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BRIEF COMMUNICATION

A novel activation-induced cytidine deaminase (AID) mutation in Brazilian patients with hyper-IgM type 2 syndrome

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Abstract Activation-induced cytidine deaminase (AID) is a DNA editing protein that plays an essential role in three major events of immunoglobulin (Ig) diversification: somatic hypermutation, class switch recombination and Ig gene conversion. Mutations in the AID gene (*AICDA*) have been found in patients with autosomal recessive Hyper-IgM (HIGM) syndrome type 2. Here, two 9- and 14-year-old Brazilian sisters, from a consanguineous family, were diagnosed with HIGM2 syndrome. Sequencing analysis of the exons from *AICDA* revealed that both patients are homozygous for a single C to G transversion in the third position of codon 15, which replaces a conserved Phenylalanine with a Leucine. To our knowledge, this is a new *AICDA* mutation found in HIGM2 patients. Functional studies confirm that the homologous murine mutation leads to a dysfunctional protein with diminished intrinsic cytidine deaminase activity and is unable to rescue CSR when introduced in *Aicda*$^{-/-}$stimulated murine B cells. We briefly discuss the relevance of *AICDA* mutations found in patients for the biology of this molecule.

Abbreviations: AID, activation-induced cytidine deaminase; wt, wild type; SHM, somatic hypermutation; CSR, class switch recombination; APOBEC1, apolipoprotein B mRNA editing catalytic polypeptide 1; GC, gene conversion; NES, nuclear export signal; NLS, nuclear localization signal; hAID, human AID; PI, propidium iodide

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1. Introduction

Hyper IgM (HIGM) syndrome is an immunodeficiency in which patients show low or absent levels of IgG, IgA and IgE, although presenting high values of IgM [1]. Patients with HIGM are susceptible to recurrent infections and may also show lymphoid hyperplasia, as well as autoimmune and inflammatory disorders. The diagnosis is made through laboratorial and clinical assessment. This syndrome may be caused by X-linked or by autosomal recessive and occasionally dominant mutations. The central defect is the inability that B cells have to undergo class switch recombination (CSR), and somatic hypermutation (SHM) is also frequently compromised. During CSR, the original constant region of the heavy chain, that forms the IgM antibody, is replaced by downstream regions encoding the constant regions of the heavy chain from the additional isotypes, IgGs, IgA or IgE, each with unique effector functions [2]. In contrast, during SHM it is the V(D)J region of the immunoglobulin genes that is edited, mostly through the introduction of point mutations that will allow the selection of high-affinity antibodies in the context of the germinal center reaction [3].

B cells need to be activated through ligand–receptor interactions in order to undergo CSR and SHM, which explains i) the X-linked form of HIGM (HIGM1), caused by mutations in the CD40L, a ligand that is normally transiently expressed on activated helper T cells, [4–8]; ii) the autosomal recessive HIGM3, caused by mutations in the CD40, a receptor constitutively expressed by B cells [9]; and iii) mutations in the nuclear factor κB (NF-κB) essential modulator gene (NEMO), that interferes with the NF-κB signaling triggered by the CD40 cross-linking [10–12]. Since CSR involves the processing of DNA lesions in the switch regions, the reaction depends on several enzymes, including uracil-DNA glycosylase (HIGM5), which has also been associated with HIGM [13]. The molecular characterization of the phenotype of HIGM patients lacking mutations in the documented candidate genes suggests that mutations in other genes encoding repair molecules or survival factors are likely to be associated with HIGM (HIGM4) in the future [14].

One last example of a gene that has been associated with HIGM is *AICDA*. Mutations in this gene origin HIGM2 [15], now also named B-cell intrinsic immunoglobulin class switch recombination (Ig-CSR) deficiency 1 [16], which is characterized by high levels of IgM, reduced or absent IgG, IgA and IgE, and lymphoid hyperplasia. *AICDA* encodes the protein activation-induced cytidine deaminase (AID), a DNA editing enzyme expressed by B cells that deaminates cytosines from exposed single-strands of DNA, which are the essential triggering lesions for both CSR and SHM [15,17,18]. Thus, the absent or low serum IgG, IgE and IgA in these patients can be explained by the intrinsic inability of activated B cells to undergo class switch recombination.

