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Recombination activity of human recombination-activating gene 2 (*RAG2*) mutations and correlation with clinical phenotype

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Abstract

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Background: Mutations in recombination-activating gene (RAG) 1 and RAG2 are associated with a broad range of clinical and immunologic phenotypes in human subjects.

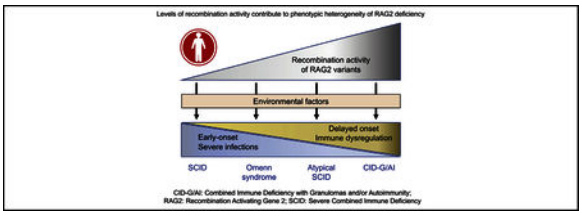
Objective: Using a flow cytometry–based assay, we aimed to measure the recombinase activity of naturally occurring RAG2 mutant proteins and to correlate our results with the severity of the clinical and immunologic phenotype.

Methods: Abelson virus–transformed *Rag2*$^{-/-}$ pro-B cells engineered to contain an inverted green fluorescent protein (GFP) cassette flanked by recombination signal sequences were transduced with retroviruses encoding either wild-type or 41 naturally occurring RAG2 variants. Bicistronic vectors were used to introduce compound heterozygous RAG2 variants. The percentage of GFP-expressing cells was evaluated by using flow cytometry, and high-throughput sequencing was used to analyze rearrangements at the endogenous immunoglobulin heavy chain (*Igh*) locus.

Results: The RAG2 variants showed a wide range of recombination activity. Mutations associated with severe combined immunodeficiency and Omenn syndrome had significantly lower activity than those detected in patients with less severe clinical presentations. Four variants (P253R, F386L, N474S, and M502V) previously thought to be pathogenic were found to have wild-type levels of activity. Use of bicistronic vectors permitted us to assess more carefully the effect of compound heterozygous mutations, with good correlation between GFP expression and the number and diversity of *Igh* rearrangements.

Conclusions: Our data support genotype-phenotype correlation in the setting of RAG2 deficiency. The assay described can be used to define the possible disease-causing role of novel RAG2 variants and might help predict the severity of the clinical phenotype.

GRAPHICAL ABSTRACT



Keywords

Recombination-activating gene 2; VDJ recombination; severe combined immunodeficiency; Omenn syndrome; autoimmunity; genotype-phenotype correlation

Recombination-activating gene (RAG) 1 and RAG2 proteins initiate the VDJ recombination process, allowing generation of T and B lymphocytes with a largely diversified repertoire of antigen receptor specificities (reviewed by Notarangelo et al[1]). In particular, RAG1 and RAG2 form a heterotetrameric complex that binds to the recombination signal sequences flanking each of the variable (V), diversity (D) and joining (J) gene segments and introduces double-strand DNA breaks that are sealed by hairpins. On opening of the hairpin by ARTEMIS, proteins of the nonhomologous end-joining machinery complete the VDJ

recombination process by mediating joining of the coding ends and formation of signal joins.[1]

Mutations of the *RAG1* and *RAG2* genes in human subjects are associated with a broad range of clinical and immunologic phenotypes, including severe combined immunodeficiency (SCID) with absence of T and B lymphocytes (T^-B^- SCID),[2] Omenn syndrome (OS),[3] atypical severe combined immunodeficiency (AS),[4] combined immunodeficiency with granulomas and/or autoimmunity (CID-G/AI),[5] and other milder presentations.

We have reported previously on a flow cytometry–based assay in which Abelson virus (v-Abl)–transformed *Rag1*$^{-/-}$ pro-B cells engineered to contain an inverted green fluorescent protein (GFP) cassette flanked by recombination signal sequences (pMX-INV) were transduced with retroviruses encoding either a wild-type (WT) or a mutant human RAG1 protein.[6] In this assay, because the *Rag1*$^{-/-}$ pro-B cells express both RAG2 and the components of the nonhomologous end-joining machinery, the proportion of GFP^+ cells can be used to measure the recombinase activity of the introduced human RAG1 protein. The results obtained supported correlation between levels of recombination activity of the mutant RAG1 proteins and the severity of the clinical and immunologic phenotype observed in patients with RAG1 deficiency.[6]

Here, using a similar assay based on a v-Abl *Rag2*$^{-/-}$ pro–B-cell line containing a single pMX-INV integrated cassette, we report on the systematic analysis of recombination activity of 41 naturally occurring human RAG2 variant proteins (including compound heterozygous variants) identified in 58 patients with various clinical and immunologic phenotypes. Our results demonstrate that genotype-phenotype correlation also exists for patients with RAG2 deficiency. Therefore this assay might be useful in determining the pathogenicity of newly identified *RAG2* mutations.

METHODS

Patients

Deidentified clinical, molecular, and immunologic data of patients with *RAG2* mutations were collected both by means of literature search and through collaboration with referring clinicians. The study was approved by the Institutional Review Board at Boston Children's Hospital and at local institutions of the referring physicians and was conducted according to protocol 16-I-N139 approved by the National Institutes of Health Institutional Review Board.

Analysis of recombination activity

The v-Abl *Rag2*$^{-/-}$ Em-Bcl2 pro–B-cell line with a pMX-INVand human truncated hCD4 cassette was generated by transducing v-Abl *Rag2*$^{-/-}$ pro-B cells with a retrovirus containing the pMX–INV–internal ribosome entry site (IRES)–hCD4 plasmid, as previously described.[7,8] Cells with successful integration of the pMX-INV-IRES-hCD4 cassette express a truncated human CD4 surface protein, allowing for selection by using anti-hCD4 magnetic beads (MACS; Miltenyi Biotech, Bergisch Gladbach, Germany). The hCD4-enriched cells

were further transduced with a retrovirus containing either the pBMN-WT-hRAG2-IRES-hCD2 or pBMN-Mut-hRAG2-IRES-hCD2 plasmid expressing WT or mutant (Mut) human RAG2 (hRAG2) protein, respectively. In parallel, *Rag2*$^{-/-}$ v-Abl pro-B cells were transduced with a retrovirus containing pBMN-empty-IRES-hCD2 construct as a negative control.

