

Impaired induction of DNA lesions during immunoglobulin class-switch recombination in humans influences end-joining repair

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Ig class-switch recombination (CSR) is a region-specific process that exchanges the constant Ig heavy-chain region and thus modifies an antibody's effector function. DNA lesions in switch (S) regions are induced by activation-induced cytidine deaminase (AID) and uracil-DNA glycosylase 2 (UNG2), subsequently processed to DNA breaks, and resolved by either the classical nonhomologous end-joining pathway or the alternative end-joining pathway (XRCC4/DNA ligase 4- and/or Ku70/Ku80-independent and prone to increased microhomology usage). We examined whether the induction of DNA lesions influences DNA end-joining during CSR by analyzing S μ -S α recombination junctions in various human Ig CSR defects of DNA lesion induction. We observed a progressive trend toward the usage of microhomology in S μ -S α recombination junctions from AID-heterozygous to AID-autosomal dominant to UNG2-deficient B lymphocytes. We thus hypothesize that impaired induction of DNA lesions in S regions during CSR leads to unusual end-processing of the DNA breaks, resulting in microhomology-mediated end-joining, which could be an indication for preferential processing by alternative end-joining rather than by classical nonhomologous end-joining.

antibody maturation | switch junction | DNA repair

During immune responses, mature B cells diversify their Ig genes through class-switch recombination (CSR) and somatic hypermutation (SHM). The latter mechanism introduces nontemplated point mutations in the variable region of Ig genes and thereby enables the selection of antibodies with increased affinity for the antigen. CSR modulates antibody effector function by replacing one constant region with another in a deletion-mediated recombination process, while retaining the binding specificity (variable region) of the B-cell receptor. Both processes are initiated by the enzyme activation-induced cytidine deaminase (AID), which deaminates cytosine to produce U:G mismatches in target DNA (1–3). GC-rich regions, so-called “switch” (S) regions, present in front of each constant region and of varying lengths, are the target DNAs for AID during CSR. The main route for processing AID-induced lesions involves uracil excision through several pathways, primarily the pathway involving uracil-DNA glycosylase (UNG2) (4, 5). Abasic sites are further processed to generate DNA double-strand breaks (DSBs), which are obligate intermediates in CSR (6, 7). The DSBs activate damage response proteins, such as PI3-like protein kinase ataxia-telangiectasia mutated, the phosphorylated histone variant H2AX, the MRN complex (MRE11, RAD50, and NBS1), MDC1, and 53BP1, all of which are known to play roles in CSR in promoting appropriate repair and efficient long-range, region-specific recombination (8–14). In CSR, the resolution step is normally mediated by the classical nonhomologous end-joining pathway (c-NHEJ); however, recent reports strongly suggest that resolution also can be mediated (albeit at a lower frequency) by Ku 70-, Ku 80-, and/or

XRCC4/DNA ligase 4-independent alternative end-joining (AEJ) pathways, which are biased toward microhomology usage, and which components are not yet well defined (15–18). The low frequency of microhomologies in S junctions from healthy individuals suggests that under normal conditions, AEJ is inefficient or even excluded from the CSR process, raising the question of how the choice between these pathways is made.

In the other lymphocyte-specific antigen receptor gene diversification process [V(D)J recombination], DNA breaks are induced by the recombinase-activating gene 1/2 (RAG1/2) in a site-specific manner. RAG1/2 core or RAG2 truncated protein (with a frameshift mutation at aa 361) enables V(D)J recombination in the absence of components of the c-NHEJ pathway, providing evidence for (i) the existence of an AEJ pathway and (ii) a role for WT RAG1/2 in recruiting c-NHEJ for repair and/or excluding AEJ components (19). This observation suggests that AEJ is excluded from the V(D)J process by the time that DNA lesions are induced.

AID and UNG can be viewed as the CSR “counterparts” of RAG1/2, given that they are responsible for inducing DNA lesions in the S regions. AID activity is essential for CSR, and the expression level of this enzyme influences the efficiency of the process. Haploinsufficiency has been reported for AID-deficient mice, although no evidence for this has been reported in humans on the basis of serum Ig levels and in vitro CSR experiments (20–22). Conversely, overexpression of AID in the CH12 murine B lymphoma cell line and in murine B lymphocytes increases the frequency of cells undergoing CSR (3, 23). We have found evidence of defective CSR in patients bearing a heterozygous deletion in which the C-terminal part of AID, including the nuclear export signal, is missing, resulting in an autosomal dominant form of AID (due to a dominant-negative effect) (20). AID^{+/-C-termΔ} mutants, together with the UNG2-deficient setting that we described previously (4), provide a unique opportunity to study DSB repair pathways in human CSR with impaired AID activity or in the absence of UNG. Using a PCR-based assay, we established that the nature of S μ -S α CSR junctions differs in AID-heterozygous (AID^{+/-}), AID-autosomal dominant, and UNG2-deficient B cells compared with controls, with a progressive, marked shift toward the use of microhomologies (≥ 10 bp), indicating unusual processing of altered DNA lesions during CSR.