AID is a 198-residue protein with a molecular mass of approximately 24 kDa that shares homology with Apolipoprotein B (apoB) mRNA-editing cytidine deaminase 1 (APOBEC-1) [17]. *AICDA* mutants isolated from HIGM2 patients [15,19], combined with functional analyses, have revealed a cytidine deaminase domain, regions important for the shuttling of AID between the nucleus and the cytoplasm, regions that are specifically required for SHM or CSR and residues involved in important post-translational modifications (reviewed in [20]).

In this article we describe AID F15L, a new recessive mutation found in two Brazilian sisters with HIGM2, whose parents are first-degree cousins. To unravel the functional implications of this mutant form of AID, we performed a functional analysis of the corresponding murine AID mutant and found it to be impaired in deaminase activity and in its ability to perform CSR. We complement the description of these findings with a brief comment on the relevance of the mutations found in patients to the biology of AID.

2. Patients

At the Children's Hospital, Faculdade de Medicina da Universidade de São Paulo, Brazil, two 9 and 14-year-old Brazilian sisters from a consanguineous family were diagnosed with Hyper IgM syndrome 2 (HIGM2): low serum IgG and IgA (≤2 standard deviation [SD] below normal values for age), normal or elevated IgM and recurrent or severe bacterial infections. Informed consent was obtained from both patients, and blood was collected under institutional guidelines [21–23]. One of the sisters presented a more severe clinical picture with pneumonia and meningitis, causing her deafness at the age of 4 years. This patient presented hyperplasia of the tonsils, bilateral cervical lymphadenopathy and bronchiectasis. Laboratorial results showed serum IgM levels of 1.5 mg/ml while the other immunoglobulins were all undetectable. The other sister presented mostly respiratory manifestations and was admitted to the intensive care unit once with *Haemophilus influenza*, isolated from respiratory secretions, and had two episodes of dental abscess. Her level of serum IgM was 2 mg/ml, while IgA and IgE were undetectable and IgG was very low (0.4 mg/ml). At a physical exam, she presented generalized lymphadenopathy and splenomegaly. Flow cytometric analysis indicated normal lymphocyte count in both patients, although the B cell counts were close to the lower limit of the normal range.

3. Methods

3.1. Detection of serum immunoglobulins

The levels of serum immunoglobulins were detected as previously described [24].

3.2. *AICDA* sequencing

The genomic sequence used to design the primers and align the amplicons was NCBI NT_009714.17. For mapping the exons and introns, we used the cDNA sequence from NM_020661.2. After genomic DNA isolation from whole blood, the five exons of *AICDA* were amplified by Polymerase Chain Reaction (PCR) using a PTC-100 Programmable Thermal Cycler (MJ Research Inc.), a Taq DNA polymerase (Fermentas, USA) and specific primers, designed to flank each of the five exons. PCR specific primers used to amplify and sequence the *AICDA* gene were the following: exon 1: sense 5′GAACCATCATTAATTGAAGTGAG-3′ and anti-sense 5′-ACGCCTCCCTAGGAAAGTGTGTC-3′; exon 2: sense 5′-TGATGAGTATCTCTCAATTGGCC-3′ and anti-sense 5′-TCAGCATCTAGAAGACAGTGG-3′; exon 3 and 4: sense

5′-TTTAGCGTGGTCCTCTCTGTCTC-3′ and anti-sense 5′-ACTG AGAGTGATTGAACAATAAG-3′; and exon 5: sense 5′-ACTCAGT TTAGAAGACTCTCCGG-3′ and anti-sense 5′-ACCACTGTTA ACTCAAATGAATC-3′. The PCR program was: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 56 °C for 30 s and 68 °C for 1.5 min; and one cycle of 68 °C for 7 min. Due to their proximity, the third and the fourth exons were amplified and sequenced as a single amplicon.

The PCR products were separated by gel electrophoresis, treated with a QIAquick® PCR Purification Kit (QIAGEN) and sequenced with a Big Dye Terminator Cycle Sequencing Kit and analyzed with a 310 Genetic Analyzer ABI PRISM (Applied Biosystems, Foster City, CA, USA), using the same primers as before.