To analyze the recombination activity of 2 distinct *RAG2* variants for which compound heterozygosity had been identified in patients, we developed bicistronic vectors (*RAG2* [allele 1]-T2A-*RAG2* [allele 2]-IRES-h*CD2*) using overlapping PCR (see the Methods section in this article's Online Repository at www.jacionline.org). Successfully transduced cells were enriched with anti-hCD2 magnetic beads (MACS; Miltenyi Biotech). As a negative control, v-Abl *Rag2*$^{-/-}$ Eμ-Bcl2 pMX-INV-IRES-hCD4 pro-B cells, hereafter referred to as *Rag2*$^{-/-}$ Abl pro-B cells, were transduced with a retrovirus containing a pBMN-empty-IRES-h*CD2* plasmid, allowing for expression of hCD2 but not hRAG2.

The *Rag2*$^{-/-}$ v-Abl pro-B cells transduced with the vectors indicated above were incubated with 3 μmol/L STI-571/imatinib (Novartis, Basel, Switzerland), an inhibitor of the Abl kinase, thereby maintaining cells in the G0-G1 phase of the cell cycle and allowing for RAG1 and RAG2 to initiate VDJ recombination more efficiently, whereas the presence of the *Bcl2* transgene prevents cell death. After 96 hours, cells were harvested and GFP expression was analyzed by using flow cytometry on gating on cells expressing both hCD4 and hCD2. The relative recombination activity of the individual RAG2 variants was calculated as a percentage of WT-hRAG2 activity.

High-throughput sequencing analysis of rearrangements at the endogenous immunoglobulin heavy chain locus

Analysis of immunoglobulin heavy chain (*Igh*) locus rearrangements in transduced *Rag2*$^{-/-}$ v-Abl pro-B cells was performed by Adaptive Biotechnologies (Seattle, Wash), as described previously.[9] Sequences were aligned to a reference genome, and *Igh VDJ* gene definitions were based on ImMuno-GeneTics system (see Table E1 in this article's Online Repository at www.jacionline.org).[10] These sequences are available in Table E1. Heat maps were generated to detail the frequency of individual *V-J* rearrangements within unique sequences of rearrangement products. The Shannon diversity index and Simpson index were calculated by using the PAST program, as described previously,[11] and according to the following formulas:

$$\text{Shannon H index} = -\sum_{i=1}^{R} p_i \ln p_i$$

$$\text{Simpson D index} = \sum_{i=1}^{R} p_i^2,$$

where R indicates total templates, i indicates unique rearrangements, p_i indicates the proportion of the total sequences belonging to the ith unique rearrangement, and D indicates dominance and unevenness.

In particular, the Simpson D index takes into account both richness and evenness. With this index, 0 represents infinite diversity, and 1 indicates no diversity.

Statistical analysis

Statistical analyses for 1-way ANOVA for nonparametric analysis (Kruskal-Wallis) and *post hoc* analysis of the unpaired, 1-tailed, nonparametric Mann-Whitney test were carried out with Prism 5 software (GraphPad, La Jolla, Calif).

RESULTS

Patients' clinical and molecular features

We compiled a list of 58 RAG2-deficient patients from published manuscripts and unpublished sources. These patients were divided into 4 different groups based on their clinical and immunologic presentation and according to the diagnostic criteria of the Primary Immunodeficiency Treatment Consortium.[12] In particular, 20 patients were given a diagnosis of T^-B^-SCID,[2,4,13–21] 28 patients were given a diagnosis of OS,[2,4,14–19,22–27] 7 patients were given a diagnosis of AS,[26,28] and 3 patients were given a diagnosis of CID-G/AI (Table I).[2,4,5,13–28]

Analysis of the distribution of *RAG2* mutations by disease phenotype revealed that some mutations were observed in multiple patients with the same phenotype, such as p.G35A, which was reported in 4 patients with AS, and the p.C478Y mutation, which was detected in 3 patients with SCID. Moreover, 6 other mutations (G95R, K127X, M285R, G451A, W453R, and E480X) were identified in 2 patients each, with the same clinical phenotype (Fig 1). Missense mutations at residues G35, T215, and R229 were identified in 24 patients who presented with either SCID or OS, indicating that these mutations are associated with a severe but somewhat variable phenotype. Furthermore, missense mutations at residues R73 and M459 were reported in patients with either OS or AS. By contrast, other mutations (F62L, T77N, and G451A) were reported only in patients with CID-G/AI (Fig 1).

Distribution and recombination activity of mutant RAG2 proteins

As a preliminary step to analyze the recombination activity of the RAG2 variants identified in the patients, we transduced murine *Rag2*$^{-/-}$ Abl pro-B cells with a vector encoding for either human or mouse WT RAG2 (WT-hRAG2 and WT-mRAG2) or with an empty vector and compared levels of GFP expression on treatment with STI-571 (Fig 2, A). As shown in Fig 2, B, similar levels of GFP expression were induced by hRAG2 and mRAG2, whereas no GFP expression was observed in cells transduced with an empty vector (Fig 2, B). Altogether, these data indicate that hWT-RAG2 can cooperate efficiently with mWT-RAG1 in mediating recombination.

Initially, *Rag2*$^{-/-}$ Abl pro-B cells were transduced with vectors encoding for various RAG2 mutant proteins (C478Y, A456T, W416L, and G451A) that had been identified in patients

with SCID, OS, OS, and CID-G/AI, respectively. As shown in Fig 2, B, no detectable levels of GFP expression were induced by the C478Y mutant protein, whereas the G451A mutant protein supported significant levels of GFP expression, although levels were lower than in the presence of hWT-RAG2. Finally, low but detectable levels of GFP expression were supported by the A456Tand W416L mutant proteins associated with OS (Fig 2, B).

