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Expression of Activation-induced Cytidine Deaminase Is Confined to B-Cell Non-Hodgkin's Lymphomas of Germinal-Center Phenotype

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Abstract

Activation-induced cytidine deaminase (AID) is essential for somatic hypermutation and class switch recombination of the immunoglobulin (*IG*) genes in B cells. It has recently been proposed that AID, as the newly identified DNA mutator in man, may be instrumental in initiation and progression of B-cell non-Hodgkin's lymphomas (B-NHL). We quantitatively measured, by real-time reverse-transcription PCR, expression of AID and of the error-prone DNA polymerase ι in normal B cells and a comprehensive panel of B-NHL entities. In pre- and postgerminal center (GC)-type B-NHLs like in normal naive and memory cells, AID did not exceed background levels. However, half of Burkitt lymphomas tested were found to express AID, at most at levels comparable with those found in normal GC B cells. Thirty percent of diffuse large B-cell lymphomas also transcribed AID, some at supraphysiological levels. Of follicular lymphoma cases, only 25% expressed significant amounts of AID. Moreover, within the group of GC-type B-NHLs, a statistically significant correlation between AID and polymerase ι expression was found. By contrast, we observed no correlation between AID expression and mutation load neither with the degree of intraclonal diversity of *IG* variable heavy chain genes. Interestingly, in two of seven follicular lymphomas with clinical and histological progression, selective outgrowth of AID-expressing clones occurred, suggestive for a role of the somatic diversification machinery in lymphoma transformation.

Introduction

To create the degree of antibody diversity necessary to combat the huge repertoire of microorganisms, *IG*[3] genes undergo several regulated alterations during B-cell development. The *IG* remodeling processes that occur during the GC stage of B-cell differentiation, albeit essential for the quality of the humoral immune response, are believed to bear an intrinsic risk of derailment (1): NHLs are in majority of B-cell origin and of GC or post-GC phenotype (2) and several *IG* gene-related chromosomal translocations of B-NHL entities now seem to result from mistakes in DNA diversification in the GC, in particular SHM and *IG* CSR (1). It has also become clear that the long-held dogma that SHM is confined to the *IG* loci is incorrect and that other genes such as *BCL-6* (3) and *CD95* (4) can be substrates even in nonneoplastic B cells. Recently, Pasqualucci *et al.* (5) demonstrated that some reported translocation partners of *IG*, *i.e.,* the proto-oncogenes *C-MYC*, *PIM-1*, *PAX-5*, and *TTF-1*, are mutated in a proportion of DLBCLs, most likely attributable to an aberrantly targeted SHM machinery. Such promiscuous hypermutation activity is assumed to increase the chance that target genes become involved in chromosomal translocations and, perhaps more importantly, implies a new form of genetic instability.

SHM and CSR are now known to be highly related molecular processes both depending on the recently identified enzyme AID (6). Absence of *AID* in knockout mice (7), as well as in hyper-IgM syndrome type 2 patients (8), leads to absent CSR and defective SHM. Although AID shares homology with the RNA-editing enzyme APOBEC-1, recent reports strongly suggested that AID acts by direct deamination of cytidine residues in DNA (9–12). Most likely, SHM and CSR both start with the AID-triggered conversion of cytidine to uracil residues in the nontemplate DNA strand of the *IG* variable and H chain switch regions, respectively, with preference for certain hot-spot motifs (12, 13). These mismatches can become fixed mutations if they are replicated without prior modification (14). Alternatively, the created dU/dG pairs can be resolved by the base excision repair (9, 15, 16) and mismatch repair systems, involving uracil glycosylase, endonucleases, and error-prone DNA polymerases. There is evidence that the polymerases Polι, Polη (17, 18), and Polζ (19) are operational in these pathways. Of note, transgenic mice, with ectopic and deregulated AID expression, were found to die early because of development of epithelial and lymphoreticular neoplasms harboring several hypermutated non-IG genes (20). This demonstrated that AID, as the first active genome mutator identified in man, has oncogenic potential.

