Activation-induced deaminase and its splice variants associate with trisomy 12 in chronic lymphocytic leukemia

Kristina Zaprazna[1], **Kamila Reblova**[1], **Veronika Svobodova**[1], **Lenka Radova**[1], **Vojtech Bystry**[1], **Jiri Baloun**[1], **Kristina Durechova**[2], **Nikola Tom**[1], **Tomas Loja**[1], **Martina Buresova**[1,2], **Kamila Stranska**[1,2], **Alexandra Oltova**[2], **Michael Doubek**[1,2], **Michael L. Atchison**[3], **Martin Trbusek**[2], **Jitka Malcikova**[1,2], **Sarka Pospisilova**[1,2]

[1] Central European Institute of Technology, Center of Molecular Medicine, Masaryk University, Kamenice 5/A35, 625 00 Brno, Czech Republic

[2] Department of Internal Medicine – Hematology and Oncology, Faculty of Medicine, Masaryk University and University Hospital Brno, Brno, Czech Republic

[3] Department of Biomedical Sciences, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, USA

Abstract

Activation-induced cytidine deaminase (AID) is a mutator enzyme essential for somatic hypermutation (SHM) and class switch recombination (CSR) during effective adaptive immune responses. Its aberrant expression and activity have been detected in lymphomas, leukemias, and solid tumors. In chronic lymphocytic leukemia (CLL) increased expression of alternatively spliced AID variants has been documented. We used real-time RT-PCR to quantify the expression of AID and its alternatively spliced transcripts (AID E4a, AID E4, AIDivs3, and AID E3E4) in 149 CLL patients and correlated this expression to prognostic markers including recurrent chromosomal aberrations, the presence of complex karyotype, mutation status of the immunoglobulin heavy chain variable gene, and recurrent mutations. We report a previously unappreciated association between higher AID transcript levels and trisomy of chromosome 12. Functional analysis of AID splice variants revealed loss of their activity with respect to SHM, CSR, and induction of double-strand DNA breaks. In silico modeling provided insight into the molecular interactions and structural dynamics of wild-type AID and a shortened AID variant closely resembling AID E4, confirming its loss-of-function phenotype.

Keywords

Activation induced deaminase; Chronic lymphocytic leukemia; Complex karyotype; Alternative splicing; Trisomy 12

Introduction

Chronic lymphocytic leukemia (CLL) is defined as the accumulation of clonal B cells in the peripheral blood (PB), bone marrow (BM), spleen, and lymph nodes (LNs). The disease manifests high clinical and biological heterogeneity and a number of biomarkers have been established to refine patient prognosis: adverse prognostic parameters include the unmutated status of the immunoglobulin heavy chain variable gene (UM-IGHV) [1, 2]; deletions of 17p13 (*TP53*) and 11q22–23 (*ATM*) [3]; mutations in *TP53, NOTCH1, BIRC,* and *SF3B1* genes [4]; and the presence of three or more cytogenetic aberrations (i.e., complex karyotype, CK) [5, 6]. CLL with intermediate prognosis often carries trisomy 12 (tri12) while favorable outcome is associated with a normal karyotype or deletion 13q14 present as the sole abnormality [3].

Activation-induced cytidine deaminase (AID) is a DNA mutating enzyme that plays a crucial role in the processes of somatic hypermutation (SHM) and class-switch recombination (CSR) of immunoglobulin genes taking place in germinal center (GC) B cells during adaptive immune response [7, 8]. SHM enhances antibody affinity towards antigens while CSR diversifies antibody effector functions. AID converts cytosines to uracils in single-stranded DNA exposed during transcription with a preference for RGYW (R = purine, Y = pyrimidine, W = A + T) hotspot motifs [9, 10]. In contrast to the important role AID plays in adaptive immunity, deregulated AID expression and activity is linked to genomic instability, off-target mutations and oncogenic translocations occurring in B cell lymphomas, CLL, Ph-positive acute lymphoblastic leukemia, and chronic myeloid leukemia in a lymphoid blast crisis [11–17]. Aberrant AID expression has also been detected in some solid tumors associated with chronic inflammation, e.g., colorectal cancer, gastric cancer, pancreatic cancer, or hepatocellular carcinoma [18].

In healthy B cells, AID is expressed almost exclusively in germinal centers, and its transcription is tightly regulated [7, 19–23]. In CLL, similar to healthy B cells in germinal centers, the proportion of AID positive cells increases during proliferation [24, 25]. CLL cells enter proliferation centers engaging their CD40 receptor with CD40 ligand presented by Th2 cells and receive other signals which activate AID transcription. Nevertheless, only about 1% of these cells eventually express AID protein [24]. After CLL cells leave the stimulating microenvironment in secondary lymphoid organs and enter peripheral blood circulation, they downregulate AID expression. The highest fraction of PB CLL cells reported to maintain AID transcription was 0.2% [26]; nevertheless, even such a small amount of AID was reported to be associated with adverse survival in CLL patients [24].

The *AICDA* gene itself as well as two genes encoding positive regulators of AID expression, HoxC4 at position 12q13.13 and STAT6 at position 12q13.3, are located on chromosome 12;

AICDA maps to position 12p13.31, and AID belongs to gene-dosage sensitive proteins [17, 27–29].

Human AID protein is encoded by the *AICDA* gene consisting of five exons, which are translated into a 198 amino acid protein. AID transcripts can undergo alternative splicing giving rise to four variants: AID E4a (deletion of first 10 amino acids from exon 4), AID E4 (deletion of exon 4 leading to a premature stop codon and C-terminal truncation of exons 4 and 5), AID E3E4 (deletion of exons 3 and 4), and AID-ivs3 (retains third intron leading to C-terminal truncation due to a frameshift) (Fig. 1) [26, 30–32]. While GC B cells and memory cells express predominantly the fully functional full-length AID transcript (AID FL), CLL cells express a much higher percentage of alternatively spliced variants [32]. Wu and colleagues reported that AID variants AID E4a and AID E4 had higher mutation activity than standard full-length AID protein [32], which led to speculation that these variants might be implicated in CLL pathogenesis. However, others have shown that AID splice variants lack deaminase activity [31, 33] and their expression at the protein level is too low to be functionally relevant [34]. Our work presented here shows that even though AID splice variants are expressed in CLL cells obtained from peripheral blood, they do not retain function. This result is supported by in silico modeling of an AID variant encompassing amino acids 1–142, which is almost identical to the AID variant E4.