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Results

S μ -S α Junctions Exhibit Trends Toward Microhomology in AID-Heterozygous, AID-Autosomal Dominant, and UNG2-Deficient B Cells. To study the impact of DNA lesion induction on DSB repair during CSR, we purified genomic DNA from peripheral blood from healthy donors (controls), AID^{+/-} patients, AID^{+/-}C-term Δ patients carrying the previously described R190X mutation (20) or the recently identified V186X mutation, and the UNG2-deficient (UNG2^{-/-}) patients described earlier. The various mutations analyzed are listed in *Materials and Methods*. Both AID^{+/-}C-term Δ mutations resulted in a dominant-negative form of AID. Individual S μ -S α fragments from the various patients were amplified using a nested PCR assay, as described previously (24). The amplification efficiency of S μ -S α fragments was similar for the AID^{+/-}, AID^{+/-}C-term Δ , and control samples, in contrast to the UNG2^{-/-} samples, in which fewer bands were amplified by nested PCR. In particular, only a few S μ -S α fragments were amplified in UNG2^{-/-} patient P1 (as identified in ref. 4) (Fig. S1). To define the S junctions, the amplified S μ -S α fragments were sequenced and aligned with the germline S μ and S α sequences. In all, we characterized 67 S μ -S α recombination junctions in blood samples from five AID^{+/-} patients, 84 from five AID^{+/-}C-term Δ patients, and 88 from three UNG2^{-/-} patients. The distribution of S μ -S α junctions for each individual patient is shown in Fig. S2. Of note, α 1 junctions were more prevalent than α 2 junctions in controls (83%) compared with AID^{+/-}, AID^{+/-}C-term Δ , and UNG2^{-/-} patients (AID^{+/-}, 66%, $P < 0.05$, χ^2 test; AID^{+/-}C-term Δ , 58%, $P < 0.001$; UNG2^{-/-}, 53%, $P < 0.0001$). Because the S α 2 sequence does not exhibit enhanced microhomology with S μ compared with the S α 1 sequence, our observation might be related to increased IgA2 production in the patients, based on recurrent bacterial intestinal stimulation (25, 26).

There was a trend toward an increased overlap between the S μ and S α sequences in recombination junctions derived from AID^{+/-} cells (6.7 ± 0.8 nucleotides vs. 4.5 ± 0.6 in controls; $P < 0.05$, Student t test) (Fig. 1). A more pronounced increase in overlap between the S μ and S α sequences was observed with recombination junctions derived from AID^{+/-}C-term Δ and UNG2^{-/-} patients (8.9 ± 0.8 and 9.8 ± 0.8 nucleotides, respectively) compared with controls ($P < 0.0001$) (Fig. 1). The increase in microhomology between S μ and S α sequences in recombination junctions from AID^{+/-}, AID^{+/-}C-term Δ , and UNG2^{-/-} patients was associated with (i) a significant decrease in the proportion of S μ -S α junctions with no microhomology (6% in both AID groups and 2% in UNG2^{-/-} patients vs. 17% in controls; $P < 0.05$ and < 0.01 , respectively, χ^2 test) (Fig. 2A) and (ii) a significantly greater proportion of junctions exhibiting a long microhomology (≥ 10 bp) for the AID^{+/-}C-term Δ and UNG2^{-/-} patients (36% and 43%, respectively, vs. 10% in controls; $P < 0.001$, χ^2 test; AID^{+/-}, 19%, $P < 0.09$) (Fig. 2A). We excluded the possibility that the increased microhomology observed in the junctions from the patients was the consequence of enhanced S α 2 usage, because when analyzed separately, S μ -S α 1 and S μ -S α 2 from AID^{+/-}, AID^{+/-}C-term Δ , and UNG2^{-/-} patients demonstrated the same preferential microhomology usage (Fig. 2B and C).