Sequences were first obtained straight from the PCR products and were then confirmed from cloned PCR products, using pGEM®-T Easy Vector Systems (Promega, Madison, WI).

3.3. Constructs and plasmids

We generated the murine AIDF15L in pASK by DpnI site-directed mutagenesis of a previously cloned murine AID wild type sequence [25], using the oligonucleotides 5′-TTCTTTAC CAATTTGAAAAATGTCCGCT-3′ (the substitution is underlined) and 5′-AGCGGACATTTTTCAAATTGGTAAAGAA-3′. The PCR program used was 95 °C for 30 s, 18 cycles of 95 °C for 30 s; 55 °C for 30 s; and 68 °C for 2 min/kb of plasmid length. The introduction of the mutation was then confirmed by sequencing. A pMX-AIDF15L construct was produced by cloning the AIDF15L with a Kozac site PCR product into the pMX vector using the BamHI and NotI restriction sites [26]. A set of murine AID and murine AIDF15L constructs with linker-3 × FLAG (-GGG-DYKDHDGDYKDHDIDYKDDDDK) fused to the C terminus was also produced by PCR and cloned in pMX. For the subcellular localization experiments, constructs encoding the wild type and AIDF15L murine open reading frame fused to the amino terminus of GFP were produced by DpnI site-directed mutagenesis from an AID-GFP fusion in pQCXIP (CLONTECH Laboratories, Inc.), a gift from Dr. Kevin McBride [27].

3.4. Cytidine deaminase assay in *Escherichia coli*

AID, AIDF15L and pASK (empty vector) were each transformed into a BH156 Uracil-DNA Glycosylase (UDG) deficient *E. coli* strain. The cytidine deaminase activity assay was performed essentially as described before [26,28]. Briefly, cultures from individual colonies were grown overnight in 50 μg/ml of ampicillin (Amp), 34 μg/ml of chloramphenicol (Cam) and 100 μg/ml of spectinomycin (Spc), with no agitation and then used to inoculate cultures that were grown to log phase before induction for 3 h with 0.2 μg/ml Anhydrotetracycline (Sigma) to express AID, and with 1 mM Isopropylthiogalactoside (IPTG, Promega), to express a kanamycin resistance gene with a premature stop codon. The cultures were then plated at appropriate dilutions in plates with Amp, Cam and Spc to count live cells and in plates with Amp, Cam, Spc, IPTG and kanamycin (60 μg/ml) to count cells that reverted the premature stop codon. Mutation frequencies were measured by determination of the average frequency of colonies resistant to kanamycin within viable cells.

3.5. CSR assay on transduced B cells

BOSC23 retroviral packaging cell line was propagated in complete DMEM (supplemented with 10% Fetal Bovine Serum, 1% Sodium Pyruvate and 1% Penicillin/Streptavidin), at 37 °C, with 5% CO_2. For transfection, 4×10^5 cells were seeded in six-well plates and grown to 50–70% confluence. Cells were co-transfected by the Ca-phosphate method with an ecotropic helper plasmid pCL-Eco (Clontech) and retroviral vectors (pMX) that express the murine AID, the AIDF15L mutant protein or no protein at all (empty vector). These experiments were also performed with the 3 × FLAG versions of these constructs. Transfected cells were incubated in complete DMEM for 36–48 h at 37 °C in a 5% CO_2 incubator.

Mouse splenic B cells from 2 to 5-month old C57Bl/6J *Aicda*$^{-/-}$ mice were isolated and separated by anti-mouse CD43 (Ly-48) beads (Myltenyi Biotech). 1×10^6 mouse splenic B cells were stimulated throughout the duration of the assay with LPS (20–25 μg/ml) and IL-4 (produced by the cell line X63) and cultured in RPMI complete medium (10% Fetal Bovine Serum, 1% Sodium Pyruvate, 1% Penicillin/Streptavidin and 50 μM β-Mercaptoethanol) for 24 h at 37 °C in a 5% CO_2 incubator. After 24 h in culture, the cells were transduced with retroviral supernatants and centrifuged for 1 h and 30 min at 2500 rpm in the presence of 10 μg/ml polybrene and 20 mM Hepes. The medium was changed 12 h post-transduction and at day 3 the cells were stained for IgG1. The GFP signal indicates that the cells are expressing the genes encoded by the retrovirus.