Material and methods

Patients

The cohort included 149 patients with CLL diagnosed at the Department of Internal Medicine—Hematology and Oncology, University Hospital Brno between 1993 and 2014. The study was performed on peripheral blood samples taken with written informed consent provided in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. Tonsillar samples were taken during routine tonsilotomies or tonsillectomies with patients' written informed consent. CLL and control B cells were negatively separated by RossetteSep kits (StemCell). Separation efficiency was verified by flow-cytometry and all tested samples contained 98% B cells, which corresponded to at least 90% CLL cell purity. Patient samples were selected based on the presence or absence of complex karyotype defined as three or more cytogenetic aberrations. The following molecular characteristics were assessed: the presence of chromosomal aberrations using fluorescent in situ hybridization (FISH) and metaphase cytogenetics, mutational status of IGHV, and mutations in *TP53, SF3B1, NOTCH1,* and *ATM* genes.

Mice

AID knockout mice on a C57BL/6 background were a gift from Michel Nussenzweig (Rockefeller University). AID knockout mice or C57BL/6 controls (WT) between 8 and 12 weeks of age were used for experiments. The mice were bred and maintained under pathogen-free conditions. All animal studies were performed in compliance with the U.S. Department of Health and Human Services guidelines and were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Functional analysis of AID splice variants

First, we isolated the individual AID splice variants AID E4a, AID E4, and AIDivs3 from peripheral blood samples of CLL patients and remaining variant AID E3E4 from a tonsil sample, and cloned an open reading frame (ORF) of each variant into a retroviral vector Pmx containing a green fluorescence protein (GFP) marker. We tagged each variant with an N-terminal HA in order to verify protein expression by WB, considering that commercial AID antibodies do not detect all tested splice variants.

Follicular B cells from the spleens of AID knockout mice were purified and stimulated with a combination of lipopolysaccharide (LPS) and interleukin-4 (IL-4) known to induce CSR to IgG1. On the next day, we infected stimulated B cells with retroviruses encoding individual AID splice variants or with an empty vector as a negative control. We cultured the cells in the presence of LPS and IL-4 for an additional 2 to 3 days and then sorted GFP-positive cells. Human MAVER-1 cells were infected with retroviral particles containing individual AID variants. GFP-positive cells were sorted 6 days after infection, cultured for 6 weeks, and then sorted again.

Levels of AID expression were assessed using real-time PCR and western blots. CSR was quantified by flow cytometry, and double-strand breaks (DSBs) were analyzed by ligation-mediated PCR. The number of single nucleotide variants in the Ig Sμ region was assessed by ultra-deep next-generation sequencing (NGS), and the results were analyzed by deep-single nucleotide variant (SNV) algorithm.

Modeling of AID

We created a homology model of human AID using the Modeller 9.11 program [35] and the crystal structure of APOBEC3G (Protein Data Bank code 3IQS) as a template. AMBER package 14 [36] and the force field parm14SB were used for molecular dynamics simulations. We also prepared a shorter model comprising only residues 1–142, which mimics splicing variant AID E4. Control simulations were carried out using a Langevin thermostat. The simulation length was 1 microsecond (μs) (for more details, see Supplementary material).

Statistical analyses

All statistical analyses were performed using R/Bioconductor environment. Two-sided tests were applied and a level of 0.05 was established as statistically significant. The Mann-Whitney U test and Kruskal-Wallis ANOVA were performed to clarify the relationships between continuous and categorical variables. Log-rank tests were applied to compare survival rates of different groups of patients, and Kaplan-Meier curves were used for visualizations.

For details on methods, see Supplementary material.

Results

The CLL cohort was selected to contain a comparable number of cases with complex karyotype (CK) (46%) and cases without complex karyotype (non-CK) (54%). Due to

this selection, 76% of the samples harbored unmutated IGHV (UM-IGHV). Clinical and biological features of the patients are summarized in Table 1. In line with previous reports [6, 37], the presence of CK was associated with worse overall survival in our cohort ($P =$ 0.007) (Suppl. Fig. 1a). Patients with CK had a median overall survival of 39.4 months from the time of sampling versus 62.5 months for the non-CK group. The median overall survival from diagnosis of UM-IGHV patients was 110 months versus 177 months for mutated IGHV (MUT-IGHV) cases ($P =$ 0.0037) (Suppl. Fig. 1b).

Alternative AID transcripts were abundantly expressed in our CLL cohort

In order to examine the expression of AID and its splice variants in leukemic peripheral blood B cells from CLL patients, we designed a quantitative real-time RT-PCR assay specific for each AID transcript. A schematic layout of AID variants with primer positions is depicted in Fig. 1. There was a continuous distribution of AID expression levels on a log scale spanning a 1000-fold range (Suppl. Fig. 2) pointing not only to different AID transcript levels in the individual cells but likely also to a different proportion of AID-positive cells in each sample. Even though there was no clear cut-off value for AID positivity, we considered samples where 2 out of 3 replicates did not yield a specific PCR product as AID-negative (cycle threshold [Ct] value 35). cDNA from HEK293T cells not expressing AID was used as a negative control.

We found four out of the five AID variants to be frequently expressed in our cohort (Table 2). The variant missing catalytic exon 3, i.e., AID E3E4, was expressed in the tonsil sample, but only rarely in CLL cells (4% of samples). Therefore, AID E3E4 was not considered in further analyses. Only 13% of the samples did not express any AID transcript. The majority of cases (75%) co-expressed several (two or more) AID variants. Of note, 49% of the samples expressed all four AID transcripts (Table 3). The proportions of AID transcript combinations expressed in CLL samples are listed in Suppl. Table 1. We observed an association between the median expression level of all four AID variants in each sample and a number of different AID splice variants present in that particular sample (Spearman $r =$ 0.8048, $P <$ 0.0001).

