Novel STAT3 gain-of-function variant with hypogammaglobulinemia and recurrent infection phenotype

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Summary

Signal transducer and activator of transcription 3 (STAT3) 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 STAT3 GOF syndrome may be presented with hypogammaglobulinemia and recurrent severe upper and lower respiratory tract infections. The patient we present here did not have multi-organ autoimmunity and lacked early-onset autoimmune manifestations. Chest CT examinations showed mild bronchiectasis with areas of non-fibrosing alveolar-interstitial disease and maldevelopment of bilateral first ribs. By using Sanger sequencing, we revealed a novel c.508G>C, p.D170H STAT3 variant affecting the coiled coil domain (CCD) of STAT3. Functional studies confirmed that p.D170H was a GOF variant as showed by increased pSTAT3 and STAT3 transcriptional activity. Our observation suggests that STAT3 GOF syndrome can manifest in early childhood with hypogammaglobulinemia and recurrent severe respiratory tract infections and may lack autoimmune manifestations. We suggest that patients with hypogammaglobulinemia and severe, recurrent infections should be screened for STAT3 variants even if autoimmune manifestations are missing.

Keywords: STAT3, gain-of-function, immune dysregulation, autoimmunity, lympho-proliferative disease; short stature.

Introduction

Autoimmune diseases comprise of a heterogeneous group of disorders considered previously to have multifactorial etiology and complex genetic traits. In the last decade, a growing number of single gene autoimmune disorders have been identified and characterized (Boisson 2015, Rieux-Laucat 2014, Tangye 2020). These monogenic immune dysregulation diseases are often caused by mutations in genes involved in central or peripheral immunologic tolerance induction and typically present with a very-early onset and follow an accelerated course.

One of the best characterized early-onset autoimmunity disease is immune dysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome caused by regulatory T cell (Treg) defects (Bennett 2001). Patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), also referred to as autoimmune polyendocrine syndrome type 1 (APS-1) have mutation in autoimmune regulator (AIRE) gene, a major regulator of central T cell tolerance (Nagamine 1997). Another prototype of immune dysregulation diseases is autoimmune lymphoproliferative syndrome (ALPS) which results from a heterogeneous group of mutations in the genes that regulate apoptosis. There is a separate group of immune dysregulation syndromes like IL-10 deficiency or IL-10R deficiency with predominant feature of colitis (Glocker 2009, Glocker 2010).

Recently, a novel monogenic defect of autoimmunity associated with severe growth failure and early-onset multiorgan dysfunction (OMIM 615952) was described, which was caused by germline gain-of-function (GOF) variants in signal transducer and activator of transcription 3 (STAT3) (Flanagan 2014, Haapaniemi 2015, Milner 2015). The specific target organs involved can differ even between patients with the same variant, like 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 (Fabre 2019). Organ system involvement appears sequentially (Figure 1). Very early-onset endocrinopathies and gastrointestinal diseases appear typically before the onset of lymphoproliferation, hematologic manifestations and lung disease. Neonatal type I diabetes, hypothyroidism and atypical celiac disease usually occur during infancy and can be for a long time the only clinical manifestations. Type 1 diabetes is usually diagnosed before 3 months which is in contrast to polygenic type 1

diabetes. Enteropathy was frequently diagnosed as pseudoceliac disease with villous atrophy, but without the usual serologic signature. Hematologic disease is frequent with an onset after infancy and most commonly includes lymphoproliferation and autoimmune cytopenia. Pulmonary diseases with a predominance of interstitial lung disease usually present in later childhood. Other autoimmune manifestations, including arthritis, autoimmune hepatitis and alopecia areata notably manifest later in life.

We describe here a novel STAT3 GOF variant in a patient who presented with hypogammaglobulinemia and severe, recurrent respiratory tract infections, but did not have multi-organ autoimmunity and early-onset autoimmune manifestations.

Methods

Genetic analysis. Genomic DNA from the patient and his mother were isolated with the Gen Elute Blood Genomic DNA kit (Sigma-Aldrich, St Louis, Missouri, USA). Mutations were analyzed by amplifying exons and flanking intronic regions of STAT3 by 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 3,130 capillary sequencer (Applied Biosystems). Sequence variants were determined compare to reference sequence, GenBank accession no. ENSG00000168610 of the STAT3 cDNA to identify the position of mutations.