3.6. Flow cytometry

Mouse splenic B cells were stained with an anti-mouse IgG1-APC antibody (BD Biosciences Pharmingen). Dead cells were excluded from the analysis on the basis of forward side scatter and Propidium Iodide (PI, Invitrogen) gating. Cells were acquired for GFP and surface IgG_1 expression by FACSCalibur and analyzed with FlowJo software (Tree Star).

3.7. Western Blot

Protein extraction was performed on CH12F3 cells [29] transduced with pMX plasmids using an NP40 Cell Lysis Buffer (Invitrogen, USA) supplemented with a protease inhibitor cocktail (Complete, Roche, USA). Samples were run on a 4–12% SDS-PAGE gel using the NuPAGE system (Life technologies, USA) and semi-dry transferred to Imobilon-P membrane (Millipore). Membranes were blocked in 5% Bovine Serum Albumin (BSA, Sigma, USA) in Tris-Buffered Saline 0.05% Tween-20 (TBS-T), incubated with Anti-FLAG M2 (F-1804, Sigma-Aldrich) for detection of AID-3 × FLAG proteins and anti-GFP (ab6556, Abcam) for loading control. Blots were developed using secondary antibodies conjugated to IR Dye 680 or 800 and imaged and quantified on a LiCor Biosciences Odyssey near-infrared imaging platform. Signal was quantified in Odyssey (Li-Cor, Biosciences).

3.8. Microscopy

293T cells were plated on glass coverslips, transfected with pQCXIP AID-GFP (the Venus variant), pQCXIP AIDF15L-GFP or pQCXIP-GFP and evaluated 32–54 h post-transfection.