AID expression is associated with complex karyotype, trisomy 12 and UM-IGHV

Next, we analyzed whether AID expression correlates with karyotype complexity and/or the presence of particular chromosomal aberrations (CAs) in CLL cells. We found an association between the presence of CK and expression levels of AID alternative transcripts AID E4a and AIDivs3 and a trend for AID FL and variant AID E4 (Table 4). Concerning links between AID expression and individual chromosomal aberrations (del17p, del11q, tri12, and del13q14), we observed that higher expression levels of all four AID transcripts correlated with the presence of trisomy 12 (Table 4; Fig. 2). This association was confirmed by Kruskal-Wallis ANOVA test (Fig. 3). We also evaluated the previously described association between UM-IGHV and AID expression and confirmed this association for each AID variant tested (Table 4; Fig. 4). Additionally, we detected a higher proportion of UM-IGHV (80% vs 50%; $P =$ 0.0028) and CK (50% vs 20%; $P =$ 0.0149) in AID-positive samples (Ct < 35) than in AID negative samples. In line with the abovementioned results, especially considering the correlation between AID transcript levels and the presence of

CK and IGVH mutation status, we expected that patients with high AID expression would have a more aggressive disease course. However, in this particular CLL cohort, we did not observe any impact of AID expression on patient survival or therapy administration need (data not shown).

AID activity inducing point mutations on a genome-wide scale is well documented in germinal center B cells and in B cell lymphomas and leukemias [12, 24, 38–41]. Therefore, as our next step, we evaluated associations between AID expression and mutations in *TP53, NOTCH1, SF3B1,* and *ATM* genes recurrently detected in CLL. We found only two statistically significant correlations: high AID E4a expression associated with wild-type *SF3B1* ($P = 0.0205$) and high AIDivs3 variant level associated with the presence of *NOTCH1* mutations ($P = 0.0487$) (Table 4).

AID splice variants manifest inactivity in mouse splenic B cells and MAVER-1 cell line

In total, the alternatively spliced AID transcripts were detected in 84% of CLL patients; therefore, we asked whether alternative splicing of AID could affect the function of the resulting AID proteins, potentially making them more mutagenic as reported by Jelinek's group [32]. We designed assays to assess AID function in primary mouse splenic B cells and the human mantle cell lymphoma (MCL) cell line MAVER-1 infected by retroviruses expressing individual AID splice variants.

Protein levels of AID splice variants in the infected murine B cells were variable (Suppl. Fig. 3) possibly in part reflecting the situation in vivo where a final amount of nuclear AID protein is regulated by several posttranslational mechanisms. AID FL was expressed the highest while variants AID E4, AID E4a and AIDivs3 reached only 3%, 21%, and 17% of AID FL expression, respectively.

In concordance with previously published data [31–33, 42–44], we demonstrated that all alternatively spliced AID variants were defective in CSR (Suppl. Fig. 4). It is widely accepted that the AID C terminus (lacking in variants AID E4 and AIDivs3) is necessary for CSR and our results were not surprising in this aspect [32, 44, 45]. An opened question remained whether AID variants are functionally active at least with respect to the induction of point mutations and/or DSBs in AID target sequences. To address this question, we used ultra-deep next-generation sequencing (NGS) to analyze mutability of the mouse immunoglobulin switch region DNA (Ig Sμ), which is known to accumulate high amount of mutations and breaks during B cell cytokine stimulation ex vivo. We infected AID knockout B cells with retroviruses expressing individual AID splice variants. Two sets of biological replicates revealed that only AID FL caused accumulation of point mutations above the background levels (Fig. 5).

To quantify the amount of DSBs in the Sμ region, we performed ligation-mediated PCR (LM-PCR) followed by a Southern blot with Sμ specific probe. As depicted in Fig. 6, only AID knockout B cells infected with AID FL variant manifested increased levels of DSBs. Hence, in our assays, AID splice variants were functionally inactive.

To employ an alternative system, we used the human MCL MAVER-1 cell line, which showed low levels of endogenous AID transcripts compared to other tested B cell lines and no detectable AID protein (Suppl. Fig. 5), while it still expressed the productively rearranged IGH gene providing a bona fide target for AID mutagenesis (Suppl. Table 2). We utilized the ultra-deep NGS approach to assess relative gain in mutation load in IGH V-D-J region between day 0 and 6 weeks after the retroviral transduction of individual AID variants. Both HA-tagged and untagged AID constructs were used; HA-AID proteins were quantified on protein level (Suppl. Fig. 6), and expression of untagged AID variants was measured by conventional PCR (Suppl. Fig. 7). Samples MAVER-5 and MAVER-11 expressed low amounts of AID FL transcripts in addition to the variant containing intron 3 suggesting that this intron has been spliced out in some infected cells.

In line with our results from mouse B cells, none of the alternatively spliced AID variants induced point mutations in the VDJ region of IGH in MAVER-1 cells (Table 5) while both HA-AID FL and untagged AID FL proteins were catalytically active. We conclude that AID splice variants expressed from retroviral vectors in MCL cell line lacked catalytic activity resulting in a complete loss-of-function phenotype.

Modeling of AID structure

To further analyze the loss-of-function phenotype on the molecular level, we modeled AID structure for wild-type protein and AID shortened variant consisting of amino acids 1–142 (AID1–142) closely resembling AID splice variant AID E4. Our in silico modeling relies on atomistic molecular dynamics simulations that provide an insight into molecular interactions and characteristic structural dynamics. Human wild-type AID structure consists of a five-stranded β-sheet flanked by six α-helices (Fig. 7a). The catalytic pocket contains zinc ion coordinated by His56, Cys87, and Cys90 amino acids. During deamination catalysis single stranded DNA is bound to AID so that deoxycytidine (dC) is positioned into the catalytic pocket [46]. Molecular dynamics simulations of the wild-type structure were perfectly stable while the shortened AID1–142 variant showed structural rearrangement in the area of the active site and the α1-helix during the first 100 ns (see Fig. 7b–e and Suppl. Fig. 8). Such structural rearrangements might propagate on longer time scales resulting in protein unfolding. We assume that the rearrangement we observed in the simulations most probably affects DNA binding and positioning of dC into the catalytic pocket (for more details, see Supplementary material).