Based on these data, we performed a comprehensive analysis of the recombinase activity of 41 naturally occurring *RAG2* mutations, including 31 for which no data of functional activity had been reported previously. Among the 41 mutations tested, 6 were nonsense mutations, and the remaining 35 were missense mutations. Both the nonsense and missense mutations were distributed broadly along the length of the hRAG2 protein (Fig 3, A, and Table II). A higher density of mutations was observed in the plant homology domain at the C-terminus of the molecule, where mutations affected 18% of the amino acids included in the region compared with 6% and 3% of the amino acids being targeted by mutations in the core and acidic regions of RAG2, respectively (Fig 3, B). Although the 6 nonsense mutations supported no or minimal levels of recombination (range, 0.1% to 2.9% of WT-hRAG2), variable levels of functional activity could be ascribed to the 35 missense mutations (mean, 25.2% [range, 0.2% to 109.1%] of WT-hRAG2; Fig 3, C). Overall, missense variants supported significantly greater recombination activity than nonsense variants ($P < .01$; Fig 3, C). Finally, no clear difference of functional activity was observed for mutations affecting the plant homology domain or other domains of the protein (Fig 3, D).

Interestingly, among the 41 RAG2 variants tested, 4 (P253R, F386L, N474S, and M502V) showed levels of recombination activity comparable with that associated with hWT-RAG2 (Table II).

For all of the RAG2 variants tested, we reported their minor allele frequency (MAF) in the Genome Aggregation Consortium (gnomAD) database,[29] which calculated the combined annotation-dependent depletion (CADD)–PHRED score[30] to predict deleteriousness and incorporated these data and the results of functional analysis of recombination activity in a scheme to assess pathogenicity according to guidelines of the American College of Medical Genetics and Genomics.[31] We reported in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) all the variants studied here (see Table E2 in this article's Online Repository at www.jacionline.org).

Correlation of the recombination activity of mutant RAG2 proteins with clinical phenotype

On excluding 3 patients whose RAG2 mutant proteins (P253R, N474S, and M502V) supported WT levels of recombination activity, we aimed to determine whether the mean recombination activity of the mutant alleles identified in the remaining 55 patients correlated with the severity of the clinical phenotype. As shown in Fig 4, mutations associated with SCID and OS allowed similar levels of recombination activity, which were significantly lower than those identified in patients with the AS and CID-G/AI phenotypes. Although only a few patients with CID-G/AI were included in this study, these data support the notion that genotype-phenotype correlation exists also for human RAG2 deficiency, as previously found for RAG1 deficiency.[6]

Analysis of recombination activity of compound heterozygous RAG2 variants

Among the 58 patients, 16 were compound heterozygous for 15 distinct combinations involving a total of 21 distinct RAG2 variants (Table I). In several of these cases, the 2 RAG2 alleles supported markedly different levels of recombination activity, making it difficult to predict the net effect of the compound heterozygous RAG2 variants on V(D)J recombination.

Furthermore, the RAG complex is expressed as a heterotetramer including 2 molecules each of RAG1 and RAG2, so that the simultaneous presence of 2 distinct RAG2 variants in the complex might have very different effects than 2 copies of each variant alone. To investigate this in greater detail, we generated bicistronic retroviral vectors expressing equimolar amounts of 2 distinct *RAG2* cDNA variants and did this for 5 cases of compound heterozygous variants that had been identified in patients P2, P9, P12, P44, and P45 included in this study. In parallel, a bicistronic vector expressing 2 copies of hWT-RAG2 was also generated. Analysis of the recombination activity supported by the bicistronic vectors produced results that were not easily predictable based on the activity of each individual variant (Fig 5, A). For example, P44 was compound heterozygous for 2 variants (T251I and R229Q) that supported 67.2% and 8.9% WT recombination activity, respectively. The bicistronic vector including both variants allowed levels of recombination activity (63%) that were very similar to those of the allele with higher residual function. By contrast, P9 was compound heterozygous for the G35A and E437K variants, which supported 22.1% and 0.9% recombination activity, respectively, whereas the bicistronic vector expressing both of these variants yielded levels of recombination activity (3.9%) that were similar to the less functional allele.

Next, we studied the richness and diversity of *V-D-J* rearrange ments introduced by the bicistronic vectors at the endogenous *Igh* locus in *Rag2*$^{-/-}$ v-Abl pro-B cells. As shown in Fig 5, B, there was a robust correlation between levels of recombination activity (as measured based on GFP expression) and the number of unique reads of *Igh* rearrangements obtained on introduction of bicistronic vectors expressing 2 copies of WTor mutant RAG2 protein. more detailed analysis of *V-J* rearrangements (Fig 5, C, and see Fig E1 in this article's Online Repository at www.jacionline.org) showed that introduction of 2 copies of hWT-RAG2 into Rag2$^{-/-}$ v-Abl pro-B cells generated a very polyclonal pattern of rearrangements, and similar results were obtained with 2 other bicistronic vectors that contained either the M502Vor T215I variants. By contrast, a more restricted pattern of *V-D-J* rearrangements was detected on transduction with the other 3 bicistronic vectors (G451A-M459L, R39G-R229Q, and G35A-E437K). Furthermore, the repertoire of *Igh* rearrangements induced by these 3 vectors revealed reduced diversity (as shown by lower Shannon index) and higher clonality (as indicated by increased Simpson index; Fig 5, D).

DISCUSSION

In this study we determined the recombination activity of naturally occurring RAG2 mutant proteins and demonstrated that mutations associated with SCID and OS have significantly lower residual activity than mutations detected in patients with less severe clinical presentations (AS and CID-G/AI). Overall, these data confirm that for human RAG2

deficiency, residual activities of the mutations correlate with the severity of the clinical presentation, as previously demonstrated for RAG1 deficiency.[6] Although numbers of both patients and mutations analyzed in our current study for RAG2 deficiency was lower than in our previous study of RAG1 deficiency (79 mutations identified in 63 patients with RAG1 deficiency vs 41 mutations identified in 55 patients with RAG2 deficiency), significant differences in average RAG2 recombination activity were observed in patients presenting with different clinical phenotypes. Specifically, mutations associated with CID-G/AI supported a recombination activity that was significantly greater than that in mutations associated with more severe phenotypes (AS, OS, and SCID). In the case of RAG2-mutated patients, the F62L, T77N, and G451A RAG2 mutations were only found in patients with CID-G/AI. In addition, recurrence of the same low-activity mutations (C41W, R229Q, R229W, and C478Y) in multiple patients presenting with either SCID or OS phenotype could be predictive of a severe clinical phenotype in newly diagnosed patients carrying these mutations.