Although it has been proposed that somatic gene diversification may be instrumental in the ongoing transformation process of B-NHLs, it is currently unknown to what extent the processes of SHM and CSR are indeed active in the group of B-NHLs. We previously noted that at least a proportion of FLs, despite their morphological and architectural resemblance to normal GC B cells and despite the somatically diversified nature of their *IG* genes, lack these typical GC functions (21–26). With the knowledge recently obtained regarding the molecular basis of the *IG* gene remodeling processes, this topic in B-NHLs can be studied in a more exact manner. We present data on mRNA expression of AID and DNA polymerase ι in human tonsillar B cells and in an elaborate set of B-NHLs, covering most clinicopathological entities. Our findings demonstrate that AID expression closely correlates with the immunopathological phenotype of B-cell malignancies and that AID, as a genome mutator, may play a role in ongoing transformation of at least part of GC stage-derived B-NHLs.

Materials and Methods

Patient Material. Tumor material was obtained from surgically removed lymph nodes that were snap-frozen in liquid nitrogen at the departments of Pathology of the Academic Medical Center in Amsterdam, the Westeinde

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[3] The abbreviations used are: *IG*, immunoglobulin; GC, germinal center; H chain, heavy chain; NHL, non-Hodgkin lymphoma; B-NHL, B-cell-NHL; SHM, somatic hypermutation; CSR, class switch recombination; DLBCL, diffuse large B-cell lymphoma; AID, activation-induced cytidine deaminase; FL, follicular lymphoma; CLL, chronic lymphocytic leukemia; RT-PCR, reverse transcription-PCR; ICV, intraclonal variation; BL, Burkitt's lymphoma; ALL, acute lymphoblastic leukemia; MCL, mantle cell lymphoma; MALT, mucosa-associated lymphoid tissue; PC, plasmacytoma; dU, deoxy uridine; dG, deoxy guanosine.

Hospital in the Hague, the Academic Hospital in Groningen, and the Spaarne Hospital in Haarlem, the Netherlands. B-CLL samples were derived from peripheral blood obtained at the Department of Hematology of the Academic Medical Center in Amsterdam. All lymphomas were classified according to the WHO classification (2) criteria and judged at least by two expert pathologists. Of all Burkitt's lymphomas studied, a translocation involving *c-MYC* [t(8;14)(q24;q32) or t(8;22)(q24;q11)] was demonstrated by classical karyotyping and/or fluorescence *in situ* hybridization (27). To evaluate the relative number of tumor cells and to exclude the presence of residual GCs, extensive immunohistochemical analyses were performed. Thus, it was ensured that in all B-NHL and B-CLL samples, the relative contribution of tumor cells was at least 80%.

B-Cell Purification and Cell Sorting. Tonsillar mononuclear cells were obtained by Ficoll density centrifugation (Pharmacia Biotech, Roosendaal, the Netherlands). B cells were purified by E-rozetting. Naive, GC, and memory B cells were sorted by a FACS-Vantage (Becton Dickinson, Erembodegem-Aalst, Belgium) based on double stainings using a biotin-labeled monoclonal antibody specific for CD38 (Caltag, Burlingame, CA), detected by Cy5-phycoerythrin-labeled streptavidin (Dako, Glostrup, Denmark), and a polyclonal FITC-labeled antibody specific for IgD (Dako; Ref. 28). All samples analyzed contained the specific B-cell subpopulations at purity of at least 90%.

RNA Isolation, cDNA Synthesis, and Quantitative PCR Analysis. Total RNA was isolated using the Trizol reagent (Life Technologies, Inc., Breda, the Netherlands), and cDNA was synthesized using $pd(N)_6$ random primers (Pharmacia) as described previously (29). Quantitative real-time PCR analysis was performed using a LightCycler (Roche, Almere, the Netherlands; Ref. 30). AID was amplified using the primers 5′-AGAGGCGTGACAGTGCTACA-3′ and 5′-TGTAGCGGAGGAAGAGCAAT-3′ matching sequences in the 3′-end of exon 2 and 5′-end of exon 3, respectively. All reported AID splice variants are detected by this PCR (but not discriminated), except for the splice variant lacking the 3′-end of exon 2 and whole exon 3 (31). To adjust for variable cDNA input, the relative levels of β-actin cDNA were determined using the primers 5′-GGATGCAGAAGGAGATCACTG-3′ and 5′-CGATCCACACGGAGTACTTG-3′. Messages of the error-prone polymerases were amplified by quantitative RT-PCR using distinct forward and reverse primer combinations, *i.e.,* 5′-CATCAGGTTGTGGAGCAGTT-3′ and 5′-GGCAAACACCCATGAACTTT-3′ for Pol β, 5′-TGGTCGTGAGAGTCGTCAGT-3′ and 5′-ACCATTGGGGTCATCACATC-3′ for Pol ι, 5′-TGTGGGCAGATGATGCTAAG and 5′-CCCGGTACTTGGTGAGGTTA-3′ for Pol η, and 5′-CGCGTCAGTTGGGACTTAAG-3′ and 5′-ACTATCGCCAACCTCAATGC-3′ for Pol ζ, respectively. The FastStart DNA Master Sybr Green I reagent (Roche) was used for amplification with $MgCl_2$ at 4 mM for AID, Pol β, and Pol ζ, 3 mM for β-actin and Pol ι, and 2.5 mM for Pol η. After incubation at 95°C for 6 min, 40 cycles of amplification were performed, *i.e.,* successively 10 s at 95°C, 5 s at 60°C, and 5 s at 72°C. In each PCR run, a dilution series of one AID-expressing FL was used to construct a calibration curve. All PCR products were positively identified by melting curve analysis. Results are expressed as ratios of the relative values of AID and β-actin in comparison to the calibrator sample.