A recent study of S μ -S α junctions recommended the use of aged-matched controls, given that S junctions from young subjects are biased toward microhomology (27). In an aged-matched analysis, we confirmed the trend toward microhomology in separately studied adult and young AID^{+/-}C-term Δ and UNG2^{-/-} patients and adult AID^{+/-} patients; young AID^{+/-} patients were not available (Fig. 3).

Together, our results indicate end-processing biased toward long microhomologies in UNG2^{-/-}, AID^{+/-}C-term Δ , and, to a lesser extent, AID^{+/-} B cells.

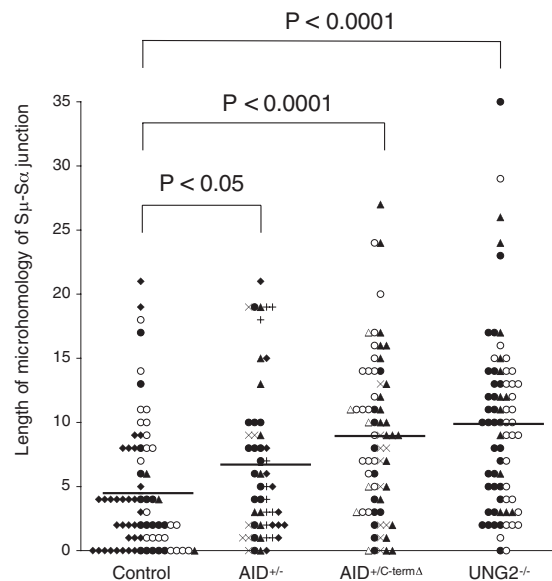


Fig. 1. Distribution of microhomology lengths for S μ -S α recombination junctions from AID^{+/-}, AID^{+/-}C-term Δ , and UNG2^{-/-} patients. Each symbol indicates a unique S μ -S α junction. The average length for each group and P values (two-tailed unpaired Student t test) are indicated. Junctions with insertions were excluded from this representation. The figure represents 72 control, 54 AID^{+/-}, 68 AID^{+/-}C-term Δ , and 72 UNG2^{-/-} S μ -S α junctions. The S μ -S α junctions obtained from the same patient in each group are marked with the same symbol. Open circles and open triangles indicate S μ -S α junctions from children.

Position of S μ and S α Breakpoints in Switch Junctions from AID-Heterozygous, AID-Autosomal Dominant, and UNG2-Deficient B Cells. We next analyzed whether the end-processing biased toward microhomologies in UNG2^{-/-}, AID^{+/-}C-term Δ , and AID^{+/-} cells also affects the position of the S μ and S α breakpoints. In DNA ligase 4-deficient B cells, significantly more S μ breakpoints were located in the stretch of the S μ region (position 275–760) that has the greatest homology with S α 1 and S α 2 (17). We observed this phenomenon for S μ breakpoints in UNG2^{-/-} and AID^{+/-}C-term Δ patients (82% and 75%, respectively, of all S μ breakpoints vs. 54% in controls; $P < 0.0001$ and < 0.01 , respectively, χ^2 test), indicating the need for donor/acceptor homology in DSB resolution, but not in AID^{+/-} patients (63%) (Fig. 4). We conclude that the recombination of S μ with S α regions in UNG2^{-/-} and AID^{+/-}C-term Δ cells depends in part on the presence of microhomologous sequences.

Mutations in S μ -S α Junctions from AID-Heterozygous, AID-Autosomal Dominant, and UNG2-Deficient B Cells. Point mutations occurring close to the recombination junction are supposedly generated during the resolving step of CSR, given that they are generally more frequent and mostly affect GC residues, in comparison with point mutations in the germline S μ region, which exhibit a pattern similar to SHM in V regions and thus likely involve a different mechanism (28, 29). A lower number of mutations around the S μ -S α junctions (± 15 bp) has been reported in patients with Artemis deficiency and Ataxia-telangiectasia compared with controls, suggesting that DNA ends are not normally processed in these individuals (27). We examined the S μ -S α junctions from the UNG2^{-/-}, AID^{+/-}C-term Δ , and AID^{+/-} cells for mutations and found significantly fewer mutations located close to the junctions in the UNG2^{-/-} and AID^{+/-}C-term Δ patients compared with controls (4.5% and 7.9%, respectively, vs. 14.2%; $P < 0.01$ and < 0.05 , respectively, χ^2 test), but not in the AID^{+/-} patients (10.4%) (Table 1). We also noted a significant shift to-

