Received: 5 March 2021 | Revised: 10 May 2021 | Accepted: 11 May 2021

DOI: 10.1111/cei.13625

British Society for immunology | Clinical & Experimental Immunology
The Journal of Translational Immunology

ORIGINAL ARTICLE

Novel STAT-3 gain-of-function variant with hypogammaglobulinemia and recurrent infection phenotype

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Abstract

Signal transducer and activator of transcription 3 (STAT-3) gain-of-function (GOF) syndrome is an early-onset monogenic inborn error of immunity characterized by multi-organ autoimmune disorders, growth failure and lymphoproliferation. We describe that STAT-3 GOF syndrome may be presented with hypogammaglobulinemia and recurrent severe upper and lower respiratory tract infections. In addition, the patient had lymphoproliferation, short stature and interstitial lung disease. Chest computerized tomography examinations showed mild bronchiectasis with areas of non-fibrosing alveolar-interstitial disease and maldevelopment of bilateral first ribs. Using Sanger sequencing, we revealed a novel c.508G>C, p.D170H STAT-3 variant affecting the coiled coil domain of STAT-3. Functional studies confirmed that p.D170H was a GOF variant, as shown by increased phosphorylated STAT-3 (pSTAT-3) and STAT-3 transcriptional activity. Our observation suggests that STAT-3 GOF syndrome can manifest in early childhood with hypogammaglobulinemia and recurrent severe respiratory tract infections. We suggest that patients with lymphoproliferation, hypogammaglobulinemia and severe recurrent infections should be screened for STAT-3 variants, even if autoimmune manifestations are missing.

KEYWORDS

autoimmunity, gain-of-function, immune dysregulation, lymphoproliferative disease, short stature, STAT-3

INTRODUCTION

Autoimmune diseases are comprised of a heterogeneous group of disorders considered previously to have multifactorial etiology and complex genetic traits. In recent decades, a growing number of single gene autoimmune disorders have been identified and characterized [1–3]. These monogenic immune dysregulation diseases are often caused by mutations in genes involved in central or peripheral immunological tolerance induction, and typically

present with a very early onset and follow an accelerated course.

One of the best-characterized early-onset autoimmunity diseases is immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome caused by regulatory T cell (T_{reg}) defects [4]. Patients with autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED), also referred to as autoimmune polyendocrine syndrome type 1 (APS-1), have a mutation in the autoimmune regulator gene, a major regulator of central T cell tolerance [5]. Another prototype of immune dysregulation diseases is autoimmune lymphoproliferative syndrome, which results from a heterogeneous group of mutations in the genes that regulate apoptosis [3]. There is a separate group of immune dysregulation syndromes, such as interleukin (IL)-10 deficiency or IL-10R deficiency with predominant features of colitis [6,7].

Recently, a novel monogenic defect of autoimmunity associated with severe growth failure and early-onset multi-organ dysfunction (OMIM 615952) was described, which was caused by germline gain-of-function (GOF) variants in signal transducer and activator of transcription 3 (STAT-3) [8–10]. The specific target organs involved can differ even between patients with the same variant, as in other monogenic disorders of autoimmunity. The most frequent manifestations are autoimmune cytopenia, lymphoproliferation, enteropathy, interstitial lung disease, thyroiditis, type I diabetes, arthritis and growth failure, with a disease onset of usually below 3 years [11]. Organ system involvement appears sequentially (Figure 1). Very early-onset endocrinopathies and gastrointestinal diseases appear typically before the onset of lymphoproliferation, hematological manifestations and lung disease. Neonatal type I diabetes, atypical celiac disease and hypothyroidism usually occur during infancy and can, for a long time, be the only clinical manifestations. Type 1 diabetes may be diagnosed before 3 months of age, in contrast to polygenic type 1 diabetes. Enteropathy was frequently diagnosed as pseudoceliac disease with villous atrophy, but without the usual serological signature. Hematological disease is frequent with an onset after infancy, and most commonly includes lymphoproliferation and autoimmune cytopenia. Arthritis and pulmonary manifestations with a predominance of interstitial lung disease usually present in later childhood (Figure 1). Other autoimmune manifestations, including autoimmune hepatitis and alopecia areata, notably manifest later in life.