We have also developed a method whereby use of bicistronic vectors allows us to test the recombination activity of compound heterozygous RAG2 mutations and have shown that this approach is particularly useful in cases in which the 2 mutant alleles exert very different levels of recombination activity. Recently, another method based on a multiple plasmid transfection assay has been published that allows simultaneous testing of RAG variants that have been reported as compound heterozygous changes in patients.[32] Compared with that method, ours has the advantage of equimolar ratios of the 2 *RAG2* cDNA variants introduced. In addition, it is flow cytometry based and thus can be scaled up easily to test the activity of multiple pairs of RAG2 variants in a single experiment and permits investigation of RAG activity in a more physiologic genomic context by analyzing rearrangements at the endogenous *Igh* locus.

Overall, use of the bicistronic vector is preferable when faced with compound heterozygous mutations because it allows us to study the net effect of the 2 mutations on the recombination activity of the heterotetrameric RAG complex and might lead to improved correlation with the clinical phenotype, as shown in the case of P9 in this study. However, even with this approach, correlation between *in vitro* recombination activity and *in vivo* clinical and immunologic phenotypes is not absolute. In particular, environmental factors, including exposure to microorganisms and use of drugs, have been shown to modulate the phenotype of RAG deficiency in both patients and mice.[33–36]

Nonetheless, functional testing of the recombination activity of newly identified occurring RAG2 variants is important to assess whether these are disease causing. In this study 4 variants (P253R, F386L, N474S, and M502V) were found to support WT levels of recombination activity. One of these variants (M502V) was detected in compound heterozygosity with the S160L variant in patient P12. When tested in combination in a bicistronic vector, the S160L and M502V variants supported robust levels of recombination activity (64.6% of WT).

To further assess possible pathogenicity of the M502V variant, we analyzed its CADD-PHRED score and evaluated its frequency in gnomAD. The latter represents a recent

evolution of the Exome Aggregation Consortium database, but compared with that database, it has removed subjects known to be affected by severe pediatric diseases and as such might serve as a useful reference set of allele frequency for severe disease studies. The M502V variant is reported in the gnomAD database with an MAF of 0.0019 (including the presence of 4 homozygous subjects) and has a low CADD-PHRED score of 10.54 (see Table E2). Altogether, these data indicate that the M502V variant is nonpathogenic.

Patient P39 with SCID and maternal T-cell engraftment was homozygous for the N474S variant, which also supported WT levels of recombination activity. Although this variant is reported in the gnomAD database with an MAF of 2.5e-5, its CADD-PHRED score is rather low (8.358). Furthermore, 2 previous studies analyzed in detail both the expression and function of this variant, demonstrating that it supports efficient V(D)J recombination.[37,38] Patient P44 was compound heterozygous for the T251I and R229Q variants. Although the latter is a well-known pathogenic variant and supports modest levels of recombination activity (8.9% of WT), the T215I variant was permissive for high levels of recombination (67.2%). The combination of the 2 was also permissive for robust recombination activity (63%) and generation of polyclonal rearrangements at the endogenous *Igh* locus when introduced into *Rag2*$^{-/-}$ v-Abl pro-B cells by using a bicistronic vector. Furthermore, although the T215I variant has a relatively high CADD-PHRED score of 21.4, it is reported in the gnomAD database with an MAF of 0.002984, although it was as high as 0.02527 in South Asians, including 11 homozygous subjects (10 of whom are from South Asia). Finally, patient P11 with OS was compound heterozygous for the P253R and K440N variants, with 95.4% and 26.7% activity, respectively. Although the P253R variant is not reported in public databases and is scored as possibly damaging by Polyphen-2,[39] the observation that it supports WT levels of recombination activity raises doubts about its possible pathogenicity. According to American College of Medical Genetics and Genomics criteria, both the T215I and the P253R variants are scored as variants of unknown significance, and additional *in vitro* data and *in vivo* observations are needed to resolve their pathogenicity.

In summary, we have performed a comprehensive analysis of the recombination activity of naturally occurring RAG2 variants. Our data support the notion that genotype-phenotype correlation exists for this disease, as previously demonstrated for RAG1 deficiency. However, this correlation is not absolute, and other genetic and environmental factors can contribute to determine the clinical phenotype. Notwithstanding this limitation, the flow cytometry–based assay to test the functional activity of RAG1 and RAG2 variants (with use of bicistronic vectors to assess the effects of compound heterozygous variants) might help assess the possible pathogenicity of newly identified RAG variants. This is particularly important because RAG deficiency has emerged as the second most common form of SCID that can be identified by using newborn screening.[40,41]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

AS:	Atypical severe combined immunodeficiency
CADD:	Combined annotation-dependent depletion
CID-G/AI:	Combined immunodeficiency with granulomas and/or autoimmunity
GFP:	Green fluorescent protein
gnomAD:	Genome Aggregation Database
IRES:	Internal ribosome entry site
MAF:	Minor allele frequency
OS:	Omenn syndrome
RAG:	Recombinase-activating gene
SCID:	Severe combined immunodeficiency
v-Abl:	Abelson virus
WT:	Wild-type

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Clinical implications: *In vitro* testing of recombination activity of naturally occurring RAG2 variants might help assess their pathogenicity and reveals correlation with the severity of the clinical phenotype.