STAT3 reporter assay. A luciferase assay was used to assess *STAT3* variants (Tanita K, 2021). The pcDNA3 expression vector containing wild-type (WT) or variant STAT3, together with the STAT3 luciferase reporter vector (Qiagen, Hilden, Germany), was transfected into A4 cells (STAT3-deficient human colon cancer cells) using Lipofectamine LTX Reagent (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's protocol. At 24 h posttransfection, the cells were treated with 10 ng/mL FP6 (a recombinant fusion protein of IL-6R and IL-6), 100 IU/mL 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, WI) in triplicate, and the data are expressed in 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 (Tanita K, 2021). Equal amounts of protein were separated on 10% SDSPAGE gel and transferred to PVDF membranes (Merck KGaA, Darmstadt, Germany). The membranes were blocked with 5% BSA (Sigma-Aldrich, St. Louis, MO) or low-fat bovine milk and were then incubated with rabbit anti-human pSTAT3 (pY705) antibody (Cell Signaling Technology, Danvers, MA), mouse anti-human STAT3 antibody (Cell Signaling Technology, Danvers, MA), or mouse anti-ß-actin antibody (Sigma-Aldrich). HRP-conjugated goat anti-mouse and anti-rabbit antibodies (GE Healthcare, Buckinghamshire, UK) were used as secondary antibodies. Antibody binding was detected using an enhanced chemiluminescence reagent (Thermo Fisher Scientific).

Results

Case history. The male patient was born at gestation week of 35 with a birth weight of 2350 g, and a length of 45 cm; 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 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 examinations of stool 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 met a bicycle accident and broke his right tibia. Cervical and abdominal lymphadenomegaly and splenomegaly was first documented at 4.5 years of age and was considered to result from the recurrent infections he had suffered from. At the same age he had surgery for mobilization and fixation of testis because of right sided retention. Since 6 years of age, he was hospitalized and treated several times with recurrent pleuropneumonia, bronchitis, sinusitis, pharyngitis and otitis. He was diagnosed with hypogammaglobulinemia at the age of 6, when serum immunoglobulin 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 MR examinations showed hepatosplenomegaly, paraaortic, mesenterial and inguinal lymphadenomegaly, signs of portal hypertension, and dilatation of the v. portae and v. lienalis. Because of the generalized lymph node enlargement and

hepatosplenomegaly, liver and lymph node biopsy were performed which showed very mild intrasinusoidal lymphoid infiltration in the liver and normal lymph node tissue. After 8 years of age he presented with recurrent loose stools, nausea, abdominal bloating and pain. Swelling of the ankles and knees occurred usually at night and especially after long-term standing. He was 9 years of age when monthly intravenous immunoglobulin therapy was started. Despite IVIG substitution, pneumonia recurred, although less frequently than before.

The patient was referred to our department for further diagnostic evaluation of primary immunodeficiency. Written and 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 (weight: 22.4 kg; 3 percentile, <9 kg, height: 128.6 cm; 3 percentile, <12 cm) were found. Serum immunoglobulin isotype levels and lymphocyte subpopulations were determined by standard immunological assays. Immunochemistry tests revealed severe hypogammaglobulinemia; IgG, <0.73 g/l (norm: 5.4-15.1 g/l), IgM, 0.14 g/l (norm: 0.52-1.5 g/l), IgA, 0.05 g/l (norm: 0.52-3.25 g/l) and normal levels of IgE (IgE <4.4 kU/l, norm: 0.0-200.0 kU/l). The patient had normal total white blood cell counts and $CD4^+$ T lymphopenia ($CD4^+$ cells: 343/µl). The $CD3^+CD4^-CD8^-$ double-negative T cell ratio was normal (2.71%). X-ray showed porotic bones, with severely decreased skeletal age. At 13 years of age the skeletal age was like of a 7 years of age child. Endocrine parameters showed normal levels of sTSH, fT4 and fT3 with decreased insulin-like growth factor-1 (IGF-I): 78.4 µg/L

Inspiratory HRCT examination was first performed at 13 years of age with conventional sequential technique in prone position (Figure 2A and 2B). It revealed 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 specific pattern, but features of desquamative interstitial pneumonia and lymphocytic interstitial pneumonia could be identified (Figs 2A and 2B).

From 14 years of age the patient started smoking and he and his parents arbitrarily withdraw 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, he refused therapy. The second CT scan was performed at the age of 21 years after resuscitation, for the exclusion of pulmonary embolism (Figure 2C and 2D). 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 specific pattern is evident on both scans, significant progression was not proved, and definitive fibrosis did not evolve. Maldevelopment of bilateral first ribs were known from chest radiographs. This is clearly demonstrated on the volume rendered images of the last CT scan (Figure 3). The anterior part 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 STAT3 affecting a highly conserved position among species. Genetic analysis of the mother by targeted sequencing (Sanger) revealed normal sequences. The father was not available for the analysis. The variant in the patient was also confirmed by Sanger sequencing. *In vitro* functional analysis was performed and STAT3 reporter assay revealed that the novel D170H variant was GOF. Luciferase assay was performed using A4 cells transiently transfected with WT or variant STAT3 together with a STAT3 reporter vector (Figure 4). E415K known GOF variant and R382W known 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 <0.01), suggesting that this variant is GOF. Western blotting also confirmed increased phosphorylation of STAT3 after FP6, fusion protein of sIL-6R and IL-6, stimulation using A4 cells (Figure 5).

