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Letter
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Heterozygous Variants in the DNA-binding Domain of c-Myb May Affect Normal B/T Cell Development

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Inborn errors of immunity (IEI) are serious immunological disorders characterized by aberrant development, proliferation, regulation, or function of immune cells.[1] The introduction of next-generation sequencing has facilitated the discovery of new causes of IEI.[2] Already >400 IEI-related genes have been identified and classified according to the affected component of the immune system.[1]

The MYB proto-oncogene (*MYB*) has previously been identified in both in vitro and in vivo models as a key player in transcription factor networks involved in stem cell and hematopoietic cell development but has not yet been related to IEI.[3] While homozygous null variants of *MYB* in mice have been shown to be lethal, heterozygous, temporal and local null models of the DNA-binding domain exon in mice and human cell lines have shown that its product, the c-Myb transcription factor, is crucial for pro- and pre-B cell differentiation by controlling the expression of interleukin-7 receptor-α, and recombinase activating gene (Rag) and the initiation of survival signals.[3,4] Moreover, c-Myb is vital for thymocyte development and regulates 2 distinct pathways in mature CD4+ and CD8+ cells; in CD4+ cells, it is involved in T helper 2 (Th2) development through GATA binding protein-3 (GATA3) regulation, while it regulates central memory stemness in CD8+ cells, through *Tcf7* and *Bcl2* upregulation and *Zeb2* repression (Figure 1A).[5,6]

Here, we describe 2 patients who presented with a combined immunodeficiency that progressed into severe bone marrow dysfunction with distinct de novo *MYB* heterozygous DNA-binding domain variants.

Participants provided written informed consent for institutional review board-approved studies in the Netherlands (National PID study, METC: NL40331.078) and Canada (Care4Rare).

Single nucleotide polymorphism (SNP)-array copy number variant (CNV) profiling and analysis of regions of homozygosity were performed on DNA isolated from peripheral blood according to standard procedures, using the Infinium Human CytoSNP-850K v1.0 BeadChip (Illumina) and the Nexus software v7 (BioDiscovery) with Human genome build February 2009 GRCh37/hg19.

Peripheral blood mononuclear cells were isolated from heparinized blood according to standardized protocols and stored in liquid nitrogen. After thawing, T cells were isolated with CD3+ magnetic beads and MS Columns (Miltenyi Biotec) and seeded in 24-wells plates at a 1×10^6/mL density and cultured for 0, 24, 72, and 120 hours with CD3/CD28 Dynabeads (Thermo Fisher Scientific).

RNA was isolated with the RNeasy Mini Kit (Qiagen) and complementary DNA was obtained using the RevertAid First Strand Kit (Thermo Fisher Scientific). Primers selected from earlier publications (Suppl. Table S1, Integrated DNA Technologies) were used for real-time polymerase chain reaction (PCR) using the SYBR Select Master Mix (Thermo Fisher Scientific).[7–9]

Antibodies used for flow cytometry are listed in Suppl. Table S2. For intracellular staining of c-Myb, Tcf7, and Zeb2, cells were fixed and permeabilized (eBioscience, 00–5524). BDFortessa II (BD Biosciences) was used for flow cytometry acquisition. Samples were analyzed with FlowJo software (TreeStar). Telomere length was measured by a 2-panel assay by flow fish technology at RepeatDx (https://repeatdx.com).