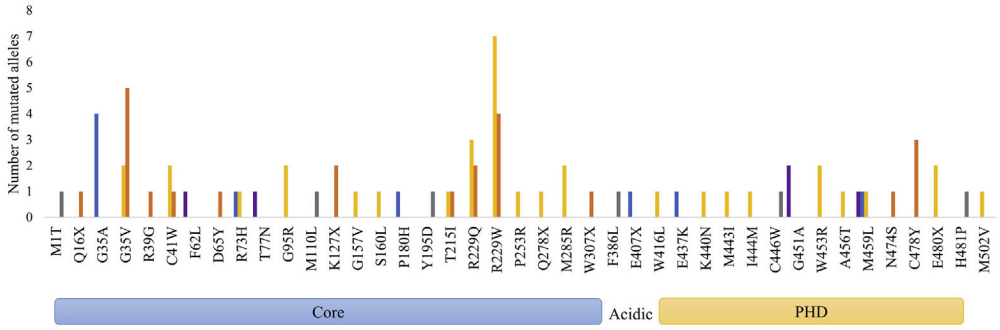

FIG 1.

Distribution of RAG2 mutations. Graphic presentation of different RAG2 mutations according to their position in the RAG2 protein and abundance of the patients according to various clinical presentations. For 5 RAG2 mutations (M1T, M110L, Y195D, C446W, and H481P), complete genetic and clinical information for the patients is not available. *PHD*, Plant homology domain.

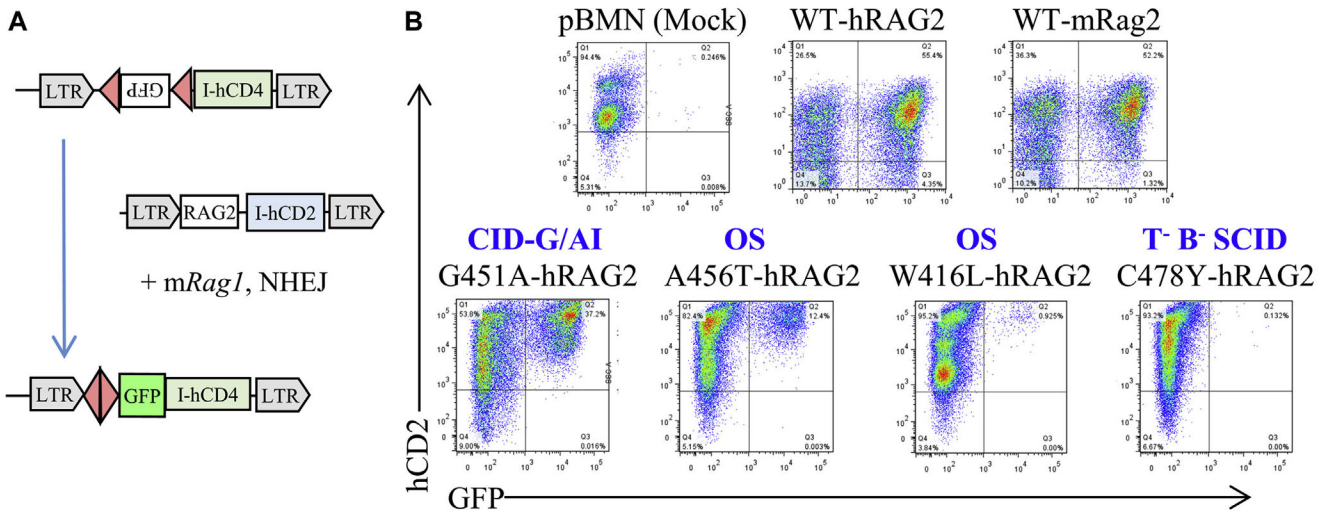

FIG 2.

Assay to measure recombination activity of human RAG2 variants. **A**, Schematic representation of the recombination assay. *Rag2*$^{-/-}$ v-Abl–transformed pro-B cells in which an inverted GFP cassette flanked by a recombination signal sequence (RSS) had been integrated stably in the genome (*top*) were transduced with a retroviral vector encoding either WT or mutant human RAG2 (hRAG2) and hCD2 as a reporter (*middle*). In this system GFP expression is measured by using fluorescence-activated cell sorting as a readout of successful recombination induced by the RAG2 protein in the presence of WT-mRag1 and the intact nonhomologous end-joining (*NHEJ*) pathway (*bottom*). LTR, Long terminal repeat. **B**, Representative fluorescence-activated cell-sorting dot plot of *Rag2*$^{-/-}$ v-Abl pro-B cells transduced with an empty vector (pBMN[mock]) or with retroviral vectors encoding either WT human RAG (WT-hRAG), WT mouse Rag2 (WT-mRAG2), or RAG2 mutants identified in patients with various clinical presentations ($T^{-}B^{-}$SCID, OS, and CID-G/AI).