Variable H Chain Gene Mutation Analysis. *IGVH* genes of B-NHLs were amplified by RT-PCR, cloned, and sequenced as described previously (29). Of each lymphoma, 3–10 clones (on average 6 clones) were sequenced. The consensus *IGVH* sequence is defined as the nucleotide sequence that is shared by >50% of the clones. The ICV was calculated as the mean number of nucleotide differences/clone compared with this consensus *IGVH* sequence.

Results and Discussion

AID/β-actin mRNA ratios were measured in a variety of normal and malignant B-cell populations by real-time RT-PCR using a LightCycler. In normal naive as well as memory B cells, originating from peripheral blood and tonsil, no significant AID was amplified yielding AID/β-actin ratios < 0.8 (Fig. 1 and data not shown). In accordance with previous articles (6, 32, 33), significant AID expression was found in sorted GC B cells by our quantitative PCR approach yielding relative values of 1.5, 2.8, and 6.3 (with an average of 3.5).

We subsequently investigated an extensive panel of B-NHLs representative for all maturational stages, *i.e.,* 2 B-ALL, 4 MCL, 36 FLs, 18BLs, 22 DLBCLs, 9 low-grade extranodal marginal zone B-cell lymphomas of MALT, 18 B-CLLs, and 5 plasmacytomas (Fig. 1). In remarkable accordance with their phenotypes, in B-NHLs of pre-GC phenotype, *i.e.,* B-ALL and MCL, and of post-GC phenotype, *i.e.,* MALT lymphomas, B-CLL and plasmacytomas, we measured AID/β-actin ratios that did not exceed those found in normal naive or memory B cells. Of note, among the 15 B-CLLs, 9 cases expressed *IGVH* genes with mutation frequencies >2%. Moreover, also after B-cell antigen receptor-mediated stimulation *in vitro* of these mutated and unmutated B-CLL variants, no AID expression was induced up to quantifiable levels (data not shown). Our findings seem to contradict recent articles reporting on AID expression in B-CLL (31–33). In most of these studies, however, AID mRNA expression was measured by nonquantitative RT-PCRs, and by consequence, the actual expression levels are difficult to judge. Recently, McCarthy *et al.* (32) reported on high AID expression in a proportion of mostly unmutated, B-CLL. The authors, however, show, by quantitative AID mRNA measurement using TaqMan technology, that the expression levels found in the B-CLL were in all cases <5% of those found in GC cells. This thus is concordant with our measurements, albeit in our RT-PCR assay, such levels are not quantifiable. We therefore think that the mutations found in a proportion of B-CLL are unlikely to result from an active or inducible mutation apparatus but are introduced before or at early stages of cellular transformation.

Strikingly, AID was only expressed at significant levels in lymphomas with a GC phenotype: in 9 of 18 BLs, AID was expressed, at most at levels comparable with those found in normal GC B cells.

