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Novel Compound Heterozygous Mutations in ZAP70 Leading to a SCID Phenotype with Normal Downstream In vitro Signaling

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To the Editor:

ZAP70 is the zeta chain of the T cell receptor associated protein kinase 70 and is a member of the protein tyrosine kinase family. Upon engagement of the T cell receptor (TCR) by antigen presented on major histocompatibility (MHC) molecules, the Src family kinase, Lck, is activated and phosphorylates immune-receptor tyrosine based activation motifs (ITAMs) of the CD3ζ chain. Phosphorylated ITAMs promote the recruitment and activation of ZAP70. The ZAP70 SH2 domain interacts with phosphotyrosines of the CD3ζ chain leading to increased intracellular calcium, transcription of IL-2, and T cell activation [1].

In humans, mutations in *ZAP70* lead to abnormal thymus development presenting with CD8 lymphopenia but normal CD4 T cell numbers associated with defective T cell receptor (TCR) signaling. Severe combined immunodeficiency is the most common phenotype reported; however, immune dysregulation, autoimmunity, malignancy, and late onset combined immunodeficiency have all been described [2]. Recent reports suggest the amount of residual ZAP70 protein expression may modulate the clinical phenotype [3]. Importantly, newborn screening for SCID with enumeration of T cell rearrangement excision circles (TRECs) has been unreliable in its ability to detect ZAP70 SCID [4].

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We report a novel mutation in *ZAP70* leading to SCID phenotype. The patient was born vaginally at term to non-consanguineous parents. Her newborn screen was normal with TRECs of 325 copies/μL (normal > 52 copies/μL). At 2 months of age, she presented with cough and was diagnosed with respiratory syncytial virus (RSV) bronchiolitis by respiratory pathogen PCR panel (RPP) and briefly required oxygen by nasal cannula. At 4 months, she developed an erythematous, papular rash over her face, and extremities that was unresponsive to topical anti-fungal and steroid creams. Fungal scraping of the rash was negative. At 6 months, she presented with cough and respiratory distress. RPP was not performed. She was treated with amoxicillin followed by azithromycin and prednisolone for presumed pneumonia/asthma with minimal response. Of note, her rash resolved on prednisolone. She was hospitalized again at 7 months and diagnosed with a second episode of RSV bronchiolitis and required oxygen by high-flow nasal cannula. ALC was 10,800/mm^3 at that time. Her cough persisted and she had poor feeding after discharge and she received a course of amoxicillin clavulanate and an additional course of 2 mg/kg/day prednisolone. At 8 months, she developed worsening respiratory distress and was hospitalized with multi-focal pneumonia and failure to thrive (weight < 1 % for age). Shortly after admission, she developed respiratory failure requiring intubation. Admission CBC demonstrated Hb 12.3 g/dL, WBC 35,800/mm^3 (ANC 12,300/mm^3, ALC 20,900/mm^3), and platelets 675,000/mm^3. C-reactive protein was 0.4 mg/dL. RPP was negative. Urinalysis was normal, and blood cultures were negative. Further work-up included a normal echocardiogram, a chest CT demonstrating multifocal pneumonia, and bronchioloalveolar lavage confirming *Pneumocystis jiroveci* pneumonia (PJP). IV trimethoprim/sulfamethoxazole (TMP/SMX) and prednisone were initiated.

An immunodeficiency laboratory evaluation was pursued. HIV 4th generation testing was negative. Immunoglobulin levels were within normal limits for age; however, titers to tetanus, diphtheria, and *S. pneumoniae* were absent despite appropriate vaccination. Repeat CBC revealed WBC of 7000/mm^3 with ALC of 3530. Total CD4 T cells were normal for age (abs 2305/mm^3) as were CD19 B cells and CD56 NK cells. CD8 T cells were profoundly low for age (abs 11/mm^3) as were naive T cells (313/mm^3, 8.9%) (Fig. 1a). Subsequent CD3 proliferation studies revealed essentially absent responses to phytohemagglutinin (PHA), pokeweed mitogen (PWM), Candida, and tetanus toxoid. There was normal activation of CD4 T cells as assessed by CD69 expression upon stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin demonstrating intact response to mitogenic stimuli that bypass the TCR as previously described in ZAP70 deficiency (Fig. 1b) [1].