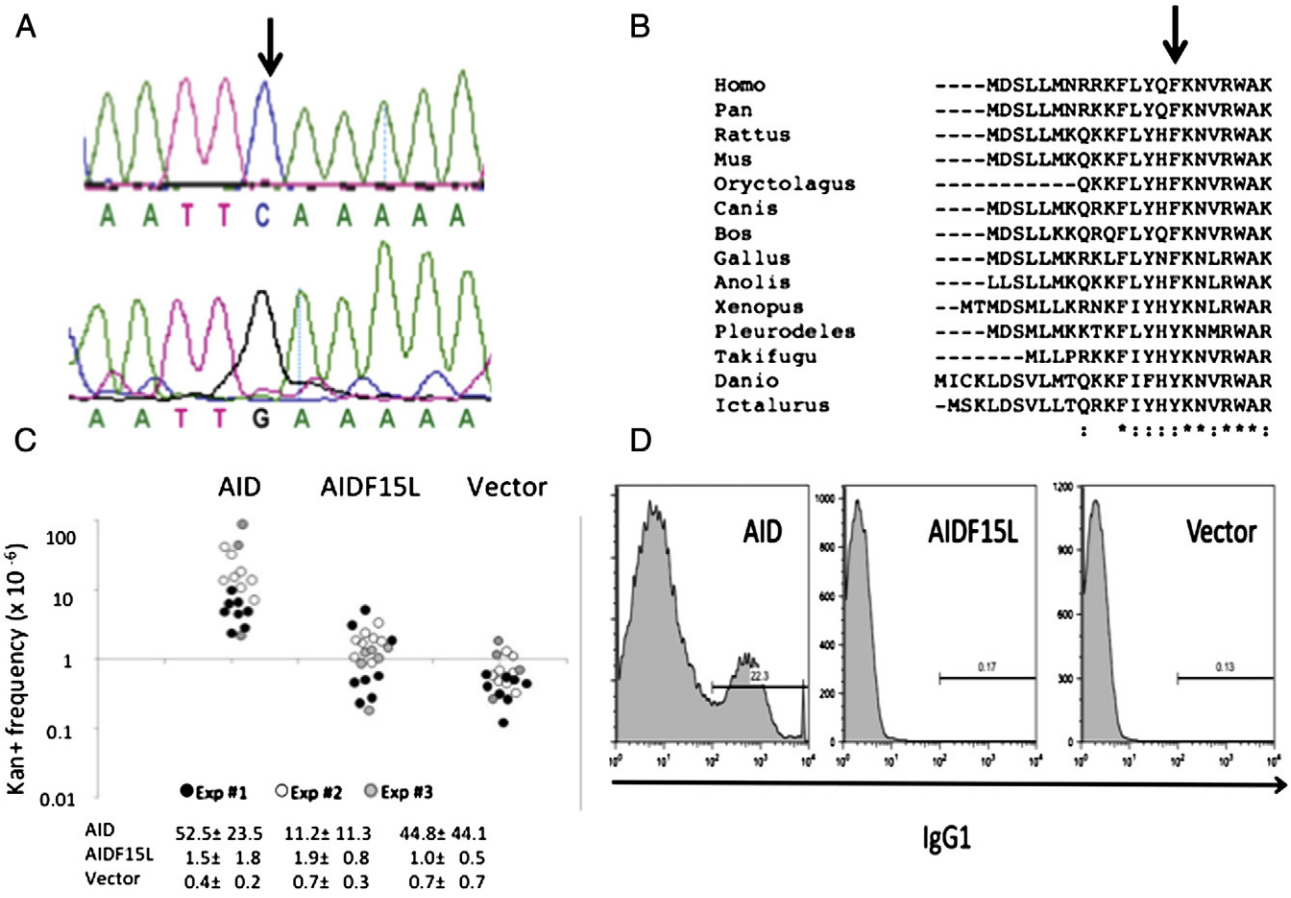


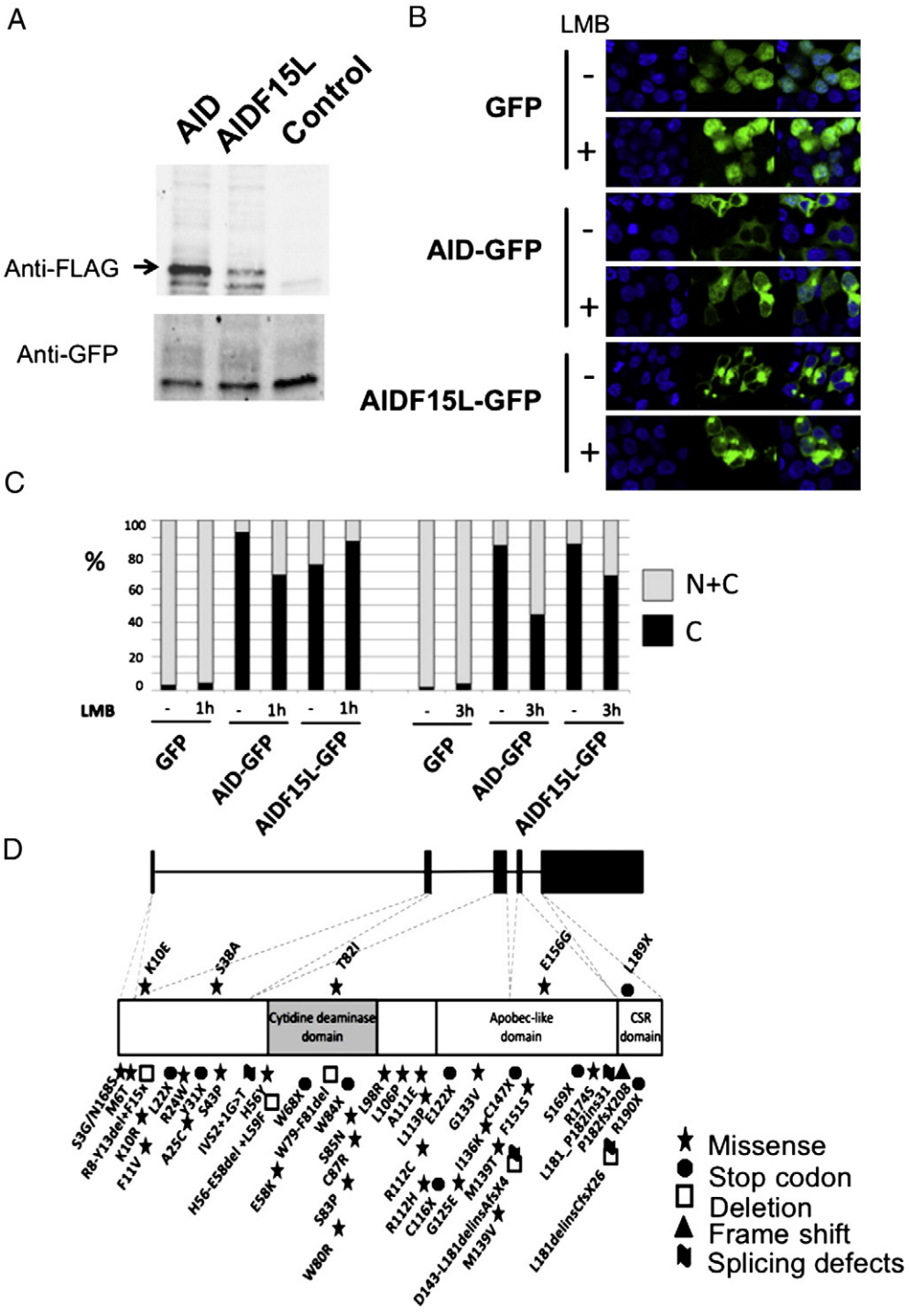
Figure 1 Mutation found in *AICDA*. A) Control's (top) and patients' (bottom) sequences showing a single non-silencing C to G transversion in the third position of codon 15 (F15L) in *AICDA* that replaces a conserved Phenylalanine with a Leucine. B) Clustal sequence alignment of the N-terminus of orthologous AID sequences from the following species (accession number): *Homo sapiens* (GenBank AAP36918.1), *Pan troglodytes* (NP_001065277.1), *Rattus norvegicus* (NP_001094249.1), *Mus musculus* (NP_033775.1), *Oryctolagus cuniculus* (GenBank AAX99151.1), *Canis lupus* (NP_001003380.1), *Bos Taurus* (NP_001033771.1), *Gallus gallus* (NP_001230151.1), *Anolis carolinensis* (XP_003216895.1), *Xenopus laevis* (NP_001089181.1), *Pleurodeles waltl* (GenBank: CBG76578.2), *Takifugu rubripes* (XP_003966295.1), *Danio rerio* (NP_001008403.1), and *Ictalurus punctatus* (NP_001187114.1). The arrow shows the position of the mutation. C) *E. coli* cytidine deaminase assay to measure the intrinsic deaminase activity of the murine AID and AIDF15L. The plotted data are from three independent experiments and the circles correspond to individual cultures. Mean and standard deviations for each experiment are shown. D) *Aicda*$^{-/-}$murine splenic B cells were stimulated with LPS and IL4, transduced with retroviruses expressing the murine AID, AIDF15L or only the GFP reporter (vector), and at day 3 post-transduction the cells were stained for IgG_1. The histograms show the percentage of switching to IgG_1 within the transduced (GFP+) live cells of one representative experiment.