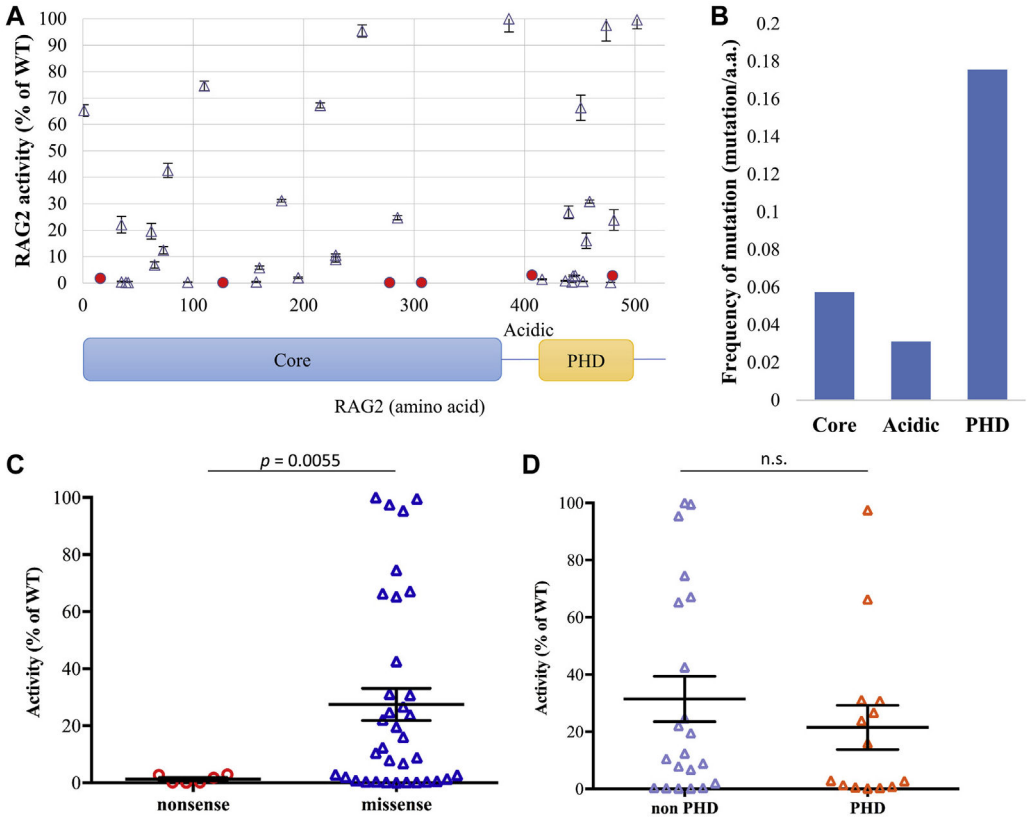

FIG 3.

Recombination activity of mutant RAG2 proteins. **A**, Graphic representation of the recombination activity of various RAG2 mutations according to their position in different domains of the RAG2 protein (core domain: amino acids 1–383; plant homology domain [*PHD*]: amino acids 414–487). Solid circles identify nonsense mutations, and triangles represent missense mutations. Means ± SEMs are shown to illustrate recombination activity. For each RAG2 variant, the assay was performed in triplicates. **B**, Frequency of pathogenic mutations (defined as recombination activity <80% WT-hRAG2) per amino acid length of various domains of the hRAG2 protein. **C**, Recombination activity (expressed as percentage of the activity of WT-hRAG2) of nonsense and missense hRAG2 mutations. *Bars* represent means ± SEMs. Statistical analysis was performed with the Mann-Whitney test. **D**, Recombination activity (expressed as percentage of the activity of WT-hRAG2) of missense mutations affecting the PHD and non-PHD domains of the hRAG2 protein. Bars represent means 6 SEMs. Statistical analysis was performed with the Mann-Whitney test. *n.s.*, Not significant.

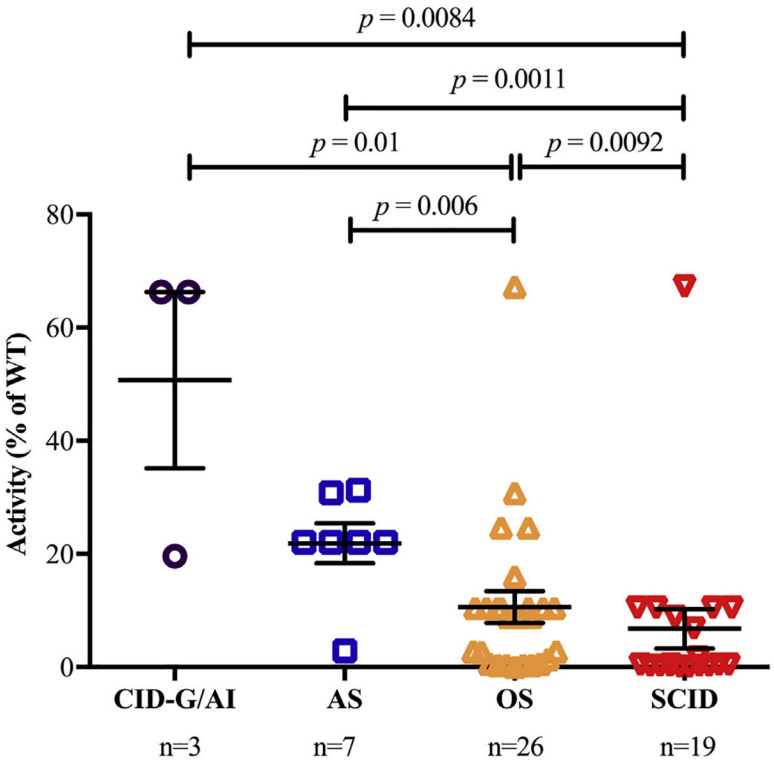

FIG 4.

Correlation between RAG2 recombination activity and severity of clinical presentation. Representation of recombination activity of mutant RAG2 alleles according to the clinical phenotype in 55 patients with RAG2 deficiency. Patients with variants resulting in values of 100% were omitted from this analysis. In the case of patients with compound heterozygous mutations, recombination activity corresponding to the allele with the higher activity is shown. Bars represent means ± SEMs.