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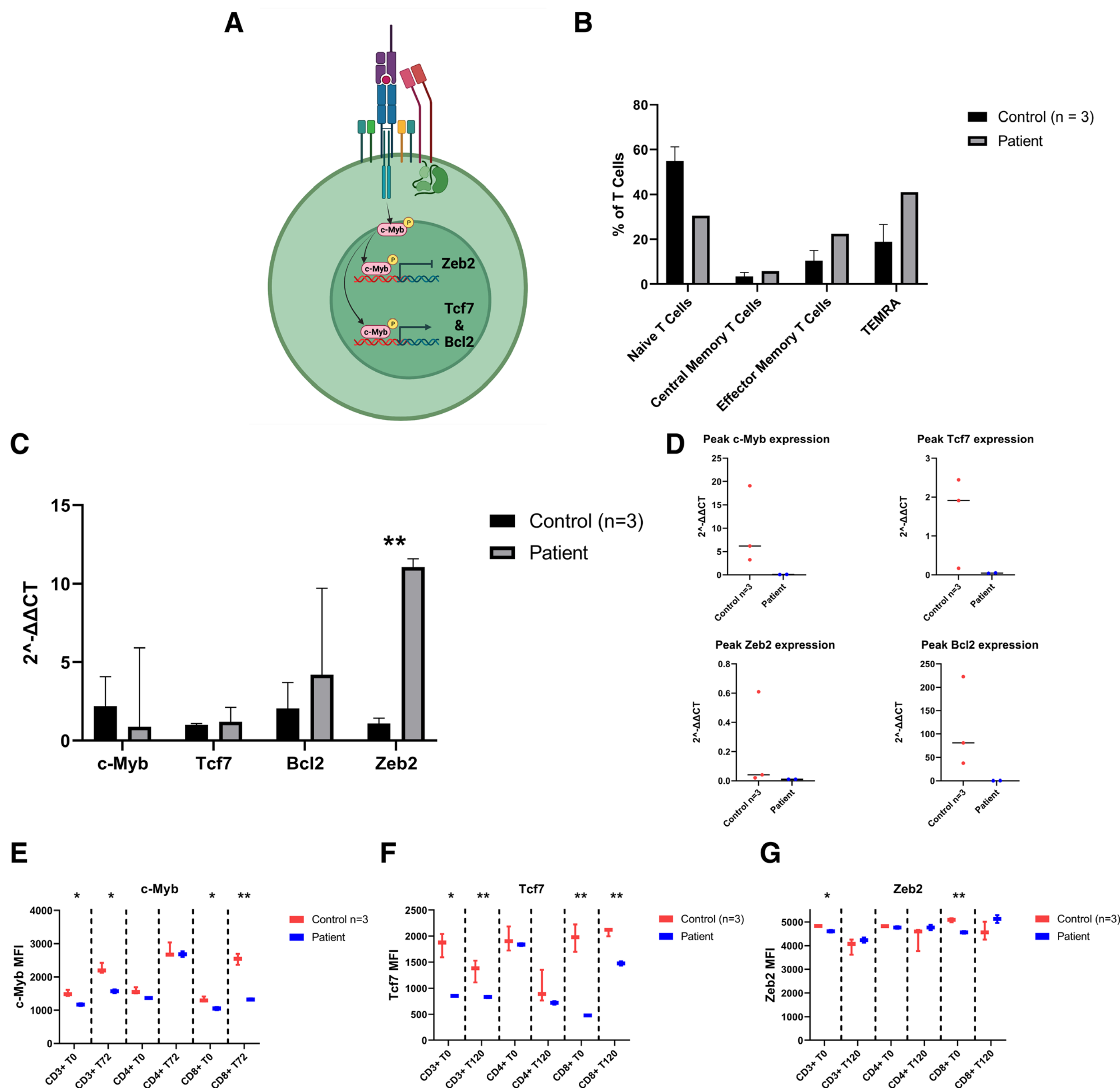

Figure 1. c-Myb and its downstream targets are differently expressed in 2 technical replicates of T cells carrying the p.Lys182Arg variant. Results were analyzed with 2-tailed Student *t* tests, *$P < 0.05$ **$P < 0.01$. (A), In activated mice CD8+ T cells, transcription factor c-Myb has been found to increase Tcf7 and Bcl2 expression, while repressing Zeb2 expression through competitive binding to the promoter.[5] (B), Proportions of CD3+ subsets in the patient vs 3 healthy controls showing that the patient has reduced naive CD3+ fractions and increased TEMRA fractions. (C), Expression of levels of c-Myb, Tcf7, Bcl2, and Zeb2 in unstimulated T cells. 2^–ΔΔCt values were obtained using the mean ΔCt values of the healthy controls. Zeb2 expression is higher in patient samples. (D), Peak c-Myb, Tcf7, and Bcl2 expression and Zeb2 repression in T cells after stimulation. 2^–ΔΔCt values were obtained using the mean ΔCt values of unstimulated T cells. Patient cells show a trend towards decreased c-Myb, Tcf7, and Bcl2 expression and similar Zeb2 expression. (E–G), MFI of c-Myb, Tcf7, and Zeb2 in CD3+, CD4+, and CD8+ cells. Peak protein expression of both c-Myb and Tcf7 was lower in CD3+ and CD8+ cells but not in CD4+ cells. MFI = mean fluorescence intensity; TEMRA = T effector memory cells expressing CD45RA.

Results were analyzed using the 2-tailed Student *t* test in GraphPad Prism v9.

A 22-year-old male of Dutch origin with a history of tetralogy of Fallot presented with recurrent lower tract infections at the age of 3 years. Laboratory test results showed reduced IgG and IgM and absent peripheral B cells. Fourteen B cell disorder-associated genes were analyzed and no disease-associated variants were identified. He was diagnosed with common variable immunodeficiency and treated with intravenous immunoglobulin replacement therapy (IgRT).