Repeat TREC screening was greater than 2 SD below the mean for the Massachusetts State Newborn Screening laboratory. High-throughput sequencing of the T cell receptor β (TRB) was performed. Consistent with previous reports, the CD4+, T regulatory, and T follicular helper cell repertoires had normal diversity and clonality but the CD8+ T cell repertoire had reduced diversity and increased clonality [5] (Fig. 1c). Invitae 18 gene SCID panel demonstrated 2 variants of unknown significance in the *ZAP70* gene. The first variant. Exon 3.c.109C>G(p.Arg37Gly), leads to a substitution of Arginine with Glycine and occurs at the N-SH2 domain affecting the phosphotyrosine binding and likely results in loss of function. This variant is not present in population databases and has not been previously reported in

the literature in individuals with ZAP70-related disease. SIFT, PolyPhen-2, and Align-GVGD all suggest this variant is likely to be disruptive. The second variant. Exon 12.c.1529_1532dupGCAT(p.lle511 Metfs*65), leads to a frameshift expected to truncate the last 109 amino acids of the protein, deleting part of the C-lobe of the catalytic domain. This variant is present in the gnomAD database at a very low frequency (minor allele frequency, $3.98e^{6}$) and has not been previously reported in individuals with ZAP70-related disease. Familial cosegregation was confirmed. Stimulation of the patient's cells with CD3/CD28 beads revealed normal phosphorylation of ZAP70 and SLP76 (down-stream substrate of ZAP70). Of note, the level of pSLP76 appears to fall off at the 10-min time point. We believe this is due to suboptimal transfer; however, there was inadequate patient sample available to repeat the blot (Fig. 1d). Whole-exome sequencing was performed and confirmed compound heterozygous *ZAP70* variants. No further candidate gene variants were identified.

The patient was treated successfully with TMP/SMX and prednisone for PJP pneumonia. She was prophylactically treated with IVIG, oral TMP/SMX, and fluconazole. At 12 months, she received an HLA-B mismatched unrelated donor bone marrow transplant after myeloablative conditioning with busulfan, fludarabine, and antithymocyte globulin. Her day 365 labs revealed 100% donor chimerism in whole blood, CD3 cells and myeloid cells, CD3 2325/mm^3, CD4 1421/mm^3, CD8 700/mm^3, CD16/56303/mm^3, CD19 841/mm^3, naive CD4 T cell 64.5%, and naive CD8 T cells 78.2%. Proliferation to PHA was 278,696 counts per minute (background 279 cpm). IgG of 741 g/L, IgA of 79 g/L, IgM of 11 g/L, and IgE of 7 g/L were all normal for age.