Discussion

Several studies have linked the presence of AID transcripts with more aggressive disease course emphasizing its connection to the activated CLL microenvironment, association with UM-IGHV, chromosomal aberrations, ongoing AID activity in vivo, and increased level of DSBs [24, 38, 47–50]. In addition, Chiorazzi's group demonstrated inferior survival of CLL patients expressing AID mRNA [24].

In our cohort, we found a significant correlation between AID FL and its splice variant expression and the presence of trisomy 12 as well as UM-IGHV and a trend towards karyotype complexity. AID transcripts were detected in 87% of patients. This percentage

is higher than previously reported in CLL (40% in Albesiano et al., 56% in Heintel et al., and 65% in Marantidou et al.) [26, 30, 47]. Differences in AID expression observed by different groups likely reflect different sensitivities of RT-PCR assays (single-round RT-PCR vs nested RT-PCR vs quantitative real-time RT-PCR performed with TaqMan probes or SYBR Green intercalating dye) as well as distinct CLL cohort compositions. In our study, about half of the cohort was intentionally selected to have complex karyotype which was naturally accompanied by a higher proportion of UM-IGHV (76% of samples) and progressive disease (84% of patients at the time of sampling). Real-time RT-PCR assays showed that the expression of one AID variant was infrequent (11%) while co-expression of all four AID transcripts occurred in almost half of the samples. Only 13% of samples did not express any AID transcript. The AID-negative samples showed a high proportion of MUT-IGHV and a low proportion of CK. On the other hand, AID-positive samples had predominantly UM-IGHV (80% of samples) and an equal proportion of CK.

The positive correlation between the level of expression and the number of expressed AID splice variants that we observed (Spearman $r = 0.8048$, $P < 0.0001$) points to deregulated splicing yielding several AID variants in the same sample rather than to a specific production, processing or selection of a particular AID variant.

The higher AID expression in patients with UM-IGHV compared to those with MUT-IGHV has been repeatedly reported [47, 49–51]. Herein, we also present a significant association between the expression of all four AID variants and UM-IGHV. Although these associations might seem somewhat counterintuitive as AID induces somatic hypermutations in IGHV, a recent proteomic study by Pettitt et al. [52] pointed to a migratory defect of UM-IGHV CLL cells accompanied by their enhanced retention and impaired egress from LNs. Our finding of increased AID transcript levels in CLL cells with UM-IGHV might, therefore, reflect their prolonged exposure to the LN microenvironment where AID expression is induced.

An unexpected result of our study is the strong association between expression of all AID variants and the presence of trisomy 12 (classified according to Dohner's hierarchical model) [3]. CLL patients with trisomy 12 constitute a heterogeneous group with an intermediate prognosis. Trisomy 12 has been associated with mutations in the *NOTCH1* gene [53] as well as with Richter transformation [54], with both events being linked to aggressive disease and inferior clinical outcome. In any case, molecular pathogenesis linked to the presence of trisomy 12 is not well understood. Liso et al. evaluated the proportion of CLL cells with trisomy 12 in three compartments: PB, BM, and LN. Interestingly, LN contained a higher percentage of trisomy 12 positive cells compared to PB or BM [55]. Consequently, it has been demonstrated that CLL cells with trisomy 12 overexpress integrin CD49d, a subunit of the late activation 4 receptor (VLA-4), involved in adhesion to endothelial cells via interaction with VCAM-1, extravasation, and homing of CLL cells to the LNs [56]. In line with these reports, Riches et al. demonstrated that trisomy 12 positive CLL cells have increased expression of β2-integrins involved in trans-endothelial migration of CLL cells from the bloodstream to tissues [57]. Notably, CLL cells infiltrating LNs in Richter syndrome had higher levels of AID as well as *c-MYC, PAX5 and RhoH* mutations in AID hotspot motives comparing to circulating leukemic cells [58, 59]. All the

abovementioned facts underscore the LN microenvironment as a key factor in induction of AID expression.

Our functional tests in murine primary B cells and a human MCL cell line did not confirm the hypothesis that AID splice variants might be translated to proteins with increased catalytic activity. We tested SHM, CSR, and DSBs in transduced AID knockout B cells ex vivo because stimulation of murine B cells with LPS and IL-4 turns on processes necessary for AID function (germline transcription and opening of switch regions to AID recruitment). These changes take place rapidly as Ig receptors switched to IgG1 can be detected within 48 h after stimulation. Nevertheless, in vitro stimulated splenic B cells usually die within 5 days of culture which left only a short window for AID induction of SHM and DSBs. Even though we used very sensitive techniques (i.e., ultra-deep next-generation sequencing and Southern blot with a radioactively labeled probe), we did not detect any mutations or DSBs in Ig switch region caused by alternatively spliced AID variants. To confirm this result and leave longer time for AID activity, we infected the human MCL cell line MAVER-1 with HA-tagged or untagged AID variants and harvested DNA after 6 weeks of cell culture. In line with the results from murine B cells, only cells infected with HA-tagged or untagged FL AID retroviruses accumulated mutations in the IGHV-D-J sequence. Interestingly, cells with untagged AID FL gained more IGHV mutations than cells with HA-AID FL, indicating a potential inhibitory effect of the HA tag.

