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Reduced immunoglobulin gene diversity in patients with Cornelia de Lange syndrome

To the Editor:

B cells rely on a broad receptor repertoire to provide protection against a wide range of pathogens. This is in part achieved through V(D)J recombination, which, by assembling various combinations of variable (V), diversity (D), and joining (J) genes, creates different IgV regions.[1] The recombination processes is initiated by recombination-activating gene (RAG) 1/RAG2 enzymes and requires a functional nonhomologous end-joining (NHEJ) machinery. B cells can further diversify their IgV regions through somatic hypermutation (SHM) to improve affinity between the antibody and antigen and switch the isotype of antibody produced by class-switch recombination (CSR). Both processes are initiated by activation-induced cytidine deaminase (AID) and rely on transcription and a number of DNA repair mechanisms.

Cornelia de Lange syndrome (CdLS) is a rare multisystem developmental disorder characterized by typical facial features, intellectual disability, and multiple congenital anomalies.[2] Most patients with CdLS have deleterious mutations in the gene encoding the cohesin loader Nipped-B-like (NIPBL), but mutations in the other cohesin-related genes *SMC1A*, *SMC3*, *PDS5B*, *RAD21*, or *HDAC8* have also been identified in selected patients. Cohesin has been implicated in regulation of sister chromatid cohesion, transcription, long-range gene interactions, and DNA repair.[3]

Aberrant CSR patterns have been observed in B cells from patients with *NIPBL* mutations.[4] Here we further investigated whether V(D)J recombination or SHM is affected in patients with CdLS. In chromosome-integrated V(D)J reporter assays, a reduced substrate recombination efficiency was observed in *NIPBL* knockdown human fibroblast or murine pro-B cells (see Fig E1 and the Methods section in this article's Online Repository at www.jacionline.org).

We next analyzed the *IGH* repertoire in 5 patients with *NIPBL* mutations and 3 patients with *SMC1A* mutations with CdLS (see Table E1 in this article's Online Repository at www.jacionline.org). The overall diversity of the *IGHV* repertoire, which is estimated by either the proportion of sequences with a unique complementary determining region 3 (CDR3) divided by the total number of sequences or by cumulative 50% of the total CDR3s in the samples (diversity 50 [D50]), which is a measurement of the evenness of the distribution of B-cell clones, was significantly lower in patients with *NIPBL* or *SMC1A* mutations (Fig 1, *A* and *B*). Thus the patients with CdLS had a reduced overall diversity of their *IGHV* regions, with an overrepresentation of large B-cell clones.

Furthermore, in patients with *NIPBL* mutations with the classical form of the disease and in patients with *SMC1A* mutations, the frequency of *IGHV* genes located in the most proximal one third of the *IGH* locus (about 250 kb) was increased (Fig 1, *C*, and see Fig E2 in this article's Online Repository at www.jacionline.org). The observed skewed pattern of *VH* genes in the patients is likely to be a result of B cell–intrinsic changes because only sequences resulting from unproductive rearrangement (successfully rearranged but out of reading frame or containing stop codons) were included in this analysis, and an influence of antigen selection can thus be excluded.

The overall mutation frequency in the unproductive and most commonly mutated *IGHV* genes was also reduced in the patients with CdLS, reaching statistical significance for the group with an *NIPBL* mutation (Fig 2, *A*). The proportion of unmutated sequences was increased in the patients with *NIPBL* mutations, especially in those with the classical form of the disease (Fig 2, *A*). However, the pattern of base pair substitution in the V regions was largely normal in the patients (Fig 2, *B*).

Our data suggest that the cohesion-associated proteins NIPBL and SMC1A are involved in regulation of V(D)J recombination in human B cells, possibly through regulation of locus contraction. Increased use of proximally located *IghV* genes has previously been observed in mice deficient in factors associated with locus contraction, such as Ikaros, Yin Yang 1, and Pax5, and the cohesin interaction partner CCCTC-binding factor has been suggested to regulate locus contraction and V(D)J recombination at the mouse *Igh* locus.[5-7]

Another possible explanation of how NIPBL/SMC1A could affect V(D)J recombination could be a change in regulation of DNA repair. We have previously observed a correlation between heterozygous *NIPBL* loss-of-function mutations and increased sensitivity to γ-radiation and a shift toward use of microhomology-based end-joining during CSR, suggesting that NIPBL regulates the NHEJ process.[4] However, the V(D)J coding junctions generated *in vivo* in patients with CdLS showed a normal repair pattern (see Table E2 in this article's Online Repository at www.jacionline.org). Furthermore, expression of a number of key V(D)J recombination factors was largely normal in *NIPBL* knockdown cells (see Fig E3, *B*, in this article's Online Repository at www.jacionline.org). Thus the core NHEJ machinery appears to be retained in patients with CdLS.