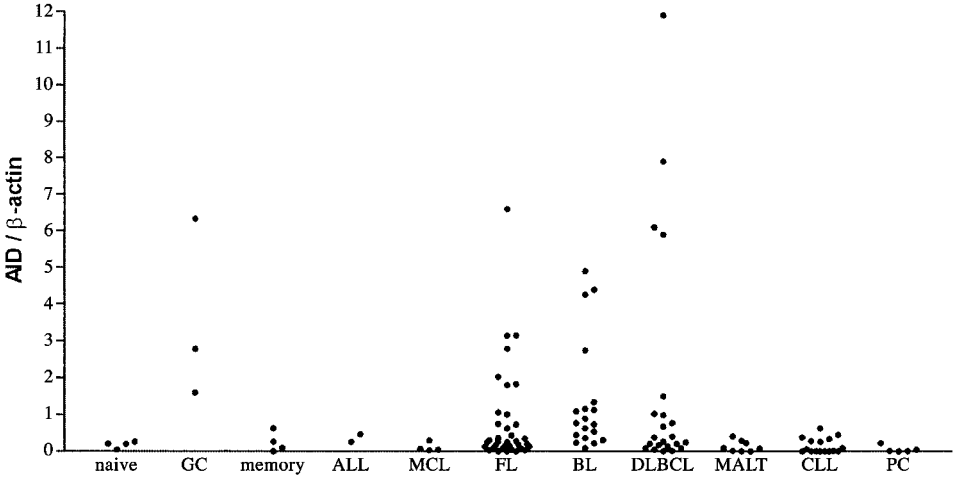

Fig. 1. AID expression in normal B-cell populations and B-NHLs. Quantitative RT-PCR analysis of AID and β-actin was performed on naive, GC, and memory B-cell populations and various primary B-NHLs. Each *dot* represents the average value of at least three AID/β-actin ratio measurements. The interexperimental variation was 30% on average.

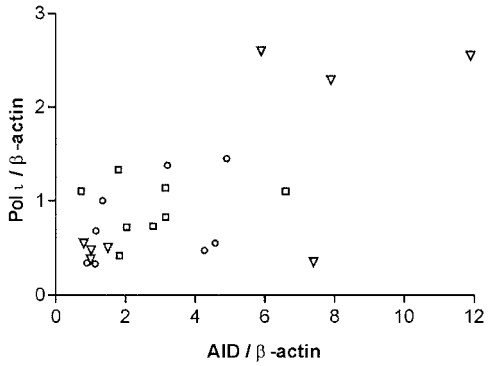

Fig. 2. Relation between AID and polι mRNA expression in the group of GC-derived B-NHL entities. Correlation between AID/β-actin and polι/β-actin ratios in AID expressing FLs (□), BLs (○) and DLBCLs (▽).

This is in agreement with evidence that in BLs (34) and in BL cell lines (35, 36), the SHM/CSR machinery is active or can be induced. Of 22 DLBCLs tested, 7 (32%) expressed measurable AID mRNA, 2 of which (overall 9%) at relatively high levels. This is an intriguing finding because artificial overexpression of AID is known to confer mutation of non-*IG* genes, also in T cells, fibroblasts (37–39), and even bacteria (9). Among the panel of 36 FLs, only 9 (25%) expressed AID above detection level. These AID-expressing FLs were, as a group, not obviously distinctive with respect to histology, immunophenotype nor stage.

We quantitatively measured in the panels of B-NHLs, mRNA expression of the DNA polymerase Polβ and members of the Y family of error-prone DNA polymerases, *i.e.,* Polι, Polη, and Polζ. Interestingly, among the AID expressing B-NHLs, a statistically significant correlation between the expression levels of AID and Polι (Spearman rank correlation coefficient ρ of 0.6, $P < 0.002$; Fig. 2), but with none of the other polymerases (data not shown), was observed. When the AID-positive B-NHLs were separated according to subtype, a statistically significant correlation between AID and Polι was observed within the group of BLs ($\rho = 0.62$, $P < 0.05$). The values calculated for both FLs and DLBCLs did not reach statistical significance. Still, it is remarkable that in the subgroup of DLBCL, the 3 cases with the highest Polι expression levels also displayed the highest mRNA levels for AID. The trend that we observed within our panel of B-NHLs seems compatible with recent RNAi-based gene inactivation studies showing that SHM depends on Polι (40) and suggests coregulation of expression of two key enzymes of the somatic gene diversification process in B-NHLs.

