IL-12Rβ1 deficiency corresponding to concurrency of two diseases, mendelian susceptibility to mycobacterial disease and Crohn's disease

Razieh Khoshnevisan[a], Nioosha Nekooei-marnany[b], Christoph Klein[c], Daniel Kotlarz[c], Mahdieh Behnam[a], Vajihe Ostadi[a,b], Majid Yaran[b], Abbas Rezaei[a,b,*], Roya Sherkat[b,*]

[a] Immunology Department, Isfahan University of Medical Sciences, Isfahan, Iran
[b] Aquired Immunodeficiency Research Center, Isfahan University of Medical Sciences, Isfahan, Iran
[c] Dr. von Hauner Children's Hospital, Department of Pediatrics, University Hospital, LMU Munich, Munich, Germany

ARTICLE INFO

Keywords:
MSMD
IBD
IL12RB1
Mutation

ABSTRACT

Background: The interleukin-12 receptor β1 (IL-12Rβ1) deficiency is a primary immunodeficiency (PID), affecting the immunological pathway of interleukin 12/interferon- γ (IL12/IFN-γ) axis and interleukin 23 receptor (IL23R). Defect in this pathway is mainly affecting the cellular immunity-related disorders. IL-12Rβ1 is a receptor chain of both the IL-12 and the IL-23 receptors and thus, deficiency of IL-12Rβ1 abolishes both IL-12 and IL-23 signaling.

Material and methods: In this study, we performed whole exon sequencing and confirmatory Sanger sequencing in *IL-12Rβ1*. Evaluation of the IL12/IFN-γ axis was performed by assessment of patients' whole blood cell to IL12/IFN-γ responding. Total and surface IL-12Rβ1expression was evaluated, in peripheral blood mononuclear cells (PBMCs) and T cell- derived PBMCs, and Th17 count was assessed.

Results: In the present study, we described a c.1791 + 2T > G mutation at a splicing site position in *IL-12Rβ1*, using whole exome sequencing, and confirmed with targeted Sanger sequencing in a 26- year-old patient with Mendelian susceptibility to mycobacterial disease (MSMD) and Crohn's disease (CD). Complete lack of IL-12Rβ1 protein expression was detected in patient's PBMCs, compared to the healthy control. Furthermore, no IL-12Rβ1 protein was expressed on the cell surface. Interestingly, IL-12Rβ1-mutant cells showed an impaired response to IL12, and *Bacillus Calmette–Guérin* stimulation, confirming that the mutation is causative in this patient.

Conclusion: A 3′splicing site mutation in *IL12Rβ1*, can be corresponding to the abolished expression of *IL12Rβ1* in patients' cells, and associated with an impaired IL12-mediated signaling, which may lead not only to MSMD, but also to inflammatory bowel disease (IBD).

1. Introduction

Primary immunodeficiencies (PIDs) are an extensively heterogeneous group of more than 300 rare inherited defects of the immune system, with increased susceptibility to chronic infection and recurrent, autoimmune and hyperinflammation manifestation, allergy and malignancy [1].

A rare subclass of PID is Mendelian susceptibility to mycobacterial disease (MSMD) [2], triggered by weak pathogenic mycobacterial species such as Salmonella species, nontuberculous environmental Mycobacteria (NTM), and Mycobacterium bovis Bacillus Calmette-Guérin (BCG) [3] The molecular basis of MSMD pathogenesis is on the functional IL-12/23-IFN- γ integrity of macrophage (monocyte/dendritic cell), correlating to T lymphocyte/ NK cells [3,4].

The interleukin-12 receptor β1 (IL-12Rβ1) receptor is a common part of IL-12R and IL23R. IL-12Rβ1 deficiency may affect signaling in both IL-12 and IL-23 [5], which is associated with MSMD pathogenesis [6], and is also implicated in inflammatory bowel disease (IBD) pathogenesis, by the defect in the differentiation of T helper 17(Th17) [7,8]. Th17 cytokine profile has a critical role in the mucosal immune response [9].

Here, we present the first coexistence of the two disorders in an IL-

12Rβ1 deficient patient from a consanguine family with an Iranian origin, initially diagnosed with Crohn's disease (CD) prior to the MSMD diagnosis.

2. Methods

Patient information was referred to the department of clinical Immunology at the Isfahan University of Medical Sciences for immunological and genetic consultation. Blood samples from the patient, his family and healthy donor controls were collected, after signing an informed consent form.

We performed this descriptive study to evaluate the patient.