A potential drawback of these functional assays was unequal protein levels of AID variants expressed from a retroviral cassette. We cannot exclude the possibility that each AID variant was translated with different efficiency. In addition, AID proteins are regulated by posttranslational mechanisms which might be responsible for lower levels of AID variants lacking C-terminal domain (AID E4 and AIDivs3). The AID C terminus encodes a conserved nuclear export signal (NES) required for CRM-1 dependent export of AID protein from the nucleus [44, 60, 61]. AID proteins lacking NES stay trapped in the nuclear compartment. Notably, nuclear AID proteins have a much shorter half-life compared to cytoplasmic AID (2.5 h vs. 18 h) as the protein is differentially polyubiquitinated and degraded in these two compartments [42]. Therefore, AID E4 and AIDivs3 lacking NES should be preferentially nuclear as shown in van Maldegem et al. [33] which might reduce their stability. Low protein levels of AID splice variants can also be seen in Rebhandl et al. [34] who expressed AID splice variants from a splice reporter construct and then thoroughly quantified levels of corresponding RNA and translated proteins, showing that AID E4 and AIDivs3 proteins do not stoichiometrically correspond to their RNA counterparts. Our results corroborate these studies suggesting low expression of AID variants at the protein level as one of the potential factors contributing to their loss-of-function phenotype observed in vitro.

To investigate how the lack of exons 4 and 5 affects AID structure, we performed atomistic molecular dynamics simulations and modeled the AID1–142 amino acid protein containing identical sequence as AID variant E4 except for the last three residues (Ala, Pro and Val) at the C-terminus (not present in our model). The comparison of variant AID1–142 with AID FL on a microsecond time scale showed structural rearrangement of the active site area that most probably cannot bind DNA and position dC into the catalytic pocket. Our structural

analysis, therefore, supports observations from functional assays, i.e., that the shortened variants highly likely do not exhibit AID deamination activity.

To conclude, we observed an association between higher AID transcript levels and trisomy of chromosome 12 as well as the unmutated IGHV status in CLL patients. However, we have also demonstrated that the alternative AID splice variants do not retain the activity in SHM, CSR, and the induction of double-strand DNA breaks.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Abbreviations