We also found a reduced frequency of mutations within the *IGHV* regions in patients with CdLS. NIPBL and SMC1A could both be involved in the SHM process through regulation of transcription or RNA polymerase pausing, which promotes formation of single-stranded DNA and AID targeting. In support of this notion, there was a reduced number of mutations observed in the RGYW motifs (containing the AID hotspot in the bottom strand) in $NIPBL^{+/-}$ and $SMC1A^{-}$ cells, suggesting an impaired mutagenesis of C residues on the bottom strand (Fig 2, *C*). We have previously observed the opposite pattern in NBS1-deficient cells.[8] Thus the targeting of AID and/or linked repair seem to be asymmetric during SHM, and this can be regulated by a number of factors, including the cohesin-associated factors.

CdLS is not traditionally considered an immunodeficiency disorder, but these patients have an increased prevalence of severe infections.[9] Immunodeficiency, including mild B-cell lymphopenia, reduced switched memory B-cell numbers, and/or reduced serum immunoglobulin levels, has been observed in previous case reports[9] and in our own patients (see Table E3 in this article's Online Repository at www.jacionline.org). Complete knockout of NIPBL is incompatible with survival, and

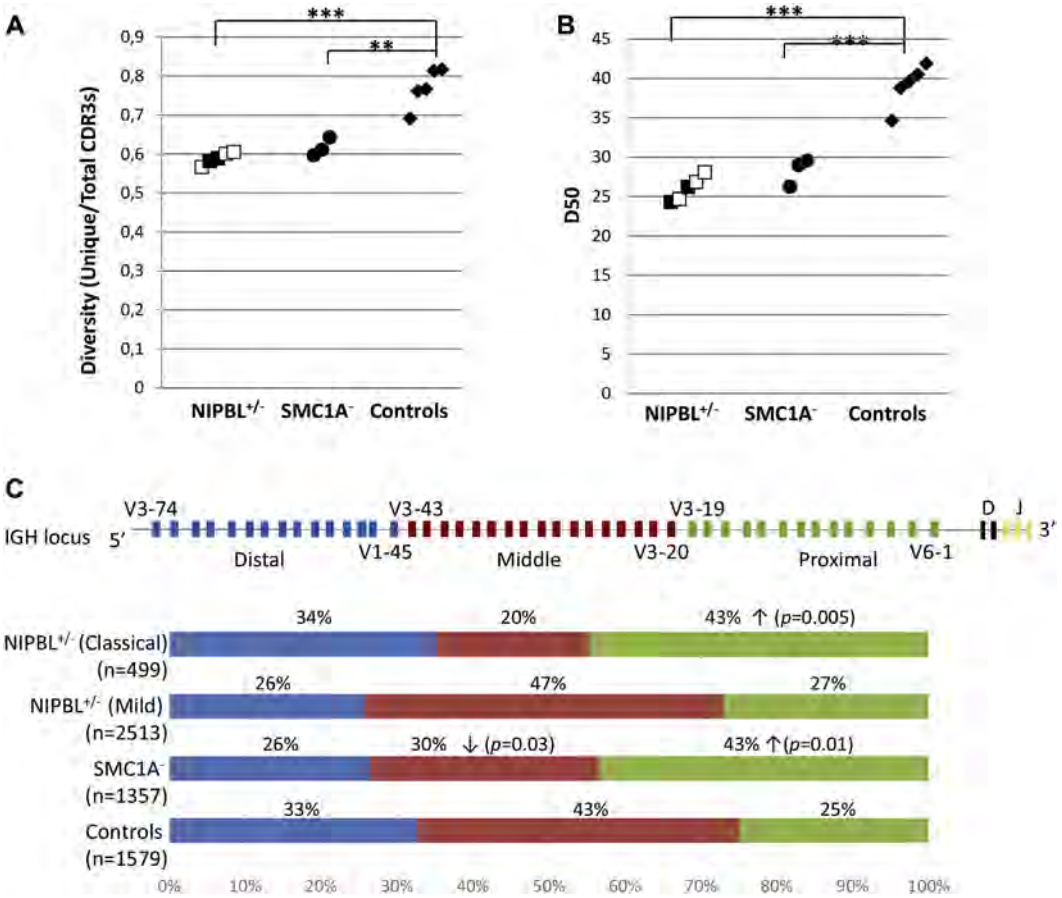

FIG 1. Reduced IGH diversities and increased use of proximal *IGHV* genes in patients with CdLS. **A** and **B,** Diversities (unique/total CDR3s; Fig 1, *A*) and the proportion of unique B-cell clones (Fig 1, *B*) that account for D50. Significant differences compared with control values: $^{**}P < .01$ and $^{***}P < .001$, Student *t* test. Each *dot* represents 1 subject. *Black* or *white squares* indicate patients with *NIPBL* mutations with the classical or mild form of