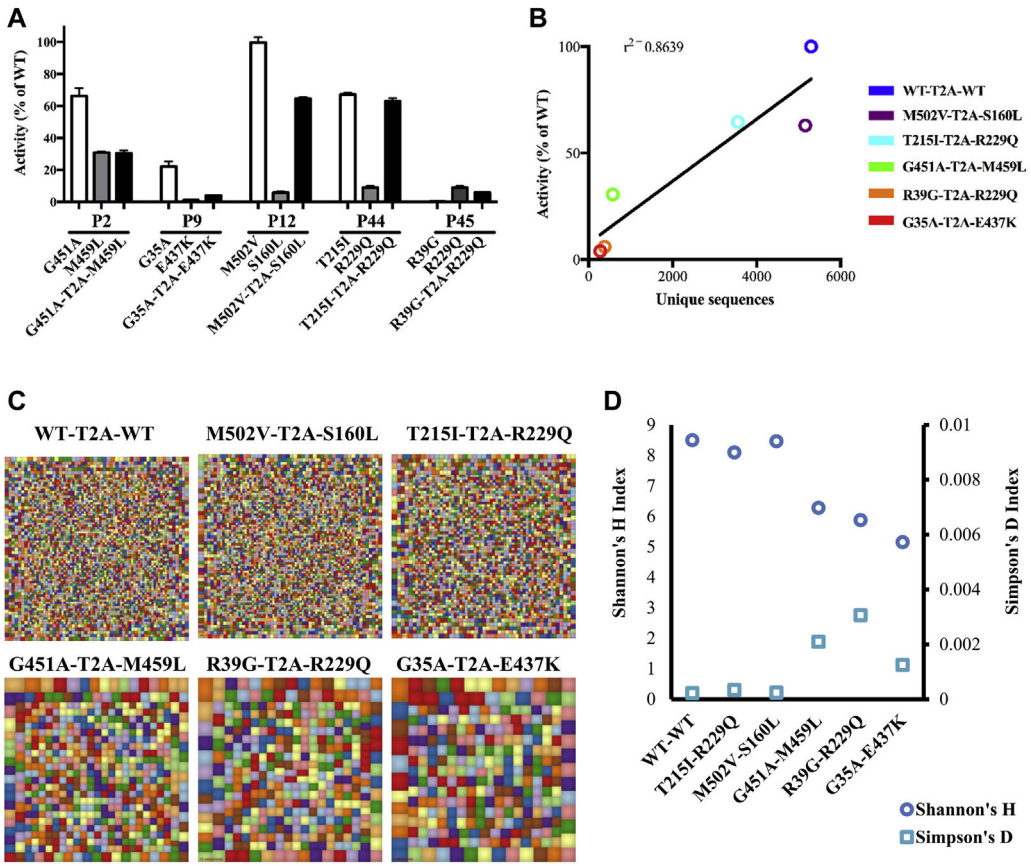

FIG 5.

Recombination activity of compound heterozygous RAG2 variants. **A**, Analysis of the recombination activity supported by bicistronic vectors simultaneously expressing 2 RAG2 variants and comparison with recombination activity of single variants. Experiments were done in triplicates. **B**, Pearson correlation analysis between recombination activity supported by bicistronic vectors versus number of unique sequences. **C**, Graphic representation of *Igh* repertoire diversity. **D**, Quantitative measurement of diversity and unevenness of the *Igh* repertoire.

TABLE I.

Clinical and genetic information of patients with RAG2 deficiency

Clinical phenotype	Autoimmunity/inflammation	Infections/malignancy	Patient no.	Mutation	Age	ALC (cells/μL)	CD3 (cells/μL)	CD19 (cells/μL)	CD16/56 (cells/μL)	References
CID-G/AI (n = 3)	Aplastic anemia; granulomas in spleen, lungs, and skin; ITP; neutropenia; splenomegaly	ARDS, disseminated and vaccine-associated varicella, meningitis, pneumonia, sinusitis, infections with *Cryptococcus* species, EBV, and RSV	P1	a. T77N; b. G451A	2 y	769–1,554	538–1,057	54–202	131–355	5
			P2	a. G451A; b. M459L	9 mo	480	138	12	86	28
			P3	a-b. F62L	5 y	687	391	78	215	28
AS (n = 7)	AIHA, alopecia areata, APS, biliary cirrhosis, granulomas in skin and bone marrow, hepatomegaly, IBD, ITP, neutropenia, splenomegaly, psoriasis, polyarthritis	Bronchiectasis, cholecystitis, chronic diarrhea, DIC, fungal nail infections, hyper-IgM syndrome, jaundice, *Klebsiella* species, meningitis, molluscum, oral thrush, otitis media, PJP, pneumonia, *Pseudomonas* species sepsis, rhinorrhea, skin rash-papules and abscesses, vaccine, associated varicella, RSV, CMV, and EBV viremia	P4	a-b. M459L	4 mo	NA	691	173	657	26
			P5	a. R73H; b. P180H	13 mo	1,479	401	524	654	
			P6	a-b. G35A	7 mo	1,027	716	105	209	28
			P7	a-b. G35A	10 mo		1,108	13	276	
			P8	a-b. G35A	12 mo	2,700	717	131	898	
			P9	a. G35A; b. E437K	5 mo	3,460	1,384	62	1,176	
			P10	a-b. E407X	18 y	3,480	2,750	0	661	28
OS (n = 28)	AIHA, eczema erythroderma, generalized edema, hepatomegaly, hepatosplenomegaly, lymphadenopathy, seborrhea-like dermatitis, splenomegaly	BCGitis, chronic diarrhea, CMV infection, failure to thrive, LAD, interstitial pneumonia, myocarditis, onychomycosis, recurrent chest infection and URTI, PJP, prolonged rotavirus infection, *Pseudomonas* pneumonia, *Staphylococcus aureus* skin infection, sepsis, severe infections	P11	a.K440N; b.P253R	7 mo	792	103	8	657	17
			P12	a. S160L; b. M502V	3 wk	9,064	7,250	0	1,360	17
			P13	a-b. T215I	NA	600	240	0	198	16
			P14	a-b. M459L	4 mo	869	149	4	279	26
			P15	a. C41W; b. M285R	1 wk	5,880	2,646	1	2,470	4
			P16	a.C41W; b.M285R	0 mo	660	554	<6	66	
			P17	a-b. A456T	0 mo	45,000	41,000	0	NA	23
			P18	a. Q278X; b. R73H	0 mo	8,339	7,071	0	NA	25
			P19	a-b. R229W	0 mo	280	162	17	53	4
			P20	a-b. R229W	4 mo		77	4	2	4
			P21	a-b. R229W	4 mo	NA	61	2	19	4
			P22	a-b. R229W	NA	287	46	6	184	16
			P23	a-b. R229W	NA	1,972	1,045	39	375	16