We describe here a novel STAT-3 GOF variant in a patient who presented with lymphoproliferation, severe hypogammaglobulinemia and recurrent respiratory tract infections.

METHODS

Genetic analysis

Genomic DNA from the patient and his mother was isolated with the Gen Elute Blood Genomic DNA kit (Sigma-Aldrich, St Louis, Missouri, USA). Mutations were analyzed by amplifying exons and flanking intronic

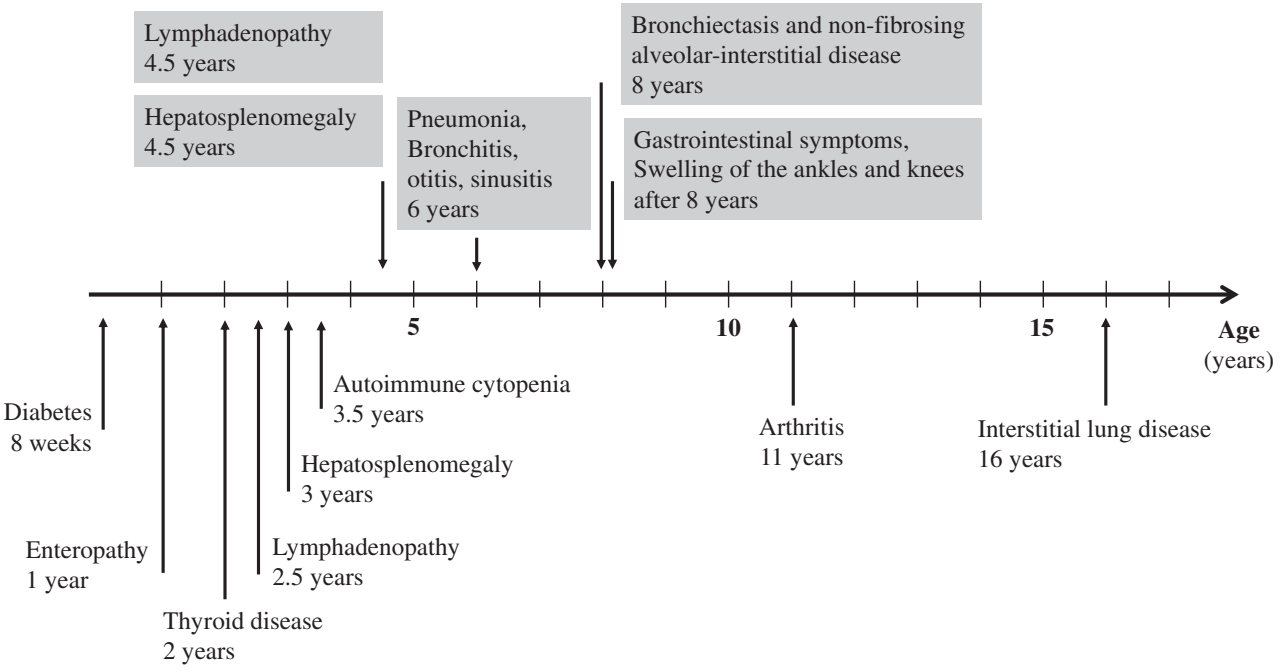

FIGURE 1 Sequence of clinical signs and symptoms of signal transducer and activator of transcription 3 (STAT-3) gain-of-function (GOF) as a function of age. The average age at onset of different manifestations in 42 previously described patients in 18 publications are shown below the bar [11]. Disease manifestations in our patient with the novel c.508G>C, p.D170H STAT-3 GOF variant are shown above the bar (shaded)

regions of STAT-3 by polymerase chain reaction (PCR). The PCR primers and sequencing primers are available on request. Amplicons were sequenced with the Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, California, USA) and targeted regions were analyzed by an ABI 3130 capillary sequencer (Applied Biosystems). Sequence variants were determined by comparison with the reference sequence, GenBank Accession no. ENSG00000168610 of the STAT-3 cDNA, to identify the position of mutations.