2.1. Whole-exome sequencing

Genomic DNA from patients and parents was isolated, using the QIAamp DNA Blood Mini Kit (Qiagen), following the manufacturer's instructions. To enrich the entire coding exons, the SureSelect Human All Exon Kit (Agilent Technologies) was utilized. The llumina Genome Analyzer II (family A) or Illumina HiSeq 2000 (family B) sequencing was used, and short sequence reads were aligned to the reference of the human genome GRCh37, using BWA software [10]. To analyze the WES data, the Genome Analysis Tool Kit (GATK) [11] was used. The functional annotation was checked, using snpEff [12] and Variant Effect Predictor (VEP) was determined with Ensembl [13] release 85 (family A) or 71 (family B). The WES data were analyzed, using SQL database (family A) or FILTUS v.0.99–934 (family B) [14]. Effects of filtered variants were predicted with a combination of software, including snpEff [12], VEP [13], SIFT [15] and PolyPhen-2 [16]. The remaining variants were compiled and filtered for rare homozygous, and compound heterozygous mutations, following a pattern of autosomal recessive inheritance. Segregation of the identified *IL12RB1* mutations was performed on family members by DNA Sanger sequencing.

2.2. Cell preparation

Diluted whole blood (1:4 in RPMI 1640 (Gibco) was divided into 1 ml/well. Stimulation was done according to the two following conditions:

1 BCG (*Mycobacterium bovis* BCG, Pasteur strain) with a multiplicity of infection (MOI) of 20, with BCG plus recombinant IFN-γ (5000 IU/mL, BioLegend).
2 BCG plus recombinant IL-12p70 (20 ng/mL, R&D Systems)

All cells were incubated at 37 °C and 5% CO_2 atmosphere. Supernatants were collected after 48 h.

2.3. ELISA

Cell culture supernatants were assayed for IFN-γ (BioLegend), TNF-α (eBioScience), IL12/IL23 p40 (eBioScience Human IL12/IL23 p40 Platinum ELISA kit), and IL-12p40 by ELISA, according to the manufacturer's recommendations.

2.4. Flow cytometry

The PBMCs were separated by density gradient, as previously described [17], and cultured in standard media (RPMI1640, supplemented with FBS 10%, penicillin 100 IU/mL and streptomycin 100 µg/mL). To evaluate the IL12RB1 expression by fluorescence-activated cell sorting (FACS), cells were stimulated by 20 µg phytohemagglutinin (Becton Dickinson) for 72 h to generate phytohemagglutinin (PHA)-activated T cells. Furthermore, isolated PBMCs were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA), 1 µg/mL ionomycin, and 10 µg/mL of Brefeldin to check the Th17 population.

The IL12RB1expression on T cells was evaluated with PE-anti-IL12RB1 mAb (BD) on CD3 positive cells (FITC-anti CD3 mAb, BD). Th17 was determined with FITC-anti CD4 (CMG) and PE-anti-IL17 mAb (Biolegend). FACS Calibur flow cytometer was used for measurement and analysis, using Cellquest pro software (BD Bioscience).

2.5. Real-time pcr

RNA was isolated with the RNA Mini plus-Kit (QIAgene), following the manufacturer's instructions. The High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher scientific) was used for reverse transcription. The cDNA was used for semi-quantitative real-time PCR, using the Fast SYBR™ Green Master Mix (Thermo- Fisher scientific). The relative mRNA expression was normalized to GAPDH, and analyzed by the $2^{-\Delta\Delta Ct}$ method. Primer sequences used for the Real- time PCR are as follows: sense: 5′-AGC AAA CAG GTG TCA GAG CAT-3′ and antisense: 5′-AAG AAG ATG AGC CAA TCA G-3′

3. Result

In this study, we investigated a 26-year-old male patient from a consanguine family (Fig. 1. I), who reportedly had a history of the right sub-auxiliary BCG lymphadenitis, recurrent mouth ulcers and chronic diarrhea in childhood. He was hospitalized with lower right quadrant pain and underwent appendectomy at 5 years old. Based on his clinical presentation with weight loss, abdominal pain, chronic and bloody diarrhea, as well as endoscopic and pathologic findings (Fig. 1. III-a), based on Montreal classification [18], he was diagnosed with CD, but severe and uncontrolled colitis resulted in colostomy surgery (only rectal part is remained), and the intestinal histopathologic staining was negative for *Mycobacterium* (Fig. 1. IIIb). At the age of 13, he presented with multifocal osteomyelitis (Fig. 1. IV), even though no bacteria were detected in the culture of biopsy samples. He did not respond to anti-bacterial treatment, but had a dramatic response to anti-tuberculosis treatment, which healed his bone pain and lesions. Even though the bone manifestations were completely controlled, the patient needed to continue the required, continuous refractory diarrhea treatment. Intestinal disorders and refractory diarrhea caused by CD, were continuously treated by Mesalamine and Prednisolone.