Figure 2 AIDF15L expression levels, subcellular localization and the collection of *AICDA* mutations found in patients with HIGM2. A) Western blot of protein extracts from CH12F3 cells transduced with pMX expressing plasmids encoding AID-3 × FLAG, AIDF15L-3 × FLAG or pMX vector. The expression of AIDF15L is 22-32% of the wild-type level. B) Microscopic fields of 293T cells transfected with plasmids encoding AID-GFP, AIDF15L-GFP or GFP (in green). DNA is visualized with Hoechst stain (in blue). C) Percentage of cells showing a subcellular localization of the GFP signal in the cytoplasm and nucleus (C + N) or exclusively in the cytoplasm (C); 140 to 615 cells were scored per condition. D) Mutations in *AICDA*. The genomic structure of the *AICDA* locus is shown with introns as lines and exons as rectangles. The broken lines connect exons to the translated open reading frame of *AICDA*. The complete collection of mutations found in AID patients is shown below the scheme of the molecule. These were found in homozygous or compound heterozygous patients (except R190X, which comes from a heterozygous patient), and include missense mutations, premature stop codons due to point mutations or to frame shifts resulting from insertions or deletions, deletions and mutations that interfere with the proper splicing of the RNA [19,37,42,45–54]. A small selection of some mutations in conserved residues identified in the context of basic research is also displayed above the molecule, including: S38A, which showed the importance of the phosphorylation of residue S38 for the activity of AID [55,56]; T82I and the combination of K10E-E156G-T82I, which showed that minor changes can produce AID mutants with enhanced mutagenic activity [43]; L189X, which showed that, of the AID-dependent DNA editing reactions, the C-terminus is only required for CSR [26,37].