STAT-3 reporter assay

A luciferase assay was used to assess STAT-3 variants [12]. The pcDNA3 expression vector containing wild-type (WT) or variant STAT-3, together with the STAT-3 luciferase reporter vector (Qiagen, Hilden, Germany), was transfected into A4 cells (STAT-3-deficient human colon cancer cells) using lipofectamine LTX reagent (Thermo Fisher Scientific, Waltham, Massachusetts), according to the manufacturer's protocol. At 24 h post-transfection, the cells were treated with 10 ng/ml FP6 (a recombinant fusion protein of IL-6R and IL-6), 100 IU/ml interferon (IFN)-α or 20 ng/ml IL-27 for 16 h. The luciferase reporter assay was performed using the Dual-Glo® luciferase assay system (Promega, Madison, Wisconsin, USA) in triplicate; the data are expressed as relative luciferase units (RLU). Three independent experiments were performed.

Western blot analysis

The transfected A4 cells were stimulated with 10 ng/ml of FP6 for 15 min and subjected to Western blotting [12]. Equal amounts of protein were separated on 10% sodium dodecyl sulphide-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to polyvinylidene fluoride (PVDF) membranes (Merck KGaA, Darmstadt, Germany). The membranes were blocked with 5% bovine serum albumin (BSA) (Sigma-Aldrich) or low-fat bovine milk and were then incubated with rabbit anti-human phosphorylated STAT-3 (pY705) antibody (Cell Signaling Technology, Danvers, Massachusetts, USA), mouse anti-human STAT-3 antibody (Cell Signaling Technology) or mouse anti-β-actin antibody (Sigma-Aldrich). Horseradish peroxidase (HRP)-conjugated goat anti-mouse and anti-rabbit antibodies (GE Healthcare, Chalfont St Giles, UK) were used as secondary antibodies. Antibody binding was detected using an enhanced chemiluminescence reagent (Thermo Fisher Scientific). Two independent experiments were performed.

RESULTS

Case history

The male patient was born at gestation week 35, birthweight 2350 g and 45 cm in length; both parents are healthy. He received all vaccines of the Hungarian mandatory vaccination program. At 7 months of age the parents recognized that he did not follow moving objects, and occasionally had trembling. Brain computerized tomography (CT) revealed bilateral subdural hygroma. He recovered spontaneously and neurosurgical intervention was not required. At 8 months of age he was hospitalized with acute enteritis; bacteriological and parasitological stool examinations gave negative results. Because of recurrent otitis and adenoid hypertrophy, adenotomy and tonsillectomy was performed at 16 months and 3 years of age, respectively. At 3 years of age he had a bicycle accident and broke his right tibia. Cervical and abdominal lymphadenomegaly and hepatosplenomegaly was first documented at 4.5 years of age and was considered to result from the recurrent infections he had suffered from (Figure 1). At the same age he had surgery for mobilization and fixation of testis because of right-sided retention. From 6 years of age he was hospitalized and treated several times with recurrent pneumonia, bronchitis, sinusitis, pharyngitis and otitis. He was diagnosed with hypogammaglobulinemia at the age of 6, when serum immunoglobulin (Ig) levels were first examined (IgG = 2.2 g/l, IgA = 0.17 g/l, IgM = 0.57 g/l). Hepatosplenomegaly and generalized lymphadenomegaly became more severe with age. Abdominal ultrasound and magnetic resonance (MR) examinations showed hepatosplenomegaly, paraaortic, mesenterial and inguinal lymphadenomegaly, signs of portal hypertension and dilatation of the vena portae and vena lienalis. Because of the generalized lymph node enlargement and hepatosplenomegaly, liver and lymph node biopsies were performed which showed very mild intrasinusoidal lymphoid infiltration in the liver and normal lymph node tissue.

At 9 years of age he was referred to our department for further diagnostic evaluation of primary immunodeficiency. Written informed consent was obtained from the parents. On physical examination mild tachypnoea, drum fingers, barrel-shaped chest, exercise intolerance, hepatomegaly, splenomegaly, generalized lymphadenomegaly and short stature (height percentile = 3; weight percentile = 3) were found. Serum Ig isotype levels and lymphocyte subpopulations were determined by standard immunological assays. Immunochemistry tests revealed severe hypogammaglobulinemia, decreased concentrations of pathogen-specific antibodies and normal levels of IgE (Table 1). The patient had normal total white blood cell counts and $CD4^+$ T lymphopenia (Table 1). The $CD3^+CD4^-CD8^-$ double-negative T cell ratio was normal. Endocrine parameters showed normal levels of sensitive thyroid-stimulating hormone (sTSH) and