disease, respectively. **C,** Average frequencies of proximal, middle, and distal *V* genes (in relation to *D* and *J* genes) in patients with CdLS. Schematic picture (not to scale) of the *IGH* locus is shown at *top*. Proximal, middle, or distal V gene groups each encompass approximately 250 kb of the *IGHV* locus and contain 15, 20, and 19 functional *V* genes, respectively. The number of sequences used in the analysis is indicated below each group. The proportion of proximal, middle, or distal *V* genes for each subject was regarded as 1 data point, and the patient and control groups were then compared by using the Student *t* test. Significant differences compared with control values are indicated by *arrows*. ↑ and ↓, Increased or decreased compared with control values, respectively.

heterozygous nonsense mutation or frameshift deletions in *NIPBL* are usually associated with a more severe clinical phenotype than those with missense mutations and in frame deletions (classical vs mild form), suggesting a dose-dependent role of the cohesin-associated proteins. The *SMC1A* mutations are missense or in-frame deletions and are generally associated with a milder CdLS phenotype. Thus mutations identified in patients with CdLS can be considered hypomorphic and/or result in haploinsufficiency, and a more significant alteration in immunoglobulin gene diversifications and a more severe immunodeficiency would be expected in the event of a complete loss of these cohesin-associated proteins.

In summary, the less diversified *VH* genes caused by reduced efficiency of V(D)J recombination together with inefficient CSR efficiency might contribute to the frequent infections in patients with CdLS.

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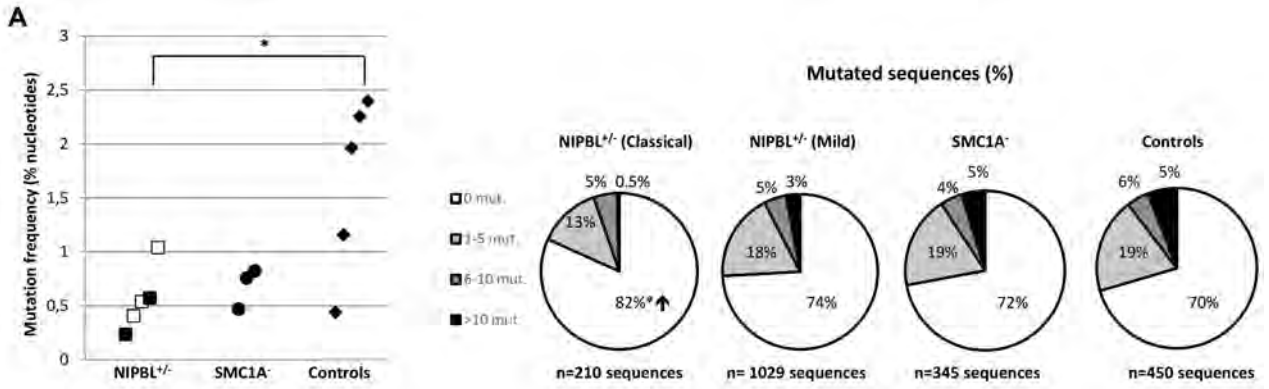