To evaluate the molecular etiology in our patients, whole exome sequencing was performed, revealing a 3′splice site mutation in *IL12Rβ1* (ENST00000600835.6, 1791 + 2T > G). Sanger sequencing confirmed a segregation with the disease phenotype in the index patient, while his mother, father and sibling showed a heterozygous genotype (Fig. 1. II).

The *IL12Rβ1* expression was evaluated by Real-time PCR. We found that the *IL12Rβ1* expression was completely abolished in PBMCs of patient, compared to the healthy control (Fig. 2. I). Furthermore, surface expression of *IL12RB1* was not detectable in T Cell PHA Blasts of the patient, compared to the healthy control (Fig. 2. IV), and the patient had low Th17 population, compared to the healthy donor (Fig. 2. V).

We also evaluated the response of patient cells to IFN-γ and IL-12 cytokine. Patient's cells did not respond to IL12 stimulation, since we could not detect any increase of INF-γ after stimulation with IL12 and BCG, using ELISA, in comparison to the healthy control (Fig. 2. II,III).

4. Discussion

In this study, we have shown a 3′splice site mutation in *IL12Rβ1,* corresponding to the abolished expression, undetectable cell surface expression of *IL12Rβ1,* impaired response to IL12 and BCG stimulation, and decreased count of Th17 that can be the cause of CD and MSMD in a patient.

Tuberculosis is a comprehensive concerning problem in healthcare, and the rate of infection with *Mycobacterium tuberculosis* (*M. tuberculosis*) shows an upward trend, worldwide [19,20].
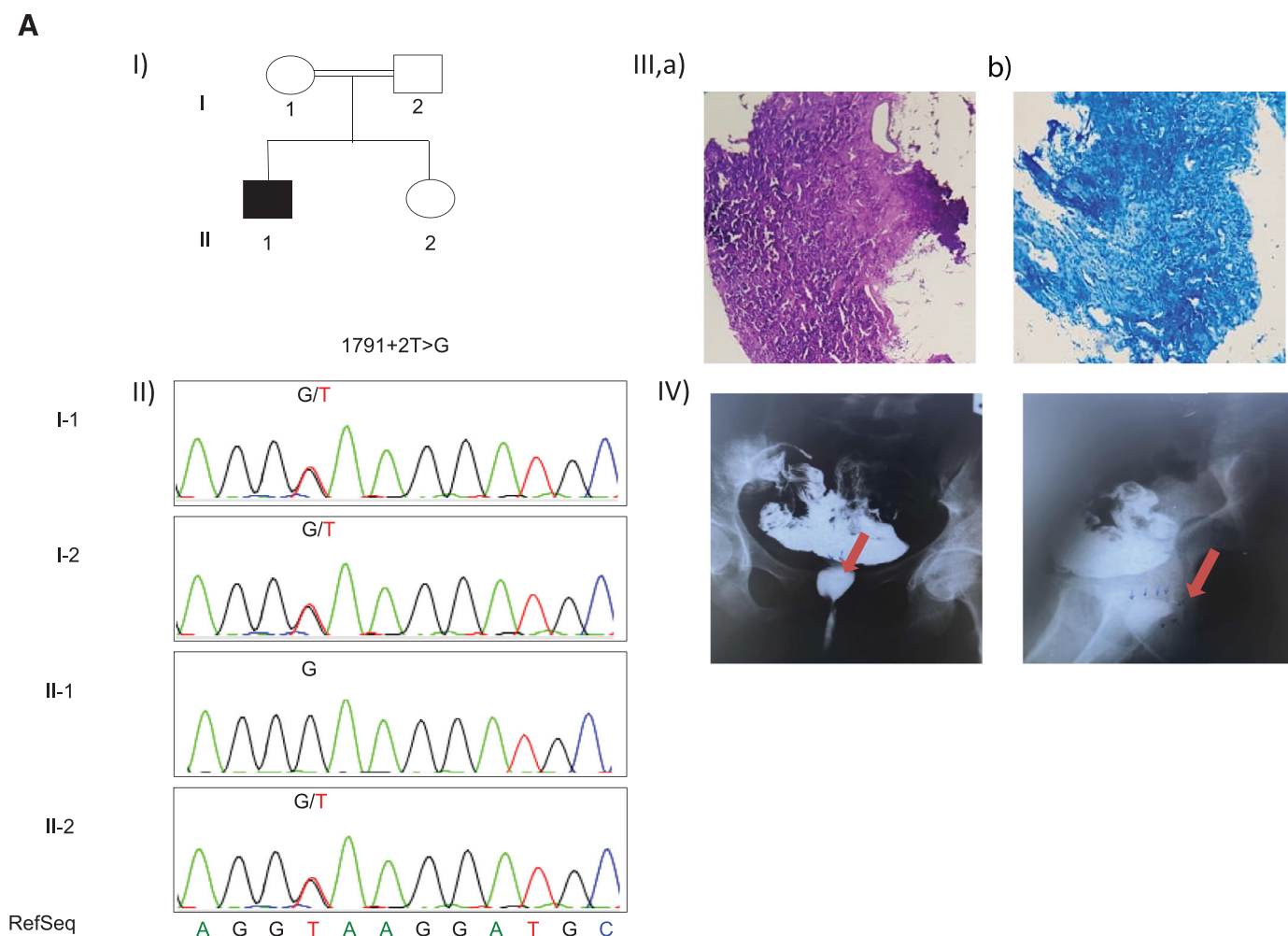
A