TABLE 1 Laboratory parameters at 9 years of age

		Patient	Normal range
Blood cell counts			
WCC		7.2	4.7–12.2 × 10^9/l
RCC		5.15	3.98–5.15 × 10^{12}/l
Hb		113.0	113.0–143.0 g/l
Hct		0.37	0.33–0.41
MCV		72	75.0–86.0 fl
Plt		416.0	187.0–415.0 × 10^9/l
Lymphocyte	%	28	22–55 %
	Absolute	2016	1700–4500 cells/μl
Neutrophil	%	61	37–70 %
	Absolute	4392	1800–7600 cells/μl
Lymphocyte subsets			
CD3	%	77.0	60.0–76.0 %
	Absolute	1552.32	120–2600 cells/μl
CD4	%	18.0	31.0–47.0 %
	Absolute	279.41	650–1500 cells/μl
CD8	%	55.0	18. –35.0 %
	Absolute	853.77	370–1100 cells/μl
CD19	%	17.0	13.0–27.0 %
	Absolute	342.72	270–860 cells/μl
CD16/56	%	9.0	4.0–17.0 %
	Absolute	181.44	100–480 cells/μl
Immunoglobulins			
IgG		< 0.73	5.4–15.1 g/l
IgA		0.05	0.52–3.25 g/l
IgM		0.14	0.52–1.5 g/l
IgE		< 4.4	0.0–200.0 kU/l
Complement			
C4		0.20	0.1–0.4 g/l
C3		1.26	0.9–1.8 g/l
CH50		48.3	38.0–69.0 CH50/ml
Specific antibody titers			
Tetanus toxin		0.07	0.1–99.9 IU/ml
Diphtheria toxin		0.06	0.1–99.99 IU/ml
Hemophilus influenzae B		0.08	0.15–99.99 μg/ml
Pneumococcus		0	9.2–225.9 μg/ml
Meningococcus A		1.58	2.0–999.0 μg/ml
Meningococcus C		0.48	2.0–999.0 μg/ml
Endocrine parameters			
sTSH		2.030	0.300–4.200 mU/l
fT4		16.5	12.0–22.0 pmol/l
IGF-1		78.4	168.0–557.0 μg/l

WCC = white cell count; RCC = red cell count; MCV = mean corpuscular volume; Ig = immunoglobulin; sTSH = sensitive thyroid-stimulating hormone; IGF = insulin-like growth factor 1.

fT4 and decreased insulin-like growth factor-1 (Table 1). X-ray showed porotic long bones with severely decreased skeletal age, but no abnormal findings were described in the knee, ankle and hip joints. The patient had normal levels of serum albumin and transferrin with normal or slightly decreased total protein level. An orosomucoid (α_1-acid glycoprotein) test was also made, which was negative. Swelling of the ankles and knees occurred usually at night and especially after long-term standing. The patient had increasing severity of pulmonary hypertension symptoms with age. He was 9 years of age when monthly intravenous Ig therapy (IVIG) was started. He was on 300 mg/kg/month IVIG substitution, but with poor adherence. Serum immunoglobulin levels were followed from the age of 6 until his death at 22 years of age. IgG trough levels remained very low (between 0.26 g/l and 1.8 g/l). The serum IgM levels were between 0.11 and 0.33 g/l. During the 16 years of follow-up the serum IgA levels remained under 0.17 g/l. Despite IVIG substitution, pneumonia recurred, although less frequently. Microbiological analyses of blood and sputum samples were performed ×3 during severe attacks of pneumonia but did not reveal possible pathogenic organisms, suggesting that the lung disease could have been, at least in part, inflammatory and due to the genetic disease.

At 13 years of age the skeletal age was that of a 7-year-old child. Inspiratory high-resolution computed tomography (HRCT) examination performed at 13 years of age with conventional sequential technique in the prone position (Figure 2 a,b) revealed a mosaic pattern with areas of hyperinflation intermixed with patchy ground glass opacities (GGO). There was some micro-reticulation within the GGO areas and incidental septal thickenings in the basal segments. Peribronchial thickening and mild cylindrical bronchiectasis was mainly seen in the hyperinflated areas. Neither honeycombing nor major architectural distortion were present. The overall impression was a mixture of airway disease with bronchiolitis and mild bronchiectasis with areas of non-fibrosing alveolar-interstitial disease. The latter did not follow a specific pattern, but features of desquamative interstitial pneumonia and lymphocytic interstitial pneumonia could be identified (Figure 2a,b). These morphological findings were in concert with interstitial lung disease characteristic of STAT-3 GOF. The patient did not receive systemic steroids or other immunomodulation, but he received inhaled corticosteroids and bronchodilators.