Leptomycin B at 10 nM or 0.1% ethanol (vehicle control) were added to cultures 1 to 3 h before fixation. Cells were fixed in 4% formaldehyde in PBS. Nuclei were stained with Hoechst (33342, Invitrogen). Preparations were analyzed on a Leica SP5 confocal microscope. Images were processed with LAS AF Lite software (Leica).

4. Results and discussion

Preliminary direct sequencing analysis of the amplified exons from *AICDA* revealed that the two patients have a single non-silencing C to G transversion in the third position of codon 15 (F15L), which replaces a Phenylalanine with a Leucine. As can be observed in the chromatogram, at that position there is a single peak (Fig. 1A), which indicates that the mutation is homozygous in both patients. Cloning and sequencing of the PCR products confirmed that both patients are homozygous for the same mutation, as all PCR-derived clones showed the same mutation. No other mutation was detected in the sequences encoding the open reading frame of *AICDA*. The patients live in the northeast area of Brazil, where the population has a unique mixed ethnic background of Portuguese, African (mainly from the Central Western



Atlantic coast) and native Indian descents, who have been merging since the 16th century [30]. To our knowledge, this is the first description of the F15L AID mutation in HIGM2 patients. A sequence alignment of the N-terminus of AID from several species shows that F15 is highly conserved, the amino acid being shared from reptiles to mammals; furthermore, from sharks to amphibians the residue at that position is a Tyrosine, which shares with Phenyalanine a hydrophobic side chain (Fig. 1B).

As the human and murine AID molecules are 198-residue highly conserved proteins that share 92.4% identity at the amino acid level, in order to test whether this mutation is sufficient to decrease AID activity and explain the HIGM2 syndrome of the two patients, we introduced the F15L mutation in the *AICDA* gene, in plasmids encoding the murine AID that we routinely use in our assays. We first tested the intrinsic cytidine deaminase activity of the AIDF15L using an *E. coli*-based assay that measures the ability of the expressed molecules to revert through cytidine deamination a premature stop codon in the gene that confers resistance to kanamycin, thus restoring the capacity of the bacteria to grow in the presence of the antibiotic. As can be observed in Fig. 1C, in three independent experiments AIDF15L showed a drastic decrease in activity compared to AID, but it nevertheless consistently kept some residual activity when compared to the empty vector, a result that is compatible with a recent biochemical analysis of an identical mutation of unknown origin [31]. We next investigated whether the AIDF15L form was still able to induce CSR. We used retroviruses to express AIDF15L in C57Bl/6J *Aicda*$^{-/-}$mouse B cells stimulated with IL-4 and LPS. Transduced B cells were identified by GFP expression and the efficiency of CSR was determined by cell surface IgG_1 expression. The percentages of class switch within the GFP+ cells were 22.3 for wild-type AID, 0.17 for AIDF15L and 0.13 for the empty vector, indicating that the AIDF15L has no activity, since it is comparable to the background level (Fig. 1D). Similar experiments performed with the 3 × FLAG versions of the molecule confirmed these results (data not shown). These are short-term assays, which may not detect the residual activity of AIDF15L observed in the *E. coli* assay. Alternatively, it is possible that the stability of the molecule in eukaryotic cells is diminished and/or that additional functions required for CSR are compromised. Thus, we first looked at the expression levels of AID in the transduced cells of the lymphoma cell line CH12F3. AIDF15L is clearly detected although it is less stable than AID, as it tends to happen with several AID mutants (Fig. 2A). Keeping in mind that the levels of AID expressed from the retroviruses are higher than the physiological levels, the slight decrease in stability does not explain the complete failure to rescue CSR (Fig. 1D). We next looked at the subcellular location of AID versus AIDF15L using fusions of these molecules to GFP. As expected, while GFP is evenly distributed in the cytoplasm and nucleus, the bulk of AID-GFP is found in the cytoplasm [27,32]. Under these conditions, we did not detect any major difference between the subcellular localization of AID-GFP and AIDF15L-GFP (Fig. 2B). Since AID is actively excluded from the nucleus by an exportin CRM1-dependent pathway, due to the presence of a nuclear export signal in its carboxyl terminus [27], it is possible to disturb the subcellular localization of the molecule with leptomycin B (LMB), a CRM1 exportin inhibitor. After 3 h of incubation with LMB, the GFP subcellular localization was not changed, but an increase in the nuclear localization was observed in both AID-GFP and AIDF15L-GFP (Figs. 2B and 2C). The F15L mutation is in a region that has been characterized as a nuclear import signal [33], although it is not one of the assigned critical residues. Furthermore, there has been some controversy over the existence or nature of the N-terminal NLS of AID [27,34,35]. In any case, we found no evidence that the subcellular localization of AIDF15L could contribute to its CSR phenotype. It should be kept in mind that one of the patients had low but detectable serum IgG levels, which is consistent with a residual cytidine deaminase activity of AIDF15L that, although undetectable in our CSR assay, could occur in vivo in a few cells that may then further expand and partially compensate for the diminished AID activity.

