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SHORT REPORT

# Activation induced cytidine deaminase expression in lymphocyte predominant Hodgkin lymphoma

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**Background:** The lymphocytic and histiocytic (L&H) cells of lymphocyte predominant Hodgkin lymphoma (HL) originate from germinal centre B cells and carry mutated V gene rearrangements, usually with intraclonal diversity. It is unclear whether intraclonal V gene diversification by somatic hypermutation, which is strictly dependent on the enzyme activation induced cytidine deaminase (AID), is restricted to the early phase of lymphoma clone expansion and later silenced, or whether it remains active throughout malignant proliferation.

**Aims:** To analyse whether AID is expressed in L&H cells as an indicator of active somatic hypermutation in the tumour cells.

**Methods:** L&H cells from lymphocyte predominant HL cases and centroblasts from lymphadenites were micromanipulated and analysed for AID expression by quantitative real time polymerase chain reaction.

**Results:** The AID transcription level was higher than background in three of the six lymphocyte predominant HL cases, although it was lower than that seen in centroblasts.

**Conclusions:** Somatic hypermutation may remain active in L&H cells in a considerable proportion of cases, increasing the risk of acquiring further transforming mutations.

Hodgkin lymphoma (HL) is subdivided into classic forms and lymphocyte predominant HL. The lymphocytic and histiocytic (L&H) cells of lymphocyte predominant HL express B cell markers and also the germinal centre (GC) marker BCL6, indicating that they are derived from GC B cells.[1] Analysis of V gene rearrangements in single L&H cells revealed the presence of somatic mutations with intraclonal diversity in functional monoclonal V gene rearrangements, in line with a GC B cell origin.[2–5]

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Somatic hypermutation requires activation induced cytidine deaminase (AID).[6] Recent studies of AID protein expression using western blot analysis showed that AID expression is largely restricted to normal and transformed GC B cells and correlates well with the expression of AID transcripts.[7] Several studies analysing AID expression and mutation patterns in V gene rearrangements in various B cell non-HLs showed that, although somatic hypermutation activity is strictly dependent on AID, the expression of this protein does not necessarily indicate ongoing somatic hypermutation.[7–10]

Intraclonal diversity in V gene rearrangements as a result of active somatic hypermutation is a typical feature of GC B cells. However, for lymphomas showing intraclonal diversity it is unclear whether intraclonal V gene diversification by somatic hypermutation is restricted to the early phase of lymphoma clone expansion, and later silenced, or whether it remains active throughout malignant proliferation of the lymphoma cells.[11] To clarify this issue for lymphocyte predominant HL we quantitatively examined the expression of AID in the L&H cells of six patients with lymphocyte predominant HL at the transcription level, because suitable antibodies for immunohistochemistry are not yet available.

## MATERIALS AND METHODS

### Micromanipulation of cells

Lymph node biopsies of six patients with lymphocyte predominant HL and three with non-specific lymphadenitis were analysed. Frozen sections (5 μm thick) were mounted on membrane covered slides (PALM, Bernried, Germany) and stained for four minutes with haematoxylin containing 200 U/ml RNase inhibitor (Roche, Mannheim, Germany). Sections were washed in water for two minutes, incubated with 2% eosin for 20 seconds, washed again, and then dried for three hours at 37°C.

For each sample, single L&H cells were microdissected and 50 of them were catapulted into one 25 μl aliquot of Purescript lysis buffer (Biozym, Hamburg, Germany) using an ultraviolet laser beam (PALM microdissection system with a Zeiss Axiovert 200M microscope). From the same sections, 250 endothelial cells in groups of five were pooled together as a negative control sample. Fifty centroblasts from each of three cases of lymphadenitis, micromanipulated in groups of five cells, were used as positive control samples. We analysed four samples and four control samples from each case.

### Quantitative real time PCR

Total RNA was isolated with the Purescript RNA isolation kit (Biozym), reducing all volumes to one tenth of the standard protocol, and adding glycogen as a carrier. Reverse transcription (RT) was performed using the First Strand cDNA synthesis kit (Roche), with a quarter of the standard volumes and the complete RNA isolated from one sample in a total volume of 5 μl. Quantitative real time polymerase chain reaction (PCR) was performed on an ABI PRISM 7900HT sequence detection system using Assays-on-Demand (Applied Biosystems, Darmstadt, Germany) for both AID and the 18S rRNA endogenous control (catalogue numbers Hs00221068_m1 and 4319413E, respectively; Applied Biosystems) and the complete First Strand reaction for each PCR.