From 14 years of age the patient started smoking and he and his parents elected from medical follow-up. At the age of 17 he presented with tachypnea and dyspnea with lower limb edema. Cardiological examination revealed severe cor pulmonale and right heart failure. Abdominal ultrasound showed hepatosplenomegaly and portal hypertension. Despite the severe symptoms of cardiac decompensation,

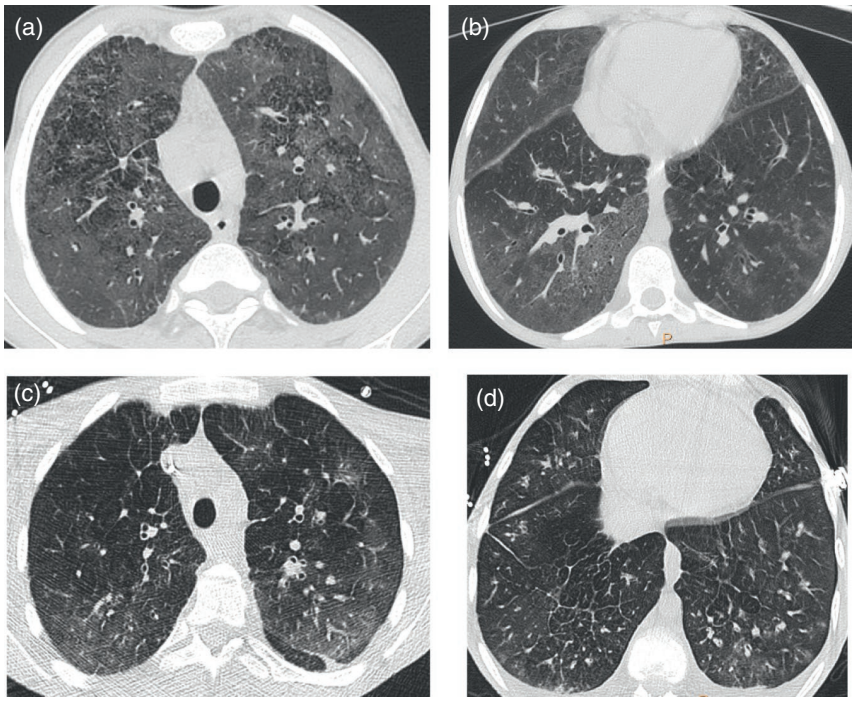

FIGURE 2 Images of sequential chest high-resolution computed tomography (HRCT) scans at the age of 13 and thin section helical chest CT scans obtained at the age of 21. Sequential chest HRCT scans obtained in prone position, at the age of 13 (a,b). Thin section helical chest CT scans obtained at the age of 21, following resuscitation are shown in (c) and (d). Inhomogeneous obstructive hyperinflation and nontraction bronchiectasis are present on both scans with a progressive tendency. Some alveolar interstitial component with patchy ground glass opacities (GGO-s) and septal thickenings are also observed but did not progress significantly in 8 years and there is no evidence of fibrosing disease

he refused therapy. The second CT scan was performed at the age of 21 years after resuscitation, for the exclusion of pulmonary embolism (PE; Figure 2c,d). There was no evidence of PE; only minor bilateral pleural effusion and mild mediastinal lymphadenopathy was seen. The lung's appearance was dominated by inhomogeneous obstructive hyperinflation and bronchiectasis with areas of GGO. Comparing the two HSCT examinations we can conclude that over the years there was a progressive tendency of inflammatory airway disease with small airway obstruction and non-traction bronchiectasis. Although some alveolar–interstitial involvement without a specific pattern was evident on both scans, significant progression was not proved and definitive fibrosis did not evolve. Maldevelopment of the bilateral first ribs was known from chest radiographs. This is clearly demonstrated in the volume of rendered images of the last CT scan (Figure 3). The anterior parts of both first ribs were missing with partial coalition of the proximal part with the second rib on left and pseudo-articulation with the second rib on the right. The patient died at the age of 22, due to progressive lung disease and heart failure.