AID   Splice variants in CLL

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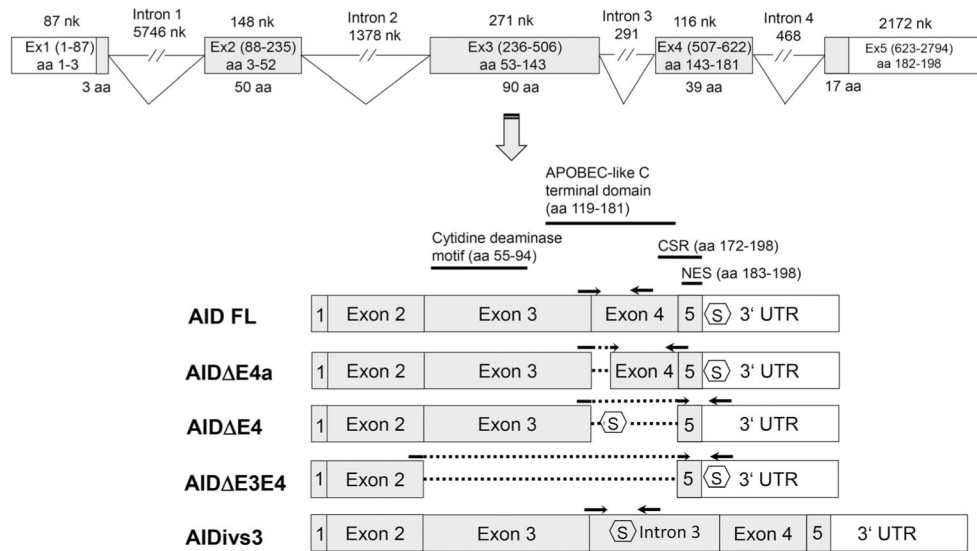
Fig. 1.
Scheme of AID variants with positions of RT-PCR primers. Dashed lines (deletions), arrows (primers), gray (open reading frame, ORF), white (untranslated region, UTR), S (stop codon)
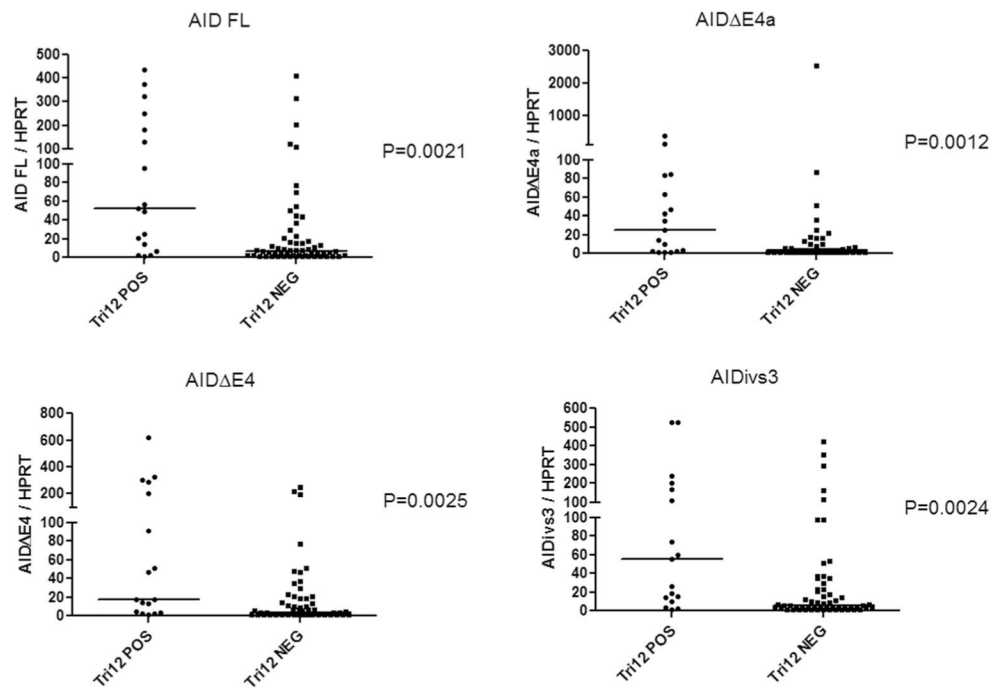
Fig. 2.
Association between AID expression and trisomy 12 as determined by Mann-Whitney *U* test. Samples with trisomy 12 were chosen based on the hierarchical model of chromosomal aberrations. Median value of expression for each group is shown
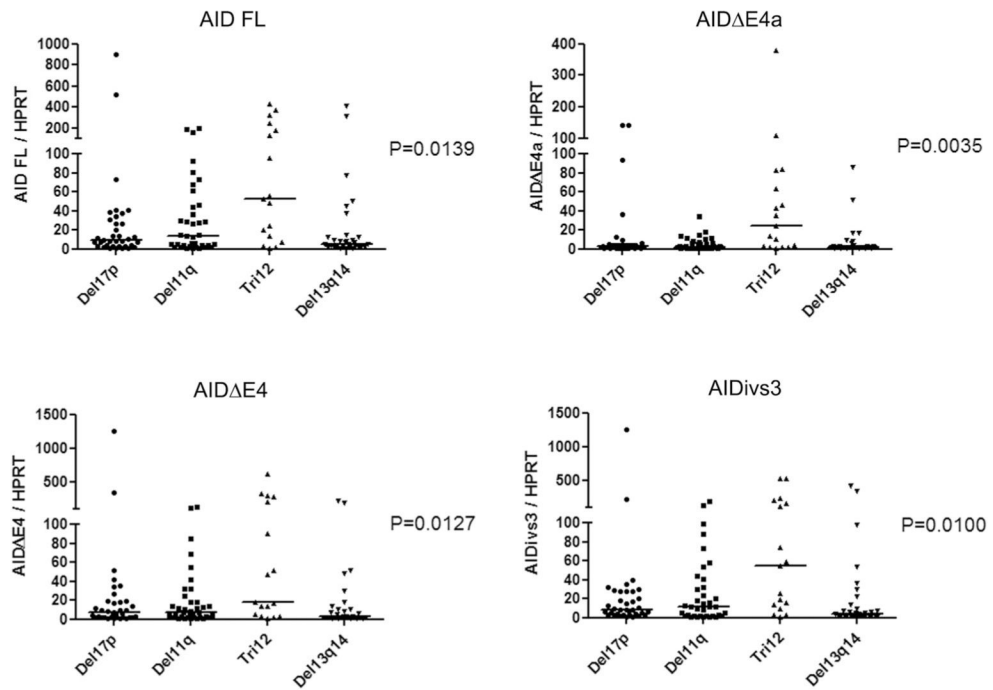
Fig. 3.
Kruskal-Wallis ANOVA comparison of AID expression in CLL cells with recurrent chromosomal aberrations. Median values of expression for each AID variant are shown. Hierarchical order of del17p (modified also for the presence of *TP53* mutations), del11q, tri12, and del13q14 aberrations specified in Table 4 was used to assign CLL patients into particular cytogenetic groups
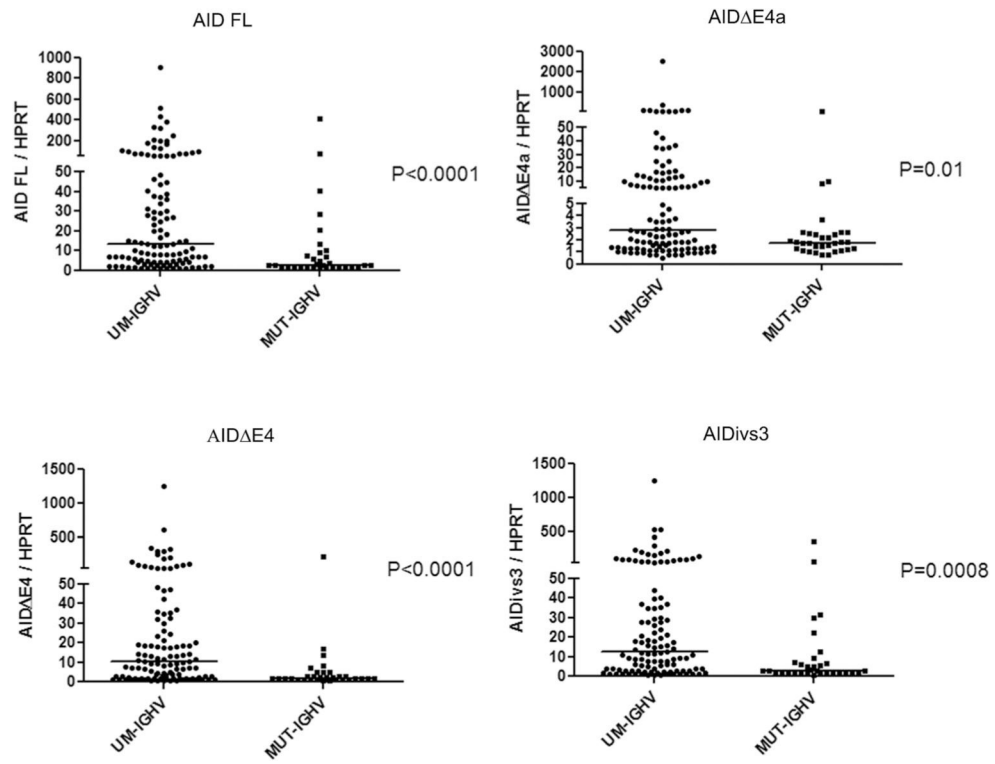
Fig. 4.
Expression of FL AID and AID splice variants according to IGHV mutation status. AID relative expression values were evaluated using Mann-Whitney *U* test. Median value of expression for each group is shown