We report novel compound heterozygous mutations in *ZAP70* leading to a T+B+NK+ SCID phenotype. Normal phosphorylation of ZAP70 and SLP76 protein in vitro following T cell stimulation with CD3/CD28 beads was unexpected in our patient with a phenotype consistent with ZAP70 SCID. We hypothesize our patient's compound heterozygous mutations lead to a ZAP70 protein which is able to phosphorylate SLP76 under supra-physiologic conditions in vitro, but in vivo is below the functional threshold for TCR signaling competence. Our patient's clinical presentation with recurrent pneumonia including PJP, profound CD8 T cell lymphopenia, markedly abnormal response to mitogens, and rescue of T cell activation when stimulated with PMA and ionomycin are consistent with previous reports of ZAP70 deficiency. Although ZAP70 expression by flow cytometry was not assessed in our patient prior to transplantation, the presence of phosphorylated ZAP70 by Western blot confirms the presence of ZAP70 protein. A previous publication by Cauwe et al. shows multiple hypomorphic ZAP70 alleles together can generate unexpected phenotypes including immune dysregulation and autoimmunity. Although our patient presented phenotypically as a classic ZAP70 SCID, our case supports the suggestion by Cauwe et al. that a ZAP70 protein level exists at which signaling capacity reverts from intact to functionally absent [3]. To our knowledge, this patient is the second case of ZAP70 deficiency born in Massachusetts since implementation of SCID newborn screen in Massachusetts in 2009. As expected, both patients had normal TRECs at birth underscoring that ZAP70 deficiency is often not detected on newborn screening for SCID and should be considered in the differential diagnosis for an infant presenting with potential symptoms of a combined immune deficiency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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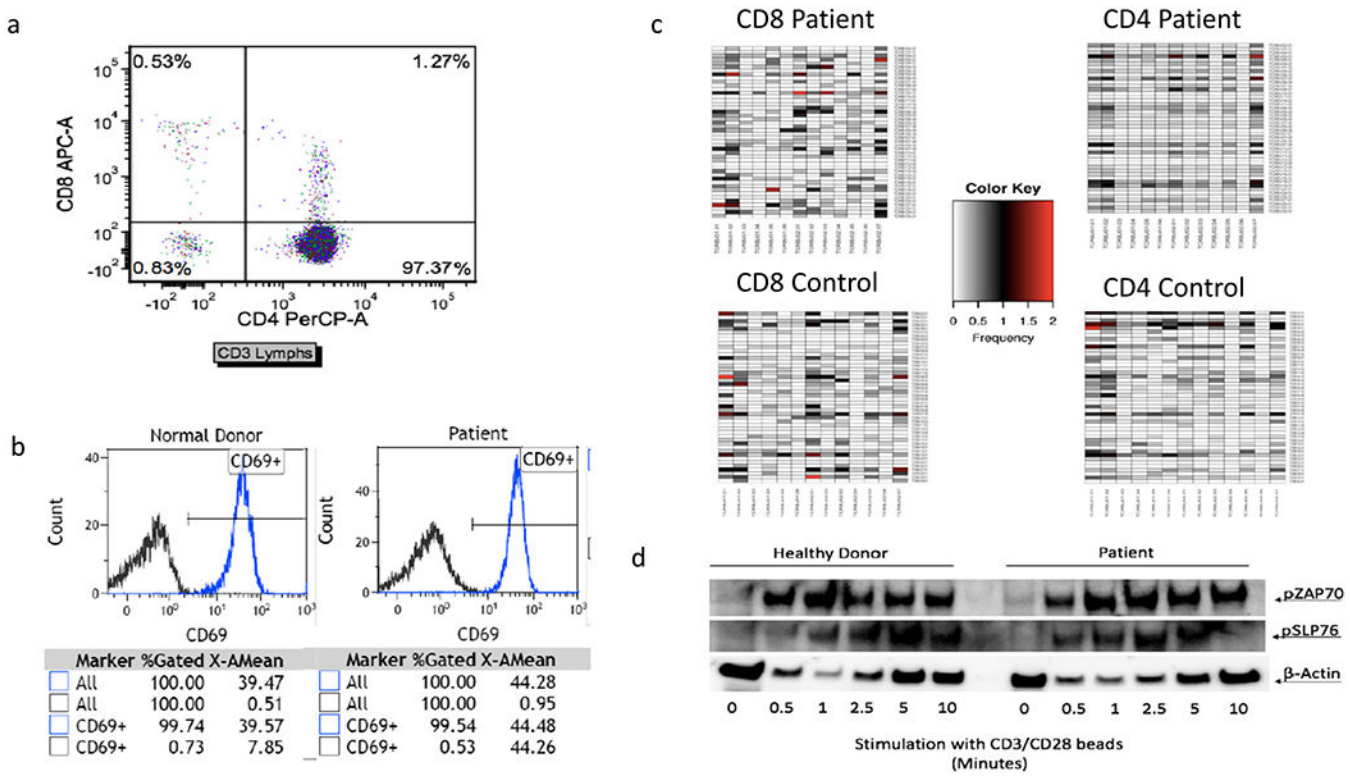

Fig. 1.

a CD4/CD8 flow cytometry of patient. **b** CD4 T cell activation after stimulation by PMA and ionomycin which was assessed as this is a mode of bypassing ZAP70 to activate T cells. **c** High-throughput sequencing analysis of the TRB repertoire in CD4 and CD8 T cell subsets; heat maps depicting Vβ and Jβ gene pairing demonstrating decreased TCR repertoire among the patient as expected in ZAP70. **d** Western blot of phospho-ZAP70 and phospho-SLP76 after stimulation with CD3 and CD28 beads. SLP76 is downstream of ZAP70 and is typically diminished in patients with ZAP70