Genetic findings

Sanger sequencing showed a novel heterozygous c.508G>C, p.D170H variant in STAT-3 affecting a highly conserved position among species. Genetic analysis of the mother by targeted Sanger sequencing revealed normal sequences. The father was not available for the analysis. *In-vitro* functional analysis was performed and STAT-3 reporter assay revealed that the novel D170H variant was GOF. Luciferase assay was performed using A4 cells transiently transfected with WT or variant STAT-3 together with a STAT-3 reporter vector (Figure 4a). E415K known GOF variant and R382W known loss-of-function (LOF) variant was used as disease control. Under unstimulated conditions, p.D170H variant showed a significant increase in reporter activity compared with the WT stimulated cells ($P < 0.05$), suggesting that this variant is GOF. Western blotting also confirmed increased phosphorylation of STAT-3 after FP6 (fusion protein of sIL-6R and IL-6) stimulation using A4 cells (Figure 4b).

DISCUSSION

In this patient with the novel c.508G>C, p.D170H STAT-3 variant, the earliest manifestation of STAT-3 GOF syndrome was lymphoproliferation with lymphadenopathy and hepatosplenomegaly at the age of 4.5 years (Figure 1). The patient had had severe and recurrent respiratory tract infections from the age of 6 years. He did not show any clinical signs of autoimmune endocrinopathy, confirming previous findings that STAT-3 GOF can present early with no or mild autoimmunity.[11] Even during his short adulthood, no laboratory or clinical signs consistent with diabetes or hypothyroidism could be detected.

After 8 years of age he presented with recurrent loose stools, nausea, abdominal bloating and pain. Although enteropathy was not fully evaluated, for example by measuring stool calprotectin or performing endoscopy/colonoscopy, it could be part of the clinical manifestation of STAT-3 GOF. However, in protein-losing enteropathies the loss of protein is non-selective affecting albumin, globulin and transferrin. The patient had normal levels of serum albumin and transferrin with normal or slightly decreased total protein level which, in

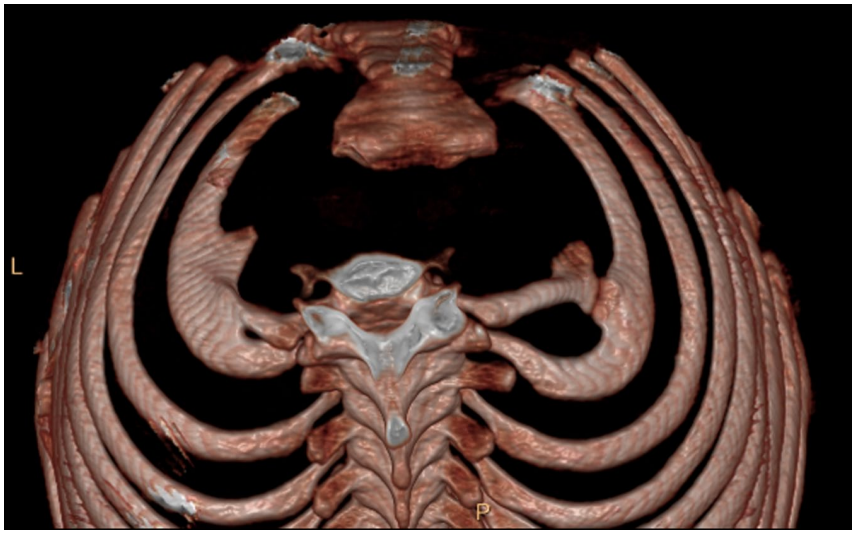

FIGURE 3 Volume rendered computerized tomography (CT) image of the thoracic inlet from a superior-posterior aspect. Volume rendered CT image of the thoracic inlet from a superior-posterior aspect shows partial absence of bilateral first ribs with a coalition with the second rib on the left and pseudoarticulation with the second rib on the right