Clinical phenotype	Autoimmunity/inflammation	Infections/malignancy	Patient no.	Mutation	Age	ALC (cells/μL)	CD3 (cells/μL)	CD19 (cells/μL)	CD16/56 (cells/μL)	References
			P24	a-b. R229W	15 d	889	595	4	231	17
			P25	a. R229W; b.G95R	NA	1,953	1,074	78	19	16
			P26	a. G95R; b. W453R	<5 mo	10,000	5,200	204	NA	22
			P27	a-b. R229Q	2 wk	8,600	3,698	<86	3,956	4, 24
			P28	a.R39G; b.R229Q	2 mo	10,000	7,400	0	NA	14
			P29	a. R229Q; b. locus del	2.5 mo	322	113	16	161	2, 4
			P30	a-b. E480X	NA	11,000	2,778	0	3,740	19
			P31	a.G95R; b.E480X	3 mo	11,000	2,871	0	3,800	27
			P32	a-b. I444M	3 mo	620	415	<6	180	18
			P33	a-b. W416L	3 mo	34,000	31,647	0	1,347	17
			P34	a.-b. W453R	NA	NA	NA	NA	NA	15
			P35	a-b. G35V	NA	1,850	537	130	370	16
			P36	a-b. M443I	1 mo	5,700	2,354	11	NA	25
			P37	a-b. G157V	NA	5,600	4,592	56	504	19
			P38	a-b. G35V	4 mo	1,320	488	0	500	27
SCID (n = 20)	Eczema, erythroderma, lymphadenopathy, hepatosplenomegaly might be present in patients with maternal T-cell engraftment	BCGitis, diarrhea, failure to thrive, oral ulcers, oral thrush, oral candidiasis pneumonia, protracted diarrhea, respiratory distress	P39[†]	a-b. N474S	1 wk	1,120	22	1	784	4
			P40	a-b. R229W	NA	110	40	0	45	16
			P41	a-b. R229W	1 mo	1,076	126	1	869	
			P42	a-b. R229W	NA	NA	NA	NA	NA	21
			P43	a-b. R229W	NA	NA	NA	NA	NA	21
			P44	a-b.T215I & R229Q	12 d	684	5	7	382	20
			P45	a. R39G; b. R229Q	NA	NA	0	0	NA	13
			P46	a-b. D65Y	NA	400	16	8	228	19
			P47	a-b. Q16X	NA	NA	NA	NA	NA	15
			P48	a-b. G35V	NA	576	110	0	432	16
			P49	a-b. G35V	NA	290	9	0	159	16
			P50	a-b. G35V	NA	NA	0	0	NA	14
			P51	a-b. G35V	3 mo	NA	NA	NA	NA	17
			P52	a-b. R41W	6 mo	NA	5	1	804	
			P53	a-b. C478Y	NA	NA	0	0	NA	2
			P54	a-b. W307X	4 mo	720	<7	<7	533	4

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Clinical phenotype	Autoimmunity/inflammation	Infections/malignancy	Patient no.	Mutation	Age	ALC (cells/μL)	CD3 (cells/μL)	CD19 (cells/μL)	CD16/56 (cells/μL)	References
			P55	a-b. C478Y	0 mo	2,000	80	<20	820	4
			P56[†]	a-b. C478Y	2 wk	5,000	3,500[*]	<50	2,050	2, 4
			P57	a-b. K127X	5 mo	810	NA	<8	760	18
			P58	a-b. K127X	1 mo	978	<10	<10	918	18

Age, Age at presentation; *AIHA*, autoimmune hemolytic anemia; *ALC*, absolute lymphocyte count; *APS*, antiphospholipid syndrome; *ARDS*, acute respiratory distress syndrome; *BCGitis*, systemic dissemination of the attenuated *Mycobacterium bovis* bacillus of the tuberculosis vaccine; *CMV*, cytomegalovirus; *DIC*, disseminated intravascular coagulation; *IBD*, inflammatory bowel disease; *ITP*, immune thrombocytopenic purpura; *LAD*, lymphadenopathy; *NA*, not available; *N*, normal; *PJP*, *Pneumocystis jirovecii* pneumonia; *RSV*, respiratory syncytial virus; *URTI*, urinary tract infection.

[*] Maternal.

[†] Maternal engraftment.

TABLE II.

Summary of RAG2 mutations and recombination activity levels

No.	Amino acid position	Domain affected by the mutation	Mutation	Mean recombination activity (% of WT hRAG2)	SEM
1	1	Core	M1T	65.3	2.2
2	16	Core	Q16X	1.7	0.4
3	35	Core	G35A	22.1	3.1
4	35	Core	G35V	0.4	0.3
5	39	Core	R39G	0.2	0.1
6	41	Core	C41W	0.2	0.4
7	62	Core	F62L	19.6	3.0
8	65	Core	D65Y	6.8	1.2
9	73	Core	R73H	12.4	1.4
10	77	Core	T77N	42.6	2.7
11	95	Core	G95R	0.3	0.2
12	110	Core	M110L	74.6	1.8
13	127	Core	K127X	0.1	0.0
14	157	Core	G157V	0.4	0.2
15	160	Core	S160L	5.8	0.6
16	180	Core	P180H	31.1	0.5
17	195	Core	Y195D	2.0	0.3
18	215	Core	T215I	67.2	1.0
19	229	Core	R229Q	8.9	1.0
20	229	Core	R229W	10.5	0.5
21	253	Core	P253R	95.4	2.3
22	278	Core	Q278X	0.1	0.1
23	285	Core	M285R	24.7	0.8
24	307	Core	W307X	0.2	0.2
25	386	Acidic	F386L	109.1	5.0
26	407	Acidic	E407X	2.9	0.4
27	416	PHD	W416L	1.4	0.2
28	437	PHD	E437K	0.9	0.2
29	440	PHD	K440N	26.7	2.4
30	443	PHD	M443I	0.4	0.2
31	444	PHD	I444M	2.7	0.3
32	446	PHD	C446W	2.9	0.1
33	451	PHD	G451A	66.3	4.8
34	453	PHD	W453R	0.6	0.1
35	456	PHD	A456T	16.0	2.9
36	459	PHD	M459L	30.8	0.6
37	474	PHD	N474S	97.5	5.9
38	478	PHD	C478Y	0.2	0.1
39	480	PHD	E480X	2.8	0.6

No.	Amino acid position	Domain affected by the mutation	Mutation	Mean recombination activity (% of WT hRAG2)	SEM
40	481	PHD	H481P	23.8	3.9
41	502	—	M502V	99.6	3.4

The activity of each RAG2 variant was determined by at least 3 independent experiments from which the mean value was calculated.

PHD, Plant homology domain.