B

		NIPBL$^{+/-}$ (%) (1346 mutations)						SMC1A^{-} (%) (690 mutations)						Controls (%) (918) mutations)				
	To	A	C	G	T	Total		A	C	G	T	Total		A	C	G	T	Total
From	A	/	5.3	13.8	7.8	26.9	A	/	6.1	12.8	6.8	25.7	A	/	6.5	11.8	6.4	24.7
	C	4.2	/	7.3	12.8	24.3	C	3.8	/	7.5	11.6	22.9	C	4.1	/	6.5	13.9	24.6
	G	17.2	10.0	/	4.5	31.7	G	17.8	10.4	/	4.2	32.4	G	18.4	12.5	/	5.6	36.9
	T	5.2	7.0	4.9	/	17.1	T	5.2	9.0	4.8	/	19.0	T	2.9	8.3	2.8	/	13.9
	Total	26.6	22.3	26.0	25.1	100	Total	26.8	25.5	25.1	22.6	100	Total	25.5	27.3	21.1	25.9	100
	Mutations at G or C site					56.0	Mutations at G or C site					55.3	Mutations at G or C site					61.4
	Transitions					50.8	Transitions					51.2	Transitions					47.1

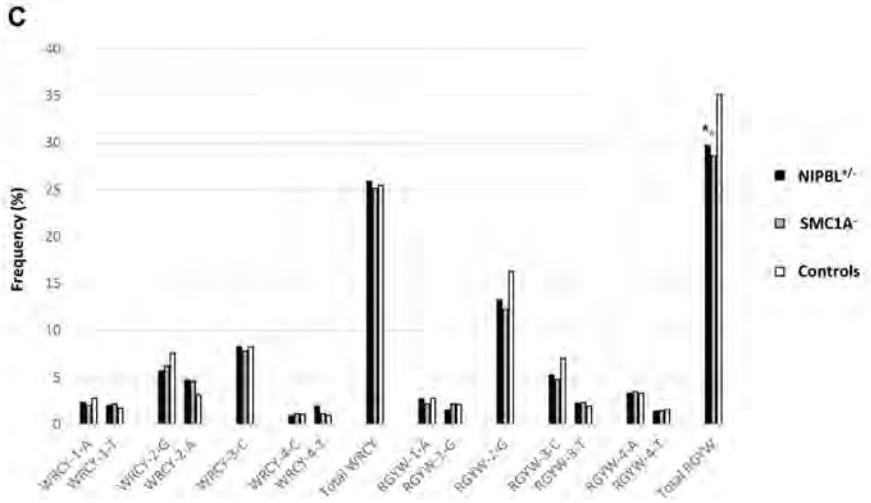

FIG 2. Mutation frequency and substitution pattern in *IGHV* regions from *NIPBL*$^{+/-}$ and *SMC1A*$^{-}$ B cells. **A,** Mutation frequencies are shown at *left*. Each *dot* represents 1 subject. *Black* or *white squares* indicate *NIPBL*$^{+/-}$ patients with a classical or mild form of disease, respectively. Percentages of sequences with 0, 1 to 5, 6 to 10, or more than 10 mutations in the IGHV regions from control, *NIPBL*$^{+/-}$, or *SMC1A*$^{-}$ cells are depicted in pie charts at *right*. *IGHV1-18*, *IGHV1-69*, *IGHV3-23*, *IGHV3-30*, *IGHV3-33*, and *IGHV4-34* sequences were included in the analysis. Significant differences compared with control values: $*P < .05$, Student *t* test. **B,** Frequencies of substitutions in the *IGHV* genes (χ^2 test, Bonferroni corrected). **C,** Frequency of mutations within W(A/T)R(G/A)CY(C/T)/RGYW motifs in the *IGHV* genes analyzed in Fig 2, *A*. *Asterisks* indicate significant differences between *NIPBL*$^{+/-}$ *(black asterisks)* and *SMC1A*$^{-}$ *(gray asterisks)* compared with control cells. $*P < .05$, χ^2 test, Bonferroni corrected.

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A new allergen family involved in pollen food-associated syndrome: Snakin/gibberellin-regulated proteins

To the Editor:

Around the Mediterranean basin, Cupressaceae pollen is considered as the primary cause of respiratory allergies with symptoms of rhinoconjunctivitis, chronic cough, and asthma.[1] The pollinosis can be severe including infectious complications, partly due to winter pathologies occurring within cypress pollinating period.[2] In Southern France, a pollen food-associated syndrome (PFAS) was described involving cypress pollen and peach and/or citrus sensitizations inducing mainly oral syndrome but also urticaria and angioedema. Up to now, the cross-reactive allergen at the basis of this syndrome has not yet been unraveled. Besides the 4 groups of allergens already described in the various Cupressaceae species, an as yet unidentified basic allergen of 14 kDa (BP14), overexpressed in *Cupressus sempervirens* pollen and different from a lipid transfer protein, was found to sensitize 37% of cypress pollen allergic patients (CPAPs) in Southern France.[3] The BP14 IgE epitopes are not related to cross-reactive carbohydrate determinants and are heat resistant but destroyed under reducing conditions.