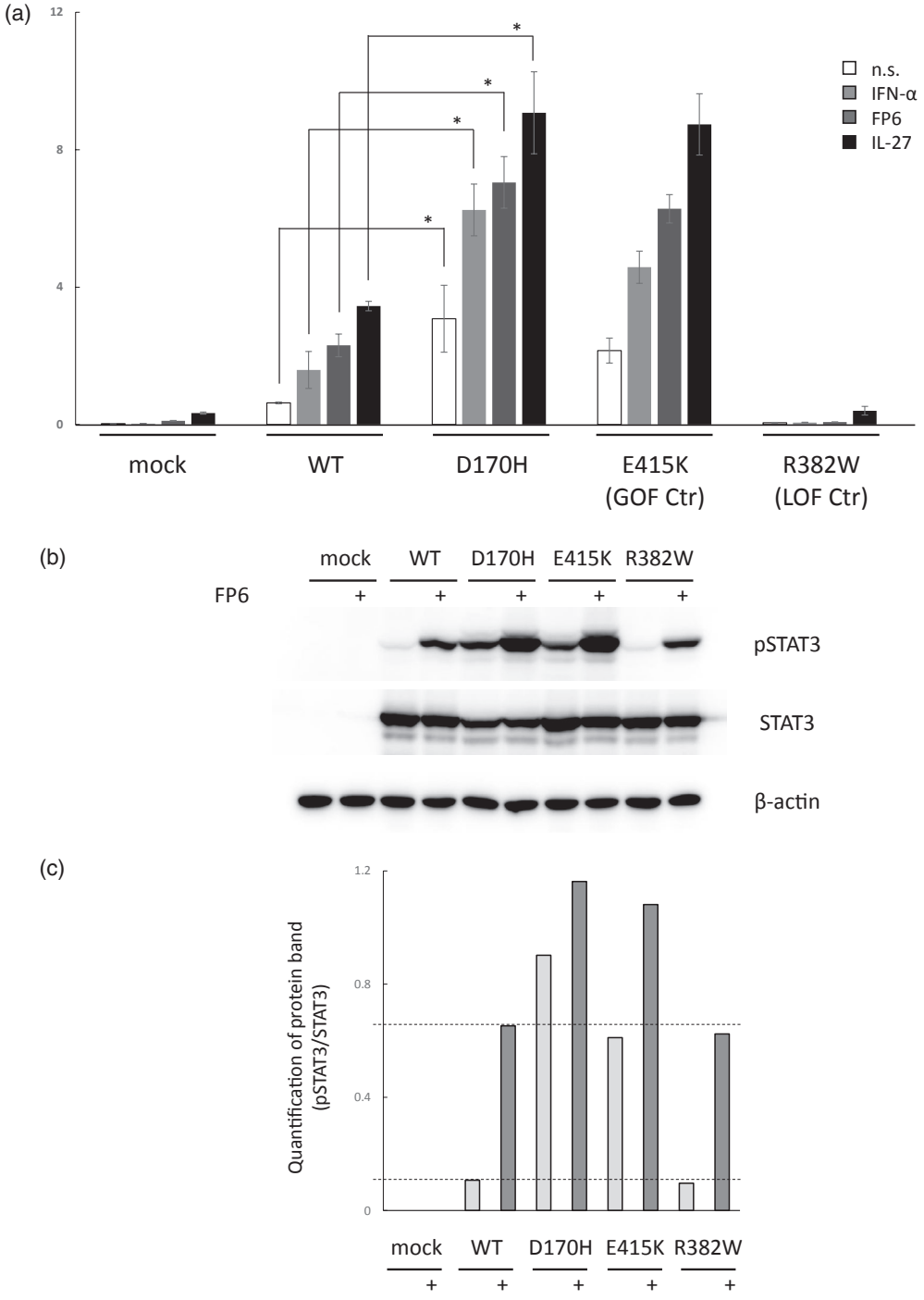

FIGURE 4 (a) Reporter assay of signal transducer and activator of transcription 3 (STAT-3). STAT-3 reporter activity was performed three times in the presence or absence of interferon (IFN)-α, fusion protein 6 (FP6) and interleukin (IL)-27. E415K and R382W are known gain-of-function (GOF) and loss-of-function (LOF) mutants, respectively. Like the E415K known GOF mutant, D170H showed increased STAT-3 activity (reporter plasmids: STAT-3 (Qiagen) IFN-α: 100 IU/ml, FP6: 10 ng/ml, IL-27: 20 ng/ml, 16 h). $^{*}P < 0.05$. (b) Western blot of STAT-3. Western blot analysis of transfected A4 cells was performed two times after stimulation with FP6. D170H showed increased phosphorylated STAT-3 upon FP6 (fusion protein of sIL-6R and IL-6) stimulation, like the E415K known GOF mutant. Increased pSTAT-3 was also found in a non-stimulated condition (FP6: 10 ng/ml, 15 min). (c) Density calculations. Quantification of protein bands was performed by Image J