Fig. 1. Pedigree of the index family, Sanger sequencing confirmed the identification of the homozygous splice site mutation in IL12Rβ1 in patient II-1. Radiographic examination of sacrum and hip joint showed osteomyelitis. RNA expression IL-12Rβ1 is completely abolished in PBMCs of patient compare to HD (n:2).

MSMD syndrome is a rarely inherited disorder, defined in 1951 as disseminated disease, characterized by the expansion of a comprehensive infection to Mycobacteria [3]. Limited defective molecules in the circuit, including IFN γ R1, IFN γ R2, STAT1, IL12β, IL12Rβ1, IRF8, ISG15, NEMO, and CYBB have been recognized in patients with the MSMD phenotype [21].

The IL-12Rβ1 deficiency, causing a profound defect in IL-12 signaling pathway, is the most shared genetic etiology of MSMD (44%). The weakly virulent Mycobacteria and invasive salmonellosis are typically the cause of frequent infections in the IL-12Rβ1-deficient patients. The deficiency of IL-12 responses impairs the production of IFN-γ, and the IL-12/IFN-γ axis is vital for defensive immunity to Mycobacterium [22]. Even though the index patient is the forth-reported patient with the IL-12Rβ1 deficiency and enteropathy, but no multifocal osteomyelitis was reported in the previous report. The first patient was a 4-year-old Mexican boy with *IL-12Rβ1*, c.1791 + 2 T > G mutation, mucocutaneous candidiasis, and *M. bovis* BCG disseminated disease, who was treated with multiple antimycobacterial therapy. The second patient was a 49-year-old Caucasian male with c.19602C > T mutation in the *IL-12Rβ1,* who suffered from severe gastroenteropathy, and the third one is a 10-year-old Colombian boy with IL-12Rβ1 c.872G > A mutation with two episodes of non-bloody diarrhea, vomiting, decreased appetite, progressive abdominal distension, and persistent fevers, presumed to be due to gastrointestinal (GI) viral infections [23].

Generally, mycobacterial osteomyelitis is a characteristic phenotype

in MSMD patients with deficiency in IFN-γR1 or STAT1 [24,25], and no mycobacterial osteomyelitis reported in all reported IL12Rβ1-deficient patients [5,23]. Interestingly, our patient presented severe multifocal osteomyelitis (Fig. 1. IV).

The cytokines IL-12 and IL-23 are heterodimeric cytokines, containing two disulfide subunits. IL-12 is composed of p35 and p40 subunits, whereas IL-23 is composed of p19 and p40. Both cytokines regulate immune responses, which are critical for adaptive and innate human immunity, including the T cell, B cell, dendritic cells and natural killer cell differentiating and responding regulation [26,27]. IL-12 and IL-23 biological activities are dependent on IL-12Rβ1, a type I integral membrane protein that physically associates with IL12 and IL23, and signals in combination with IL12Rβ2 or IL23R, respectively [28].