AIDF15L is one of over 40 different mutations in the coding region of *AICDA* that have been identified in HIGM2 patients (Fig. 2D). In the seminal work of Revy et al. the study of these mutations was decisive to firmly establish AID as the main player in both CSR and SHM [15]. The position of some mutations is relatively trivial to explain. For instance, H56Y and C87R flank and make part of a group of mutations that are predicted to affect the catalytic site of the molecule, which must have an intact Zn(2+) coordination and nucleotide binding to the deaminated base. The changes introduced by mutations located elsewhere in the molecule are less obvious, because the structure of the AID protein has not been solved. Several mutations located outside of the catalytic site, such as F15L, are also known to decrease the intrinsic deaminase activity of the molecule and attempts have been made to relate the effects to loss of structural integrity or compromised interaction with the substrate [31,36]. Interestingly, AID forms with mutations in the N-terminus were shown to be competent for CSR but not SHM, whereas AID forms with mutations in the C-terminus are able to drive SHM but not CSR [37,38]. This led to the proposal that AID is interacting with SHM-specific and CSR-specific factors [26,37,38]. While no progress has been made to identify the former, there is some evidence that the CSR-specific factor(s) interacting with AID belong to the DNA repair pathways recruited after the introduction of the lesions by the cytidine deaminase [39–41], although the identity of the molecule(s) directly interacting with AID in the context of this reaction remains to be elucidated.

AID mutants from HIGM2 patients are necessarily null forms or hypomorphs. These are typically homozygous, although several examples of compound heterozygosity have been reported, as well as a single case of a heterozygous mutation that seems to encode a dominant negative form [42], which is consistent with the hypothesis that AID acts as a dimer or a tetramer. As our knowledge of AID and the collection of AID mutants from patients increase, the chances of finding interesting null or hypomorphic mutants associated with HIGM2 decreases. In turn, it may be promising to explore the possibility of discovering AID mutants with enhanced or deregulated activity. In vitro work has shown that very few mutations are required to drastically boost the activity of AID [43] and the molecular signature of AID activity has been found in a number of human lymphomas [44]. Thus, although the sequencing of *AICDA* yielded no results [44], it is possible that the failure to find AID mutants associated with lymphomas and other tumors is due to a limited number of patients sequenced so far for the *AICDA* gene.

Conflict of Interest statement

The authors declare that there are no conflicts of interest.

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