addition to the negative orosomucoid (α_1-acid glycoprotein) test, was against severe enteral protein loss. From the age of 8 years swelling of the ankles and knees sometimes occurred, but usually at night and especially after long-term standing. X-ray and ultrasound examination of the ankle, knee and hip joint did not indicate arthritis. Taken together, the authors think that the lower extremity swelling was mainly due to the heart failure/lung disease. The patient had increasing severity

of pulmonary hypertension symptoms with age, which probably explains the lower extremity swelling.

The patient had recurrent and severe respiratory infections and severe hypogammaglobulinemia, which was more severe than in previously reported patients. Some of the patients seem to display hallmarks of B cell dysfunction, such as hypogammaglobulinemia and decreased switched memory B cells [9,10,11]. Hypogammaglobulinemia was reported in approximtely half of patients and usually not in a severe form [9,10]. It is still unclear if there are B cell intrinsic effects of STAT-3 overactivation or whether this is secondary to defects in other cells, such as T_{regs}. The patient did not have autoimmune cytopenia, which is atypical for STAT-3 GOF.

As a unique manifestation our patient had developmental bone abnormality, including missing anterior parts of both first ribs and partial coalition and pseudo-articulation of the proximal parts with the second rib. It is well known that STAT-3 is a critical mediator of bone growth and maintenance of bone structure. The loss of STAT-3 in mature osteoclasts has detrimental effects on bone structure, as STAT-3 affects bone formation and mineralization irrespective of sex [13]. The loss of STAT-3 causes a decrease in trabecular bone volume and may result in a severe osteoporosis phenotype [13,14].

All previously reported patients were heterozygous for the activating STAT-3 variant, and most of them had a *de-novo* variant. The novel c.508G>C, p.D170H STAT-3 GOF variant affects an amino acid of the coiled coil domain (CCD) of STAT-3. The CCD mediates the interaction of STAT-3 with cytokine receptors and is required for subsequent STAT-3 phosphorylation [15]. Germline GOF variants are found throughout the protein except the N-terminal domain, and several patients with STAT-3 GOF variant affecting the CCD of STAT-3 were reported [10,11,16]. STAT-3 GOF has also been found in association with common variable immunodeficiency and three of the four variants described lie within the CCD [17,18].

Our observation suggests that STAT-3 GOF syndrome can manifest in early childhood with lymphoproliferation, recurrent and severe respiratory infections and hypogammaglobulinemia. Moreover, we suggest that patients may lack early-onset endocrinopathies, autoimmune cytopenia or present only mild and/or delayed-onset autoimmunity. Although the patient had enteral symptoms and lower extremity swelling, we have not proved directly that enteropathy and arthritis were preferentially part of the STAT-3 GOF disease, even if it was consistent with the activating phenotype. Therefore, in the right clinical context, we propose that patients with lymphoproliferation, hypogammaglobulinemia and recurrent infections should be screened for STAT-3 variants.

ACKNOWLEDGEMENTS

This research was supported by grants from the Foundation for Children with Immunodeficiencies to M Erdős and L Maródi, and from the European Society for Immunodeficiencies to M Erdős. The authors thank the parents of the patients for helpful collaboration.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

M. Erdős, J. Kállai, Á. Lányi, M. Tsumura and Gy Balázs conducted the experiments. M. Erdős and Á. Lányi performed Sanger analysis. M. Erdős, Z. Nyul, L. Maródi and Gy Balázs performed the clinical investigation of the patient. M. Erdős and L. Maródi wrote the manuscript with the contribution of S. Okada.

DATA AVAILABILITY STATEMENT

Data and materials used in this study are available on request.

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How to cite this article: Erdős M, Tsumura M, Kállai J, Lányi Á, Nyul Z, Balázs G, et al. Novel STAT-3 gain-of-function variant with hypogammaglobulinemia and recurrent infection phenotype. *Clin Exp Immunol*. 2021;205:354–362. https://doi.org/10.1111/cei.13625