Furthermore, IL12RB1 signaling impairment caused defects in differentiation of Th17 [7,8], which has a critical role in pathogenesis of CD (clinical features include mucosal erosion, diarrhea, weight loss and formation of granuloma) [29,30]. Th17 has a role in intestinal homeostasis through production of IL-6, TGF-β, IL17 and IL-1β in a mucosal damage situation, defense against extracellular pathogens, and induction of anti-microbial peptides, releasing from intestinal epithelial cells [31]. In the IL12rb1$^{-/-}$ mice, the ratio of Th17 was decreased and the mice were unresponsive to both IL-12 and IL-23 [32]. In the present study, the Th17 population was decreased in the patient, suggesting that it may have a role in the CD incidence in our patient. Furthermore, there are some studies about the role of atypical Mycobacterium as a trigger for CD, using a population-based approach. Some studies used
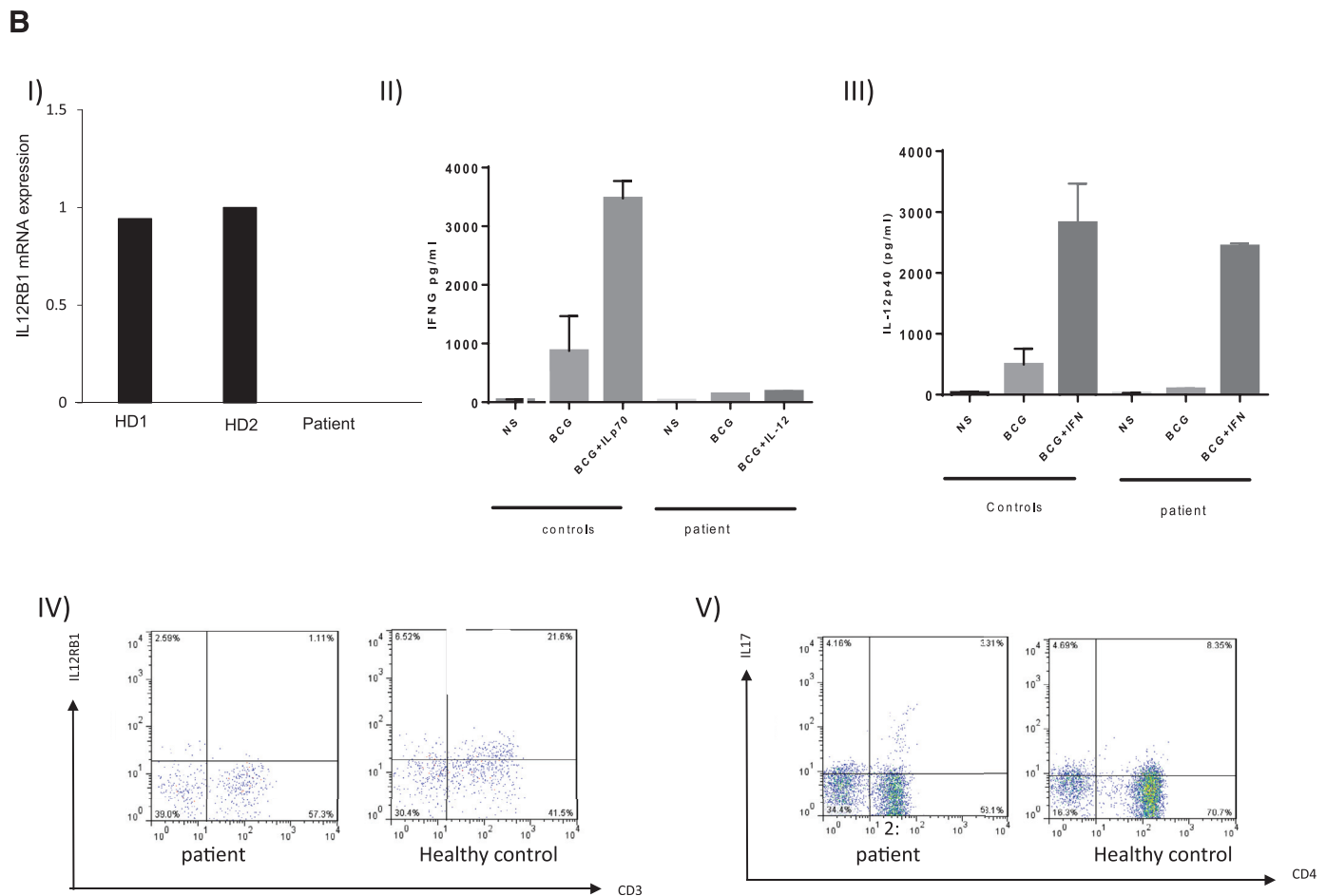
B