The finding that a minority of FLs express AID is in support of our previous proposal that, in contrast to the general belief, at least a part of these B-NHLs have not retained GC-specific functions like SHM and CSR (21, 22, 29). To substantiate this notion, we compared individual FLs, BLs, and DLBCLs the measured AID levels with the total number of consensus mutations and the degree of intraclonal sequence variation (ICV) in their *IGVH* genes, as far as these could be assessed (Fig. 3). It was striking that for all three B-NHL types, this comparison revealed no obvious correlation between either of these three parameters. With respect to the group of FLs (Fig. 3*A*), different categories could be discerned: FLs lacking ICV, with or without significant AID expression. Next, FLs with significant ICV but no detectable AID expression. This subgroup is of importance because it proves our notion that ICV is not necessarily a reflection of active SHM during the tumor stage but rather a remnant of earlier stages of transformation. By consequence, the mutation patterns found in these

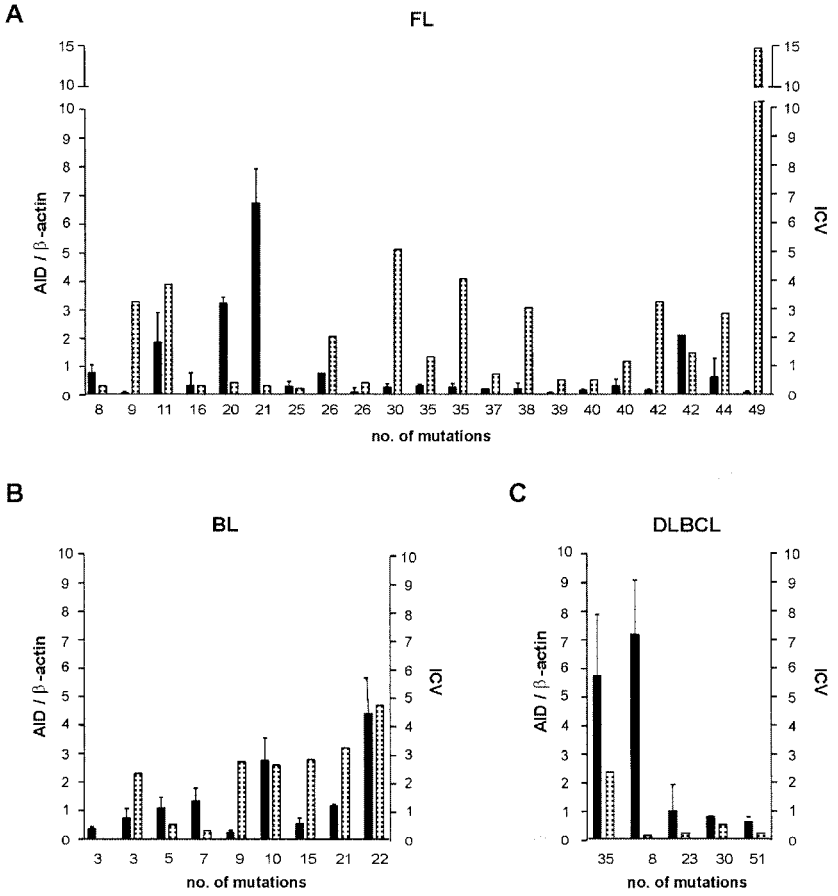

Fig. 3. AID expression compared with intraclonal *IGVH* gene diversity as measured in individual B-NHLs. AID/β-actin ratios (*solid bars*) and the values of intraclonal *IGVH* gene sequence variation (*dotted bars*) as determined in individual FLs (*A*), BLs (*B*), and DLBCLs (*C*). On the *X* axis are plotted the total number of consensus mutations present in the different B-NHLs.

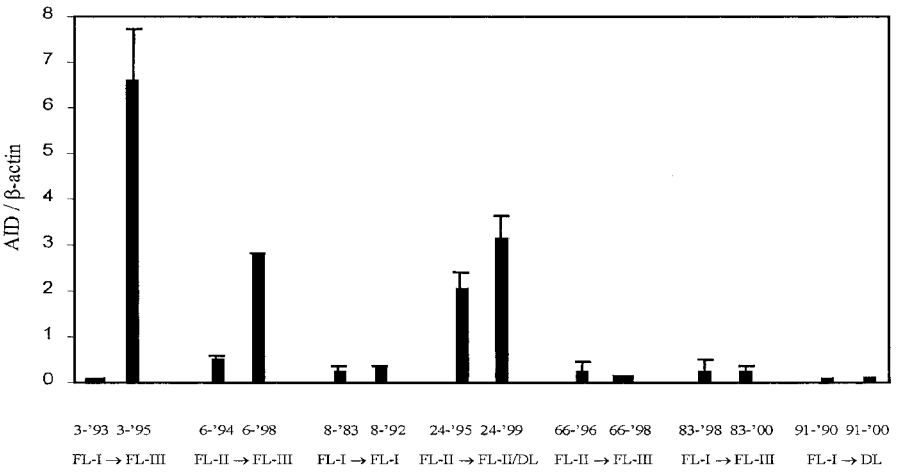

Fig. 4. AID expression in FLs with progression. AID/β-actin ratios at successive time points of 7 FLs that showed clinical progression. Histological grading, according to the WHO classification (2), is denoted below the time points of each lymphoma. FL-II/DL below FL24-'99 signifies a partially follicular, partially diffuse growth pattern. The increase in AID expression as observed in 3 FLs was statistically significant in FL3 and FL6.