**Abbreviations:** AID, activation induced cytidine deaminase; Ct, threshold cycle; GC, germinal centre; HL, Hodgkin lymphoma; L&H, lymphocytic and histiocytic; PCR, polymerase chain reaction; RT, reverse transcription

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**Table 1** Analysis of V gene rearrangements of micromanipulated L&H cells

| Case | Cells positive in PCR | Repeatedly amplified rearrangements | Unique rearrangements | Clonally related cells |
|---|---|---|---|---|
| 1 | 6/30 | 4×$V_\kappa$1–9<br>2×$V_H$4–34 | $V_\kappa$3–11<br>$V_H$3–66 | 4/6 |
| 3 | 14/30 | 5×$V_\kappa$1–17<br>9×$V_\kappa$2D–28 | $V_\kappa$4–1<br>$V_\kappa$1D–39 | 12/14 |
| 6 | 6/15 | 6×$V_H$1–69 | | 6/6 |

Taking the results from all three cases together, most micromanipulated cells (22 of 26) were clonally related and thus belong to the L&H cell tumour clones. The fraction of clonally unrelated V gene rearrangements is in the same range as previously seen when micromanipulated cells were identified by immunohistochemical staining, showing that L&H cells were reliably identified based solely on morphology. Although the fraction of clonally unrelated B cells was low, these cells could cause false positive results if most of them were centroblasts expressing large amounts of AID. However, in lymphocyte predominant HL, centroblasts form only a minor fraction of the B cells. Furthermore, in case 3, where about 15% of cells carried unique V gene rearrangements, AID expression was not detected in the L&H cells, indicating that the contaminating cells did not result in false AID positivity.
In our analysis we found no intraclonal diversity, although case 1 showed intraclonal diversity in a previous analysis.[3] This may be because of the low numbers of rearrangements analysed and because two of the $V_\kappa$ rearrangements ($V_\kappa$1–9 in case 1 and $V_\kappa$1–17 in case 3) were unmutated, and hence probably exempt from somatic hypermutation by inactivation of the $Ig_\kappa$ locus.
AID, activation induced cytidine deaminase; L&H, lymphocytic and histiocytic; PCR, polymerase chain reaction.

### Analysis of V gene rearrangements

In three cases of lymphocyte predominant HL we performed single cell PCR to analyse V gene rearrangements. The staining procedures were the same as described above. L&H cells were cut with the laser beam, isolated with a hydraulic micromanipulator, and subjected to PCR for V gene rearrangements at the IgH (immunoglobulin heavy chain), Igκ, and Igλ (κ and λ light chain) loci as described previously.[2 3 5]

## RESULTS

L&H cells for micromanipulation were selected on the basis of morphological criteria, and we performed V gene rearrangement analysis on single micromanipulated cells from three patients to confirm that the cells belonged to the tumour clones. This analysis revealed that most micromanipulated cells were clonally related (table 1). AID and 18S rRNA transcripts were measured using quantitative RT-PCR with Assays-on-Demand and RNA isolated from groups of 50 (L&H cells and centroblasts) or 250 (endothelial cells as negative controls) micromanipulated cells. For each case and type of cell four samples were analysed and average Ct values (threshold cycle—the number of PCR cycles when the reporter fluorescence reaches a value that is significantly above background fluorescence for a specific PCR assay) were calculated. The Ct values for 18S rRNA as an endogenous control confirmed that PCR with RNA isolated from 50 cells was consistently successful, and the average Ct values varied only within a range of three cycles within each group of samples (table 2).

The average ΔCt values for AID (Ct of AID − Ct of 18S rRNA) in centroblasts were around 10 cycles, with only minor variations (9.3–11.9 cycles) between the three cases, whereas endothelial cells from the lymphadenites negative controls had ΔCt values between 22.6 and 29 cycles. The negative controls from the lymphocyte predominant HL cases had ΔCt values within a similar range (24.6–30.3 cycles). For the L&H samples, there were large variations in the ΔCt values for AID, which ranged from 14.3 to 25.9 cycles, and only those obtained from cases 1, 4, and 6 (14.3, 16.8, and 20.1 cycles, respectively) were indicative of AID transcript expression above background. However, these values were clearly higher than those seen for centroblasts and indicate that AID expression in L&H cells is lower than that seen in