Single nucleotide variants in Ig Sµ switch region
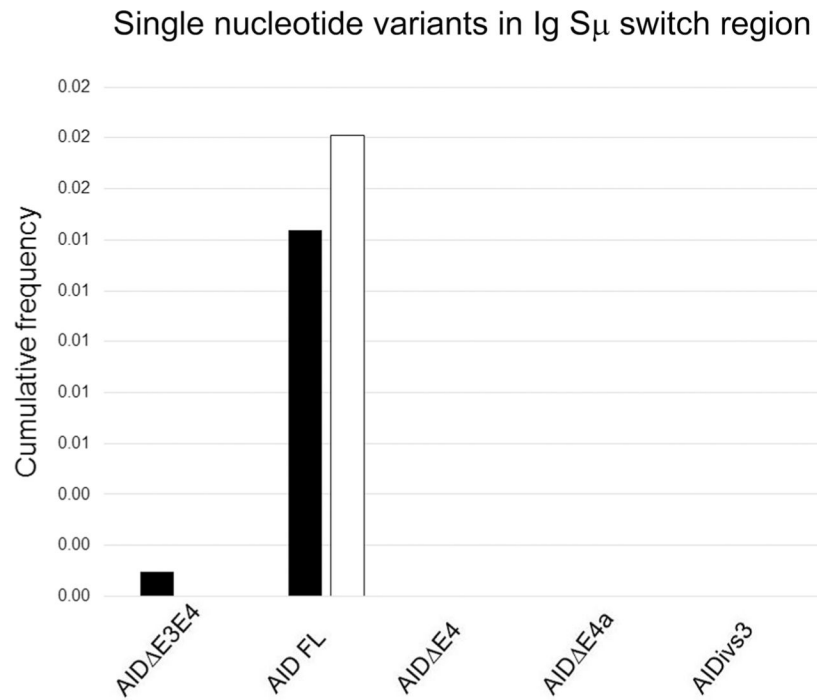
Fig. 5.

AID splice variants do not induce point mutations in Ig Sµ, switch region. Murine AID knockout B cells were stimulated ex vivo with LPS and IL-4, infected with retroviral AID splice variants, cultured for 2 days, and then sorted for highly positive GFP cells. DNA was subjected to ultra-deep next-generation sequencing to assess the amount of point mutations in each sample. The results were analyzed by deep-SNV algorithm and the cumulative frequency of variant reads was plotted. The experiment was performed in two biological replicates (shown as black and white bars)
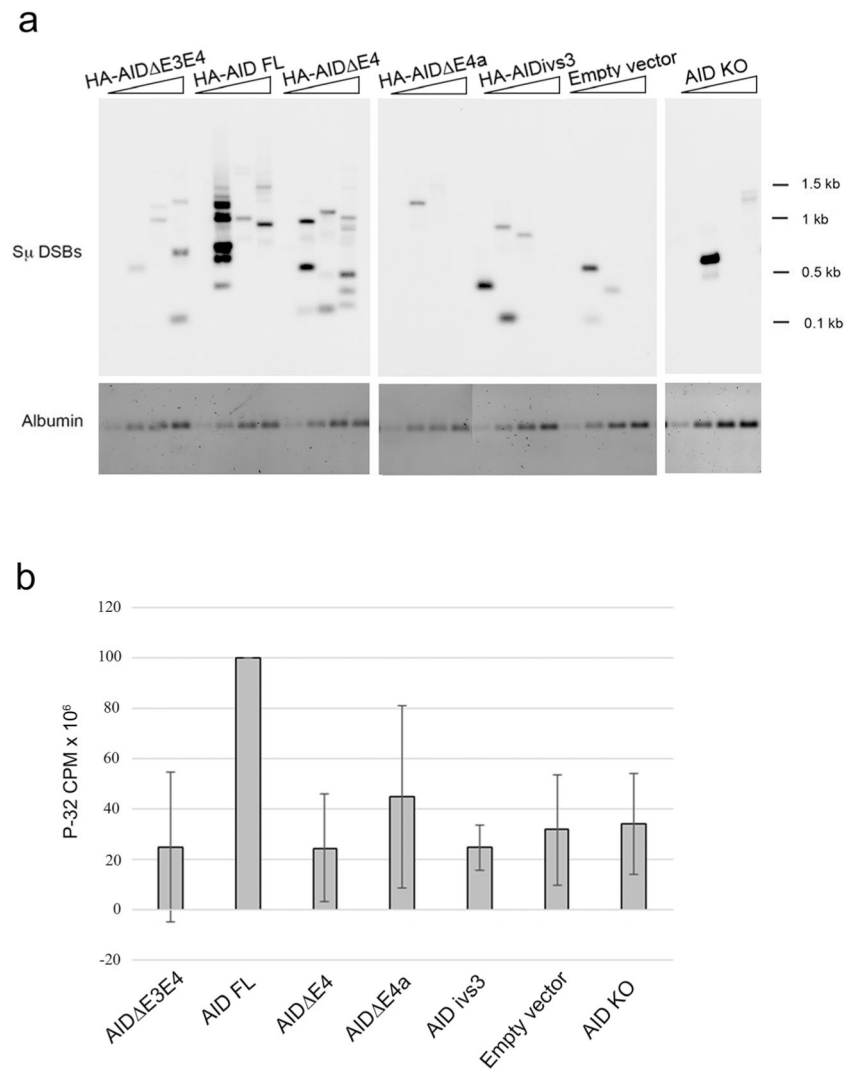
Fig. 6.
AID splice variants do not induce DSBs in Ig Sμ switch region. **a** Murine AID knockout
B cells were infected with AID splice variants and stimulated with LPS and IL-4. Highly
positive GFP cells were sorted the third day of stimulation. DNA samples were normalized
to albumin. DSBs were amplified by ligation-mediated PCR followed by hybridization to
a radioactively labeled Sμ probe. A representative blot is shown. **b** [32]P signals from 3
different experiments were quantified by Typhoon FLA 9000 laser scanner. Error bars =
STDEV
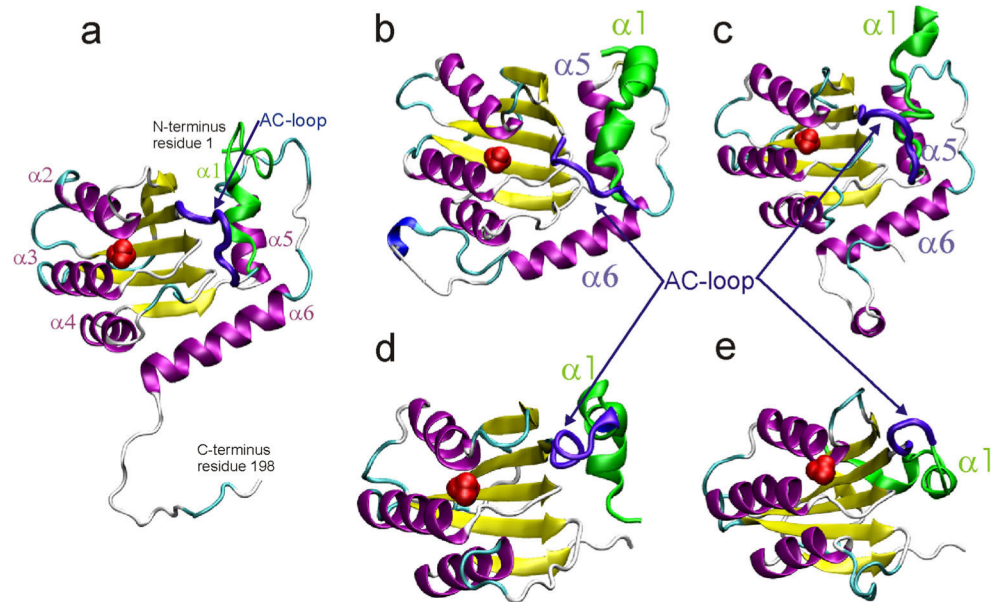
Fig. 7.