At the age of 9 years, he developed a slowly progressive thrombocytopenia and at the age of 19 years, bone marrow examination showed normal cellularity with dysplastic features in all lineages. Furthermore, he developed oral aphthous lesions and leukoplakia. Immunological investigations showed monocytopenia, neutropenia, reduced natural killer (NK) cells and low fractions of naive CD8+ T cells with impaired proliferation upon antigen stimulation and a reduced V-beta repertoire (Table 1).

Trio exome sequencing (ES) demonstrated a de novo heterozygous c.545A>G variant in *MYB* causing a p.(Lys182Arg) substitution within the DNA-binding domain of c-Myb. This variant had never been reported in genome aggregation database (gnomAD) and prediction software predicted the variant to be damaging. Hence the variant was classified as a variant of unknown

Table 1

Baseline Characteristics, CBC at Onset, Immunoglobulins at Onset, Immunophenotyping at Onset, and Functional Tests of Both MYB Cases

Parameter	Case 1	Case 2
Baseline characteristics		
Sex	Male	Male
Age at presentation	3 y	3 y
Current age	23 y	11 y
MYB variant	c.545A>G	c.383A>G
CID classification	$T^+B^-NK^-$	$T^+B^-NK^+$
CBC		
Hemoglobin (mmol/L)	8.1	8.3
Platelets (×10^9/L)	326	148
Leukocytes (×10^9/L)	4.6	2.1
Neutrophils (×10^9/L)	1.32	2.1
Lymphocytes (×10^9/L)	2.39	0.2
Igs		
IgM (g/L)	<0.04	0.25
IgA (g/L)	0.55	<0.0667
IgG (g/L)	5.19	0.34
Immunophenotyping		
CD3+ (×10^9/L)	2.3	0.726
CD3+CD4+ (×10^9/L)	1.2	0.440
CD3+CD8+ (×10^9/L)	1.1	0.165
CD19+ (×10^9/L)	0	0.011
CD56+ (×10^9/L)	0.03	0.319
Functional tests		
TREC	NA	Absent
v-beta repertoire	Reduced	Normal
T cell proliferation	Reduced for CD8+	Normal
Lymphocytic telomeres	<p2	NA

CBC = complete blood count; CID = combined Immunodeficiency; Ig = immunoglobulin; NA = not available; TREC = T-cell receptor excision circles.

significance and was submitted to the Matchmaker Exchange in order to identify other unrelated individuals with rare variants in the same gene and overlapping phenotypes.[10] Other pathogenic variants in known genes associated with primary immunodeficiencies were not found and SNP-array analysis showed a normal array profile without indications for pathogenic CNVs in 22q11.

T cell phenotyping showed decreased naive T cell fractions and increased TEMRA fractions in the patient compared with healthy controls and age-adjusted reference values (Figure 1B). Real-time reverse transcription PCR showed that c-Myb, Tcf7, and Bcl2 expression was comparable to that of healthy controls in unstimulated T cells, while Zeb2 expression was increased 11-fold (Figure 1C; $P < 0.01$). After 72 hours of CD3/CD28 stimulation, peak c-Myb and Bcl2 expression was reached, while Tcf7 expression and Zeb2 repression peaked after 120 hours (Suppl. Figure S1). There was a trend towards lower peak expression of c-Myb, Tcf7, and Bcl2 ($P = 0.19$, $P = 0.19$ and $P = 0.21$) in patient cells, while peak repression of Zeb2 was not significantly different ($P = 0.42$) (Figure 1D). Protein analyses using flow cytometry for c-Myb, Tcf7, and Zeb2 showed similar results (Suppl. Figure S2), and additionally indicated that expression of c-Myb and Tcf7 was especially hampered in the patient's CD8+ T cells ($P < 0.01$ and $P < 0.01$) (Figure 1E–G).

A telomere length assay demonstrated extremely short lymphocytic telomeres (<<p2).[11] Based on the combination of clinical features (immunodeficiency, bone marrow failure, leukoplakia, congenital heart defect), a nonclassical telomeropathy was suspected. Trio ES and Mitomycin C culture were unremarkable, aside from a shared heterozygous variant in *CTC1* ($c.3136_{del}$) between the father and the patient.

Due to significant thrombocytopenia, elective hematopoietic stem cell transplantation (HSCT) was considered for progressive bone marrow failure at age 21. However, since his thrombocytopenia was responsive to a course of dexamethasone and complete blood count was stable, HSCT has been postponed thus far.

