before and then relapsed 8 years before ISFN)</td>
<td>-</td>
<td>-</td>
<td>Breast Ca, Basal cell Ca</td>
</tr>
<tr>
<td>3</td>
<td>R4 LN</td>
<td>Mediastinal LAD</td>
<td>M</td>
<td>61</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Smoldering multiple myeloma (IgA kappa)</td>
<td>EBV+ mild polycltypic plasmacytosis</td>
</tr>
<tr>
<td>4</td>
<td>Right breast</td>
<td>Axillary LAD</td>
<td>F</td>
<td>58</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Breast Ca</td>
<td>Follicular / paracortical hyperplasia</td>
</tr>
<tr>
<td>5</td>
<td>Mediastinal and periortic LN</td>
<td>LAD, lung nodules and thrombocytopenia, lymphopenia</td>
<td>F</td>
<td>65</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Non necrotizing granulomas /sarcoildosis</td>
</tr>
<tr>
<td>6</td>
<td>Level 3, 4 LN</td>
<td>Fever of unknown origin, thoracic and abdominal mass and LAD</td>
<td>M</td>
<td>60</td>
<td>-</td>
<td>MGUS/concurrent B cell lymphoma, not biopsy proven</td>
<td>-</td>
<td>-</td>
<td>None</td>
</tr>
<tr>
<td>7</td>
<td>Left nasopaharynx</td>
<td>Nasopharyngeal mass</td>
<td>F</td>
<td>76</td>
<td>-</td>
<td>FL-G1 of another site</td>
<td>-</td>
<td>-</td>
<td>None</td>
</tr>
<tr>
<td>8</td>
<td>Left inguinal LN</td>
<td>n/a</td>
<td>M</td>
<td>67</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>BCC and melanoma of skin</td>
<td>Dermatopathic changes and sinus histiocytosis</td>
</tr>
<tr>
<td>9</td>
<td>Periduodenal LN</td>
<td>1.4cm enhancing mass involving the proximal small bowel</td>
<td>F</td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>EBV + DLBCL, IGH-BCL2 negative (7 years after IFN)</td>
<td>None</td>
</tr>
<tr>
<td>10</td>
<td>Neck LN</td>
<td>Neck mass</td>
<td>F</td>
<td>72</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Breast Ca</td>
<td>None</td>
</tr>
<tr>
<td>11</td>
<td>Level 4 LN</td>
<td>Fever of unknown origin, splenomegaly and neck LAD</td>
<td>M</td>
<td>66</td>
<td>-</td>
<td>DLBCL with underlying NLPHL in same LN</td>
<td>-</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Spleen (290 g)</td>
<td>Splenic mass</td>
<td>F</td>
<td>80</td>
<td>FL-G1 (4.5 years before ISFN)</td>
<td>-</td>
<td>-</td>
<td>Papillary serous borderline ovarian cancer</td>
<td>Sclerosing angiomatoid nodular transformation of the spleen</td>
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<tr>
<td>13</td>
<td>Left inguinal LN</td>
<td>n/a</td>
<td>F</td>
<td>53</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>None</td>
</tr>
<tr>
<td>14</td>
<td>Sigmoid colon polyp</td>
<td>Colon polyps</td>
<td>F</td>
<td>59</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>CMML</td>
</tr>
<tr>
<td>15</td>
<td>Spleen (425 g)</td>
<td>Pancytopenia and splenomegaly</td>
<td>M</td>
<td>66</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>CMML</td>
<td>None</td>
</tr>
<tr>
<td>16</td>
<td>Spleen</td>
<td>n/a</td>
<td>F</td>
<td>73</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>Follicular hyperplasia, focal EMH</td>
</tr>
</tbody>
</table>

Legends: LN- Lymph node, M- Male, F- Female, n/a- Not available, LAD-Lymphadenopathy, FL-Follicular lymphoma, DLBCL, GC- Diffuse large B cell lymphoma of germinal center phenotype, MGUS- Monoclonal gammopathy of unknown significance, NLPHL- Nodular lymphocytic predominant Hodgkin lymphoma, BCL- B cell lymphoma, BCC- Basal cell carcinoma, CMML- Chronic myelomonocytic leukemia, EMH- Extramedullary hematopoiesis