a Homology model of wild-type human AID (residues 1–198). Zinc is represented as tetrahedron-shaped ion in red, α1-helix is green, α-helices 2–6 are marked, and AC-loop (residues 20–26) is blue. In the shorter variant AID1–142, α-helices 5, 6, and the C-terminus are missing. **b–e** Wild-type AID and shortened variant AID1–142 after simulating 1 µs, specifically, we visualized structures averaged in the time period 900–1000 ns. **b** Wild-type human AID. **c** Wild-type human AID control. **d** AID1–142 variant. **e** AID1–142 variant control

Table 1

Clinical and biological characteristics of CLL patients

Parameter	Total *n* = 149	(%)
Male vs female	98 vs 51	66 vs 34
Median age at diagnosis (years)	62	
Median age at sampling (years)	67	
Treated vs untreated patients	71 vs 78	48 vs 52
Patients alive 5 years from diagnosis	52	35
Patients alive 10 years from diagnosis	17	11
Complex karyotype yes vs no	69 vs 80	46 vs 54
IGHV unmutated vs mutated vs mixed[a]	113 vs 32 vs 4	76 vs 22 vs 3
Del17p yes vs no[b]	20 vs 129	13 vs 87
Del11q yes vs no[b]	44 vs 100	30 vs 70
Tri12 yes vs no[b]	22 vs 127	15 vs 85
Del13q14 yes vs no[b]	87 vs 62	58 vs 42
Normal karyotype yes vs no	14 vs 135	9 vs 91
Del17p[c]	36	24
Del11q[c]	37	25
Tri12[c]	17	11
Del13q14[c]	37	25
TP53 mutated from total assessed	34/149	23
Notch1 mutated from total assessed	15/99	15
SF3B1 mutated from total assessed	26/127	21
ATM mutated from total assessed	12/38	32

[a]CLL patients with mixed IGHV have both UM-IGHVand MUT-IGHV

[b]Regardless of other aberrations

[c]Hierarchical classification of CAs according to Dohner et al. [3]

Table 2

Summary of AID expression in the studied CLL cohort

AID variants	AID FL	AID E4a	AID E4	AIDivs3	AID E3E4
Number of cases	109	88	98	117	6
Percent	73	59	66	79	4

Table 3

Summary of AID transcript co-expression in CLL samples

Number of AID transcripts expressed	Cases	Percent
No transcript	20	13
1 transcript	17	11
2 transcripts	14	9
3 transcripts	25	17
4 transcripts	73	49
Total	149	

Table 4

Associations between expression of AID transcripts and biological characteristics of CLL patients

Characteristics of CLL samples	P values AID FL	AID E4a	AID E4	AIDivs3
Presence of complex karyotype	0.0745	*0.0184*	0.0992	*0.0382*
Presence of UM-IGHV	*0.0001*	*0.0100*	*0.0001*	*0.0008*
(1) Del17p/TP53 defect	0.7562	0.3313	0.9240	0.9894
(2) Del11q except (1)	0.7878	0.1582	0.8328	0.7480
(3) Trisomy12 except (1, 2)	*0.0021*	*0.0012*	*0.0025*	*0.0024*
(4) Del13q14 (except 1, 2, 3)	0.4814	0.2259	0.2667	0.5274
Presence of TP53 mutation	0.9207	0.6282	0.6283	0.5519
Presence of NOTCH1 mutation	0.1031	0.1485	0.1688	*0.0487*
Absence of SF3B1 mutation	0.8931	*0.0205*	0.6999	0.7133
Presence of ATM mutation	0.7098	0.3623	0.7774	0.4604

CAs are hierarchically ordered [3]

Numbers in italics are statistically significant (*P*<0.05)

Table 5

AID splice variants did not induce mutations in the variable region of IGH gene rearrangement in MAVER-1 cell line

Sample	Number of variants	Sample	Number of variants
HA-AID E3E4	0	AID E3E4	0
HA-AID FL	2	AID FL	9
HA-AID E4	0	AID E4	0
HA-AID E4a	0	AID E4a	0
HA-AID ivs3	0	AID ivs3	0
HA-Empty vector	0	Empty vector	0

Number of induced single nucleotide variants in the V-D-J region of IGH in the evaluated samples is shown. Control sample DNA was harvested at time 0 before retroviral infection. The evaluated DNA samples were harvested 6 weeks after the retroviral infection from cells sorted for high GFP expression. The counted variants were identified as different from the control sample using customized "tumor/normal" variant calling analysis (specified in Supplementary methods)