Discussion

In this patient with the novel c.508G>C, p.D170H STAT3 variant the earliest manifestation of STAT3 GOF syndrome was lymphoproliferation with lymphadenopathy after 1 year of age and hepatosplenomegaly at the age of 4.5 years. The patient had severe and recurrent respiratory tract infections since the age of 6 years. He did not show any clinical signs of autoimmune endocrinopathy. Even during his short adulthood no laboratory or clinical signs consistent with diabetes or hypothyroidism could be detected. After 8 years of age he sometimes experienced loose stools, nausea, abdominal bloating and pain, but infectious origin could not be defined. Since the age of 8 swelling of the ankles and knees sometimes occurred but usually at night and especially after long-term standing.

The patient had recurrent and severe respiratory infections and severe hypogammaglobulinemia which was more severe than were in previously reported patients. Immunodeficiency including lymphopenia or hypogammaglobulinemia is not a predominant feature of STAT3 GOF syndrome. STAT3 GOF patients are predicted to exhibit increased B cell activity. However, some of the patients seem to display hallmarks of B cell dysfunction such as hypogammaglobulinemia and decreased switched memory B cells (Haapaniemi 2015, Milner 2015). Hypogammaglobulinemia was reported only in about half of patients and usually not in a severe form (Haapaniemi 2015, Milner 2015). It is still unclear if there are B cell intrinsic effects of STAT3 over activation or whether this is secondary to defects in other cells such as regulatory T cells (Tregs).

As a unique manifestation our patient had developmental bone abnormality, including missing anterior part of both first ribs and partial coalition and pseudo-articulation of the proximal parts with the second rib. It is well-known that STAT3 is a critical mediator of bone growth and maintenance of bone structure. The loss of STAT3 in mature osteoclasts has detrimental effects on bone structure as STAT3 affects bone formation and mineralization irrespective of sex (Davidson 2020). The loss of STAT3 cause decrease in trabecular bone volume and may result in a severe osteoporosis phenotype (Davidson 2020, Zhang Z 2005).

All previously reported patients were heterozygous for the activating STAT3 variant, and most of them had de novo variant. The novel c.508G>C, p.D170H STAT3 GOF variant affects an amino acid of the coiled coil domain (CCD) of STAT3. The CCD mediates the interaction of

STAT3 with cytokine receptors and is required for subsequent STAT3 phosphorylation (Zhang T 2000). Although germline GOF variants are found throughout the protein except the N-terminal domain, up to date only one other patient with STAT3 GOF variant (R152W) was reported affecting the CCD of STAT3 (Milner 2015). This, 25-year-old male patient had autoimmune hemolytic anemia, autoimmune thrombocytopenia, insulin-dependent diabetes mellitus, lymphadenopathy, hepatosplenomegaly, alopecia and lung nodules, but did not show short stature (Vogel 2015). In contrast malignancy-associated, somatic GOF variants are clustered in the SH2 domain, while most LOF variants causing hyper-IgE syndrome cluster in the DBD and SH2 domain (Vogel 2015).

Our observation suggests that STAT3 GOF syndrome can manifest in early childhood with recurrent and severe respiratory infections and hypogammaglobulinemia. Moreover, patients may lack early-onset autoimmune manifestations or present only mild and/or delayed-onset autoimmunity. Therefore, patients with hypogammaglobulinemia and recurrent infections should be screened for STAT3 variants even if autoimmune manifestations are missing or not severe.

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. We thank the parents of the patient for helpful collaboration.

Consent statement: Parent signed an informed consent to conduct the study and for data publication

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

Competing interests: The authors declare no conflict of interest.

Availability of data and materials: Data and materials used in this study are available on request.

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Figure legends

Figure 1

Sequence of clinical signs and symptoms of STAT3 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 line (Fabre 2019). Disease manifestations in our patient with the novel c.508G>C, p.D170H STAT3 GOF variant are shown above the line (shaded).

Figure 2

Images of sequential chest 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 (2A and 2B). Thin section helical chest CT scans obtained at the age of 21, following resuscitation are shown in 2C and 2D. Inhomogeneous obstructive hyperinflation and nontraction bronchiectasis is 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 eight years and there is no evidence of fibrosing disease.

Figure 3

Volume rendered 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

Reporter assay of STAT3. STAT3 reporter activity in the presence or absence of IFN-α, FP6 and IL-27. E415K and R382W are known GOF and LOF mutants, respectively. Like E415K known GOF mutant, D170H showed increased STAT3 activity. (Reporter plasmids: STAT3 (QIAGEN) IFN-α: 100 IU/mL, FP6: 10 ng/mL, IL-27: 20 ng/mL, 16 hours).

Figure 5

Western blot of STAT3. Western blot analysis of transfected A4 cells after stimulation with FP6. D170H showed increased pSTAT3 upon FP6 (fusion protein of sIL-6R and IL-6) stimulation like E415K known GOF mutant. Increased pSTAT3 was also found in non-stimulated condition. (FP6: 10 ng/mL, 15 min)