An unrelated patient of French-Canadian origin presented with recurrent otitis media and invasive pneumococcal disease (sepsis, pneumonia, and empyema due to *Streptococcus pneumoniae*, serotype 19A) at the age of 3 years. Immunological workup revealed profound hypogammaglobulinemia with absent vaccine responses, including pneumococcal responses (14 serotypes tested). He had T cell lymphocytopenia and near absence of CD19 B cells. Moreover, T-cell receptor excision circles were undetectable with decreased recent thymic emigrant naive T cells. V-beta repertoire, as well as T cell proliferation studies (phytohaemagglutinin/muromonab CD3 [PHA/OKT3]), were normal. Limited targeted genetic testing including a severe combined immunodeficiency - B cell "negative" (SCID-B) negative panel and testing for *GATA2* deficiency was negative. He was treated with intravenous IgRT. He subsequently developed polyarthritis and rubella-positive necrotizing granulomatous dermatitis.[12] Bone marrow examination showed normocellular bone marrow with active trilinear hematopoiesis. Given his combined immunodeficiency syndrome, he underwent a successful matched unrelated HSCT at age 6.

Trio ES analysis detected a de novo heterozygous DNA-binding domain missense variant c.383A>G p.(Lys128Arg) of *MYB*. This variant was at a conserved nucleotide (genomic evolutionary rate profiling [GERP] 5.81) and also had not been previously reported in presumed healthy controls. In silico analysis programs predicted that the missense had damaging impact on protein function and/or structure (combined annotation dependent depletion [CADD] 29, sorting intolerant from tolerant [SIFT] 0, polymorphism phenotyping [PolyPhen] 0.996, variant effect scoring tool 3 [Vest3] 0.914).

Here, we report on 2 patients with heterozygous DNA-binding domain variants of c-Myb, presenting with combined immunodeficiency-like symptoms and bone marrow failure. Similar to in vivo models, these patients both showed severe B cell lymphocytopenia and hypogammaglobulinemia.

Moreover, like earlier research showing that deletions in c-Myb cause defects in CD8+ cell signaling, we here show similar defects in CD8+ cells carrying the p.(Lys182Arg) variant.[5] However, post-stimulation peak Zeb2 repression was not significantly reduced. Instead, Zeb2 messenger RNA expression was significantly higher in unstimulated p.(Lys182Arg) T cells, while protein expression was lower in the unstimulated condition. This could suggest that the p.(Lys182Arg) does not influence peak Zeb2 repression but does play a role in long-term Zeb2 repression, which is in turn counteracted by Zeb2 specific translational control. In non-small-lung cancer, specific translational control of Zeb2 has been shown through microRNA-342-3p binding.[13]

Previously, one other patient with a more extensive phenotype consisting of immunodeficiency, progressive bone marrow failure, short stature and dysmorphic facial features and a heterozygous 3.4 Mb deletion of chromosome 6, including *MYB*, has been described, further implicating the association between *MYB*, IEI, and bone marrow failure.[14]

Furthermore, deficiency of proteins that express MYB-like DNA-binding domains, telomeric repeat binding factor 1 and 2 (TERF1/2), have been implicated in telomere disease biology.[15] TERF1/2 protect telomeres from damage, inhibit the lengthening of telomeres and monitor the cell-aging process through inhibition of telomerase activity by binding to TTAGGG repeats.[15] Pathogenic variation in TERF1/2 has been shown to cause a nonclassical telomeropathy associated with severe aplastic anemia.[11]

DNA-binding domain variants might lead to potential polymorphic effects of c-Myb interactions with the binding sites of TERF1/2, triggering competition for the TTAGGG binding site, hampering telomeric TERF1/2 binding, leaving the telomeres unprotected.[15]

However, without in silico, in vitro and specific in vivo models, to prove the effect of heterozygous DNA-binding domain variants

of c-Myb, these putative mechanisms remain highly speculative and the criteria for novel IEI, outlined by the International Union of Immunological Societies (IUIS) consortium, have not yet been fulfilled.[1] It is imperative that in the future such models are developed, to further investigate the role of DNA-binding domain variants of c-MYB in IEI, and telomere dynamics.

In conclusion, heterozygous variants in the DNA-binding domain of c-Myb should be further investigated as a possible candidate for IEI, since they are potentially related to defects in B and T cell development, telomeropathy, and consecutive myelodysplasia.

AUTHOR CONTRIBUTIONS

TH, MB, KMB, KDK, DAD, EH, OJ, JvM, AvR-K, MdW, AP-H, and HLL provided patient care and their clinical insight on these cases. KMB, JCG, and LTvdV performed the analysis of whole exome sequencing (WES) data. SN performed the analysis of immunophenotyping data. BMS, ED, and SN designed the experiments & BMS and ED carried out the experiments. BMS drafted the article under supervision of AP-H, SN, and HLL. BMS, TH, ED, MB, KMB, KDK, JCG, EH, OJ, JvM, SN, AvR-K, LTvdV, MdW, AP-H, and HLL proofread the article and edited accordingly.

DISCLOSURES

The authors have no conflicts of interest to disclose.

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