**Table 2** Quantitative real time RT-PCR for AID and 18S rRNA of micromanipulated centroblasts and L&H cells

| Sample | | Ct 18S rRNA | Range | Ct AID | Range | ΔCt | Range |
|---|---|---|---|---|---|---|---|
| L&H cells | Case 1 | 21.3 | 9.8–23.3 | 35.7 | 33.8–38.6 | 14.3 | 11.3–17.6 |
| | Case 2 | 19.5 | 18.3–20.3 | 41.5 | 37.9–50 | 22.0 | 18.3–30.2 |
| | Case 3 | 20.2 | 19.3–21.9 | 46.1 | 42.1–50 | 25.9 | 20.4–30.5 |
| | Case 4 | 18.8 | 18.2–19.1 | 35.6 | 34.8–37 | 16.8 | 15.8–18.1 |
| | Case 5 | 20.6 | 17.6–22.6 | 43.1 | 40–50 | 22.6 | 18.9–27.4 |
| | Case 6 | 18.6 | 16.8–19.8 | 38.6 | 35.9–41 | 20.1 | 17.3–22.0 |
| Centroblasts | LN 1 | 24.6 | 23.6–25.6 | 34.8 | 33.7–36.2 | 10.3 | 8.4–12.6 |
| | LN 2 | 27.3 | 24.8–29 | 36.6 | 34.7–38.2 | 9.3 | 9.1–9.9 |
| | LN 3 | 25.4 | 23.7–28.2 | 37.3 | 35.9–38.6 | 11.9 | 10.3–14.9 |
| Negative controls | Case 1 | 18.9 | 17.5–20.3 | 47.3 | 39.1–50 | 28.4 | 20.8–32.5 |
| | Case 2 | 17.2 | 16.9–17.7 | 41.9 | 38.3–50 | 24.6 | 21.1–32.3 |
| | Case 3 | 18 | 16.8–19.2 | 48.3 | 43.15–50 | 30.3 | 24.9–33.2 |
| | Case 4 | 18.6 | 17.8–19.6 | 45.4 | 40.32–50 | 26.8 | 21.7–31.6 |
| | Case 5 | 20.3 | 19.3–21.3 | 50 | 50–50 | 29.7 | 28.7–30.7 |
| | Case 6 | 18 | 16.8–19 | 47.3 | 39.4–50 | 29.4 | 21.8–33.2 |
| | LN 1 | 23.2 | 21.9–25.4 | 50 | 50–50 | 26.8 | 24.6–28.2 |
| | LN 2 | 23.3 | 21.7–24.0 | 45.9 | 40.9–50 | 22.6 | 17.2–28.3 |
| | LN 3 | 20.2 | 18.4–21.7 | 49.2 | 47–50 | 29.0 | 26.4–31.6 |

The values are averages of four samples, and the highest and lowest values are shown as the range of Ct values. In addition to the results shown, for each case negative controls (water) were analysed in parallel and were consistently negative. In cases where AID transcripts were not detected, a Ct value of 50 was used for calculations, because a 50 cycle PCR was performed (a Ct of 50 cycles was seen for the L&H cells of case 2 once, case 3 twice, and case 5 once, and for the negative controls of case 1 three times, case 2 once, case 3 three times, case 4 twice, case 5 four times, case 6 three times, LN1 four times, LN2 twice, and LN3 three times).
AID, activation induced cytidine deaminase; Ct, threshold cycle; L&H, lymphocytic and histiocytic; LN, lymph node; RT-PCR, reverse transcription polymerase chain reaction.

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## Take home messages

- We analysed whether activation induced cytidine deaminase (AID) is expressed in lymphocytic and histiocytic (L&H) cells as an indicator of active somatic hypermutation in Hodgkin lymphoma (LH) tumour cells
- Transcription of AID was higher than background in three of six lymphocyte predominant HL cases, although it was lower than that seen in centroblasts
- Somatic hypermutation may remain active in L&H cells in a considerable proportion of cases, increasing the risk of acquiring further transforming mutations

centroblasts. Whether the L&H cells in the other cases did not express AID, or expression was below the sensitivity of our assay, or only a fraction of the lymphoma cells expressed AID remains unclear.

## DISCUSSION

Taken together, we found that the AID transcription level was significantly higher than background, but below that seen in centroblasts, in a proportion of lymphocyte predominant HL cases, and that this expression pattern resembles that seen in other GC derived B cell non-HLs, such as follicular lymphoma, Burkitt lymphoma, and GC-type diffuse large B cell lymphoma, where significant amounts of AID transcripts were also found only in a proportion of the cases.[7 8] The detection of AID in half of the cases analysed suggests that somatic hypermutation may remain active in the established L&H clone in at least some cases of lymphocyte predominant HL. Because AID activity has been associated with an increased risk of acquiring transforming events,[8] the sustained expression of AID in L&H cells may affect the risk of accumulating additional transforming mutations that play a role in transforming lymphocyte predominant HL into diffuse large cell lymphoma.

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