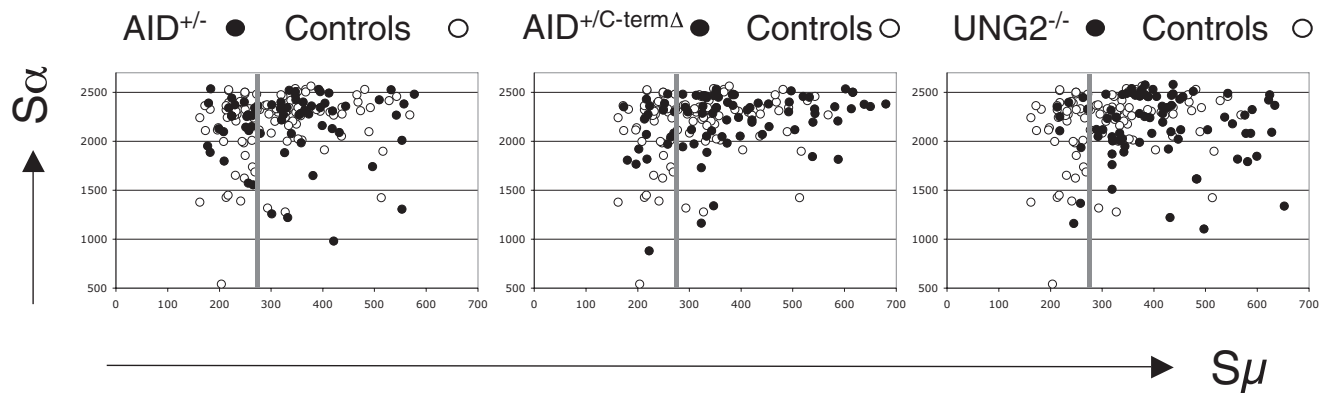


Fig. 4. Scatterplot analysis of S_{μ} and S_{α} breakpoints. The x-axis and y-axis indicate the position of the S_{μ} and S_{α} breakpoints, respectively, with respect to the annotated S sequences. The vertical line indicates the start of the S_{μ} region displaying the highest degree of homology with $S_{\alpha 1}$ and $S_{\alpha 2}$. For clarity, only the S_{μ} breakpoints upstream of position 700 are shown. One $AID^{+/-}$ switch-fragment and three $AID^{+/C-term\Delta}$, seven $UNG2^{-/-}$, and five control switch fragments were located in the more 3' part of S_{μ} (i.e., downstream of position 800) and are thus not shown.

deficient B cells (4, 20). Fewer AID^{-} and/or UNG -induced DNA lesions likely leads to staggered and long single-stranded DNA breaks that may require extensive processing by polymerases, such as polymerase μ or ζ (34, 35). Polymerase-mediated repair might lead to the insertion of nucleotides at the junctions due to template-independent synthesis, or to microhomologies due to fill-in synthesis (34, 36). The polymerase-mediated repair is likely associated with the components of c-NHEJ (34); however, the microhomology length of >10 nucleotides and the location of the S_{μ} breakpoints observed in the analyzed patients argues for another pathway involving DNA end-resection to search for donor/acceptor homology. DNA end-resection facilitates microhomology-mediated end-joining/recombination and has been recently associated with AEJ (37). Fewer AID^{-} and/or UNG -induced DNA lesions also could result in the absence of abasic sites located close to the DSB, which could favor AEJ processing, as suggested by a recent report demonstrating that abasic sites close to DSBs can be processed by Ku70's lyase activity and exclude AEJ (38).

Microhomology-mediated end-joining also could be the result of impaired recruitment of cofactor(s) involved in the processing/recombination of AID -induced lesions. Several studies have strongly suggested that the C-terminal part of AID could recruit a repair cofactor or target AID to specific areas, such as nuclear pores, thereby providing a special DNA repair/recombination environment (32, 33, 39). The detection of DSBs in the S_{μ} region from $AID^{+/C-term\Delta}$ B cells supports the impaired recruitment hypothesis for the $AID^{+/C-term\Delta}$ group. Other data suggest that UNG acts in CSR not only through its enzymatic activity, but