To identify BP14, proteins extracted from *C sempervirens* pollen were run in SDS-PAGE and the corresponding band was submitted to mass spectrometry analysis (see this article's Methods section in the Online Repository at www.jacionline.org). Except a contaminant peptide of polygalacturonase, 2 overlapping peptides of the gibberellin-regulated protein (GRP) peamaclein (Pru p 7 from peach) were found: R.CLKYCGIC-CEK.C and K.YCGICCEK.C. Furthermore, 1 very similar peptide of the GRP of *Theobroma cacao* was identified: R.CLKYCGICCK.K (Fig 1, *A*). Using the Basic Local Alignment Search Tool algorithm, the identified peptides were evidenced in proteins of the snakin/GRP family from various plant species. One of them, the snakin-1 from potato, produced in recombinant form[4] and sharing 82.5% sequence identity with Pru p 7 and 79.4% with the GRP of *Theobroma cacao* (Fig 1, *B*), was tested in direct and inhibition western blots for its binding with serum IgE from CPAP. Out of 30 CPAP sera, all those expressing IgE to BP14 exhibited IgE reactivity to snakin-1 (n = 15) (Fig 2, *A* and *B*). No IgE binding was observed in 14 of the 15 sera tested from BP14-negative CPAPs. One serum (no. 31), tested negative for BP14, was positive for snakin-1. Available clinical data from patients are given in Table E1 in this article's Online Repository at www.jacionline.org. Similarly to BP14, the reduction of snakin-1 resulted in decreased apparent molecular mass and abolished IgE reactivity (Fig 2, *C*), pointing out the presence of conformational IgE epitopes, which is in agreement with the highly folded

A

PROTEIN							PEPTIDE		
Accession	Function	Species	MW [kDa]	pI	#Peptides	SC [%]	Sequence	Range	Score
Q9FY19	Polygalacturonase	*Juniperus ashei*	55.7	10.0	1	2.6	K.VDGTIAAYPDPAK.W	121 - 133	65.1
P86888	Peamaclein	*Prunus persica*	6.9	8.6	2	17.5	K.YCGICCEK.C	25 - 32	27.2
							R.CLKYCGICCEK.C	22 - 32	21.2
A0A061FGF7	Gibberellin-regulated protein	*Theobroma cacao*	11.4	10.1	1	9.3	R.CLKYCGICCK.K	47 - 56	27.1

B

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Peamaclein:---------- ---------- -----GSSFC DSKCGVRCSK AGYQERCLKY CGICCEKCHC 35
Snakin-1†: ---------- ---------- -----GSSFC DSKCKLRCSK AGLADRCLKY CGICCEECKC 35
                                  ***** **** **** **   ***** ****** * *
GRP Theo:  MKLILVTFLL VSLVLSSSFF EVSMAGSGFC DSKCKVRCSK AGAKDRCLKY CGICCKKCKC 60
                                  ***** **** ***** **   ***** ***** ** *

Peamaclein:VPSGTYGNKD ECPCYRDLKN SKGNPKCP-- -----  63
Snakin-1†: VPSGTYGNKH ECPCYRDKKN SKGKSKCP-- -----  63
           *********  ******* ** ***  ***
GRP Theo:  VPSGTYGNKQ ECPCYRDMKN SKGQLALAGL NCTQR  95
           *********  ******* ** ***
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FIG 1. A, Mass spectrometry analysis of in-gel–separated BP14 proteins from *Cupressus sempervirens* pollen extract. The gel was run under reducing condition. For detailed information, see this article's Methods section. **B,** Sequence alignment of peamaclein from *Prunus persica* (P86888), snakin-1 from *Solanum tuberosum*, and GRP from *Theobroma cacao* (GRP Theo, A0A061FGF7). The enlighted sequences correspond to the peptides identified in mass spectrometry analysis. *MW*, Molecular weight; *pI*, isoelectric point. SC (%) percent coverage. *Amino acid identity with peamaclein sequence. †Snakin-1 sequence without signal peptide as described in Kuddus et al.[4]