FLs, which resemble those of normal antigen-selected B cells, neither result from continued antigen receptor-based selection processes but more likely are left over from the initial GC reactions in which the respective progenitor tumor cells had been engaged. Of note, this subgroup also included the previously reported FL8, which harbored both IgM- and IgG-expressing tumor cells of the same clonal origin (with 30 and 35 consensus mutations, respectively; Refs. 21, 22, 29). Despite the abundance of H chain isotype switch variants among the neoplastic cells, we have not been able to detect CSR excision circles in the tumor tissue (22). Moreover, in this FL, the degree of ICV had decreased instead of increased over a 9-year interval (21, 22). Our interpretation that in this FL and most likely also other FLs, neither CSR nor SHM proceeds is again corroborated by the finding of absent AID expression. In 2 FLs (with respectively 11 and 42 consensus mutations), significant ICV as well as AID expression was detected. A similar pattern was obtained for BLs (Fig. 3*B*), with cases showing significant ICV without quantifiable AID levels (with 3, 9, and 15 consensus mutations, respectively) and *vice versa* (with 5 and 7 consensus mutations, respectively). One BL, with 3 consensus mutations, lacked AID and ICV, whereas in 3 BLs (with 10, 21, and 22 consensus mutations, respectively), both AID expression and ICV of the *IGVH* genes was found. In these latter cases, the somatic diversification machinery is thus potentially active.

For reasons not determined, we managed to resolve the *IG* gene configuration only of a limited number of DLBCLs (Fig. 3*C*). Although this may thus well provide a biased view, it is apparent that AID is not preferentially expressed in cases with the highest number of consensus mutations; 2 of 5 DLBCLs (with 35 and 8 consensus mutations, respectively) harbored significant AID mRNA levels. Interestingly, the first case also displayed ICV of the *IGVH* gene and was 1 of 3 DLBCLs with the highest levels of Pol ι expression (Fig. 2). By contrast, the second AID$^+$ DLBCL, with 8 consensus mutations, lacked ICV and Pol ι expression. We conclude that the hypermutation machinery is likely to be active in at most a minority of the FLs and DLBCLs. In BLs the relative number with ongoing gene diversification may be higher. Furthermore, knowing that AID expression is mandatory for the hypermutation process, the current findings confirm our previous notion that ICV alone is an inappropriate parameter to read out hypermutation activity in B-NHLs.

Finally, we measured AID mRNA in 7 FLs with clinical progression over time (Fig. 4). In 6 of these lymphomas (FL3, FL6, FL24, FL66, FL83, and FL91), progression was also evident histologically. In FL24, AID expression was measurable at both time-points (FL24-'95 and FL24-'99) with a slight increase over time. Interestingly, in 2 cases, significant AID expression was only detected at the second time point (FL3-'95 and FL6-'98). The fact that in these 2 cases AID was not expressed at presentation (FL3-'93 and FL6-'94, respectively) indicates selective outgrowth of subclones with AID transcription and thus potentially with an active mutation machinery. This subgroup among the FLs is of interest because it may harbor a clue for the questions whether AID plays a role in the process of tumor progression and, if so, at what stage the mutations found in the proto-oncogenes in DLBCLs, as described by Pasqualucci *et al.* (5), are being introduced.

In conclusion, AID is expressed in a proportion of B-NHLs of GC phenotype only. The finding of DLBCL with abnormally high AID levels as well the observed selective outgrowth of AID-expressing tumor cells in FL suggest that SHM-related genetic instability is not only essential in tumor initiation but may also be instrumental in ongoing transformation of GC derived B-cell malignancies. It is envisaged that if lymphomas with a mutator phenotype are more prone to progress to higher grade malignancies, assessment of SHM activity of low-grade B-NHLs will become of clinical relevance.

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