also as an important docking protein (40). Interestingly, AID , UNG , and $PMS2$ involved in the generation of DNA lesions or breaks seem to exert further activity during S region end-joining. Why microhomology-mediated repair occurs in $PMS2$ -deficient cells remains unclear (31, 41). A recent study with $PMS2$ endonuclease-deficient mice indicated that the presence of the $PMS2$ protein, but not its endonuclease activity, was required for proper S region end-joining, but that $PMS2$ endonuclease activity was still necessary for efficient CSR (42). These results suggest that an as-yet unknown interaction of $PMS2$ with a putative partner could recruit the c-NHEJ pathway and/or counteract microhomology-mediated repair. Thus, AID , UNG , and $PMS2$ could be involved in cofactor recruitment to facilitate c-NHEJ, a hypothesis reminiscent of the known complex role of $RAG1/2$ in V(D)J recombination (19). The nature of the cofactor(s) required for proper end-joining during CSR remains unknown; $53BP1$ is a possible candidate, given the recent demonstration of its involvement in the decision between AEJ and c-NHEJ during the CSR process (37).

Whether the microhomology-mediated end-joining that we observed in $AID^{+/-}$, $AID^{+/C-term\Delta}$, and $UNG2^{-/-}$ cells is the actual result of AEJ (Ku70/Ku80- and/or XRCC4/DNA ligase 4-independent) or the consequence of modified c-NHEJ remains unresolved. The extent to which AEJ reflects a distinct bona fide repair mechanism or a compensatory pathway when c-NHEJ is defective is unclear (16, 36, 43).

In conclusion, the present study strongly suggests a direct link between DNA lesion induction in S regions during CSR and

Table 1. Mutations in S_{μ} - S_{α} fragments

	AT vs. GC	GC transition (G→A; C→T)	Total number of mutations	Number of bp sequenced	Frequency per 1,000 bp
Close to the junction (± 15 bp)					
Controls	6 vs. 38 (14% vs. 86%)	22 (58%)	44	3,090	14.2
$AID^{+/-}$	6 vs. 15 (29% vs. 71%)	10 (67%)	21	2,010	10.4
$AID^{+/C-term\Delta}$	9 vs. 11 (45% vs. 55%)**	9 (82%)	20	2,520	7.9*
$UNG2^{-/-}$	2 vs. 10 (17% vs. 83%)	8 (80%)	12	2,640	4.5**
Within the S_{μ} core (>15 bp upstream of the junction)					
Controls	35 vs. 73 (32% vs. 68%)	40 (55%)	108	16,442	6.6
$AID^{+/-}$	33 vs. 38 (46% vs. 53%)	26 (68%)	72	11,316	6.4
$AID^{+/C-term\Delta}$	44 vs. 36 (55% vs. 45%)**	25 (69%)	80	16,650	4.8*
$UNG2^{-/-}$	34 vs. 55 (38% vs. 62%)	52 (95%***)	99	20,313	4.9*

Statistical analyses were performed using the χ^2 test. Statistically significant differences are shown in bold (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). The S_{μ} core was defined as the 300-bp region starting from position S_{μ} 142 (X54713).

the DNA repair machinery subsequently involved in the end-joining process.

Materials and Methods

Patients. The study included the three UNG patients described previously (4), five AID-heterozygous adults (with a G203A, C441A, or C70T mutation or deletion of the entire coding region) from four independent families, three AID–autosomal dominant patients (with the previously described heterozygous R190X mutation) from two unrelated families [including patients 2-I-1 and 2-II-2 from a previous study (20)], and two patients from the same family with a heterozygous V186X mutation as the result of a 4-nucleotide insertion at the beginning of exon 5. Blood samples were drawn after informed consent was obtained from the patient or parents. The study was approved by the local institutional review board (CCPPRB 05632; Paris Saint Antoine) and was conducted in accordance with the Declaration of Helsinki.

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Amplification and Analysis of S μ –S α Recombination Junctions. Genomic DNA was purified from peripheral blood, and 30 ng of DNA was used as a template in each individual PCR. Amplification was performed as described previously (24), but using Go-Taq (Promega). S μ –S α fragments were gel-purified (Roche), cloned into Topo-TA vector (Invitrogen), and sequenced with an automated fluorescent sequencer (MilleGen). CSRs were identified by aligning the S fragment sequences with S μ (X54713), S α 1 (L19121), and S α 2 (AF030305). Microhomology was defined as successive nucleotides shared by S μ and S α at the S junction, with no mismatches. A nucleotide at the breakpoints that was not identical to either of the S regions was defined as an insertion. Polymorphisms in the S region were excluded from the mutation analysis.

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