Fig. 2. Flowcytometry analysis of IL-12Rβ1 expression in the T cells blasts from patient and Healthy donor(HD) indicate impaired expression of the gene in patient. The IL-12 and IFN-γ responses on whole blood activation, 1:imparment production of INFG in IL12 +/− BCG in patient, Undetectable IL12p40 in BCG stimulation compare to HD, Normal range of IL12p40 production after BCG + INFG stimulation and decrease Th17 population.

colonoscopic biopsies, and were able to detect atypical Mycobacterium in CD patient, showing that it might be a relation between the CD incidence and atypical Mycobacterium [33–39]. These studies might highlight the role of BCG vaccination in the CD and MSMD incidence, in the IL12Rβ immuno-deficient patients [23] that needs more investigation, even though we did not detect Mycobacterium in patient intestinal biopsies (Fig. 1. IIIb).

Since the pathophysiology of MSMD and IBD is different, so choosing an appropriate treatment is challenging. The therapeutic goal in the CD is to induce and maintain remission, heal the mucosa and optimize quality of life for the patient. Treatment with initial trials of immunosuppressive treatment (corticosteroid and steroid sparing immunomodulators), and anti-TNF agent are suggested for the CD treatment [40,41]. However, using anti TNF-α treatment increases the risk of Mycobacterium and granulomatous infection [42]. TNF- α is a pleiotropic cytokine with a critical role in protection against *M. tuberculosis,* produced by macrophages and T cells. TNF-α facilitates transition from innate to acquired immunity by enhancing antigen presentation and T cell co-stimulation [43]. Index patient had a concurrent incidence of MSMD and IBD, so choosing an appropriate therapy approach, following clinical and laboratory criteria was critical.

5. Conclusion

Here, we describe two different features of the IL12Rβ1 deficiency in one patient. To the best of our knowledge, this case is the first report of the concurrent prevalence of CD and MSMD in one patient in Iran. Early differential diagnosis of the patient is critical in the disorder management, and genetic evaluation would provide a beneficial attitude in the process of treatment.

Declaration

Ethical approval and consent form: The informed consent of the subject was approved by the Institutional Review Board Standards of Isfahan University of Medical Sciences.

Consent to publish: A consent form was signed by the subject in order to publish the data.

Availability of data and material: All data, analyzed or generated during this study are included in this published article.

CRediT authorship contribution statement

Razieh Khoshnevisan: Conceptualization, Data curation, Investigation. **Nioosha Nekooei-marnany:** Investigation. **Christoph Klein:** Writing - original draft. **Daniel Kotlarz:** Writing - original draft.

Mahdieh Behnam: Project administration. **Vajihe Ostadi:** Investigation. **Majid Yaran:** Project administration. **Abbas Rezaei:** Writing - review & editing. **Roya Sherkat:** Writing - review & editing.

Declaration of Competing Interest

The authors declare no competing interests.

Acknowledgement

We are grateful to the patient for participating in the study.

References

[1] Bousfiha A, Jeddane L, Picard C, Ailal F, Bobby Gaspar H, Al-Herz W, et al. The 2017 IUIS phenotypic classification for primary immunodeficiencies. J Clin Immunol 2018;38(1):129–43.

[2] Staines-Boone AT, Deswarte C, Venegas Montoya E, Sánchez-Sánchez LM, García Campos JA, Muñiz-Ronquillo T, et al. Multifocal recurrent osteomyelitis and hemophagocytic lymphohistiocytosis in a boy with partial dominant IFN-γR1 deficiency: case report and review of the literature. Front Pediatr 2017;5:75.

[3] Lee W-I, Huang J-L, Yeh K-W, Jaing T-H, Lin T-Y, Huang Y-C, et al. Immune defects in active mycobacterial diseases in patients with primary immunodeficiency diseases (PIDs). J Formos Med Assoc 2011;110(12):750–8.

[4] Altare F, Ensser A, Breiman A, Reichenbach J, Baghdadi JE, Fischer A, et al. Interleukin-12 receptor β1 deficiency in a patient with abdominal tuberculosis. J Infect Dis 2001;184(2):231–6.

[5] van de Vosse E, Haverkamp MH, Ramirez-Alejo N, Martinez-Gallo M, Blancas-Galicia L, Metin A, et al. IL-12Rβ1 deficiency: mutation update and description of the IL12RB1 variation database. Hum Mutat 2013;34(10):1329–39.

[6] Ramirez-Alejo N, Santos-Argumedo L. Innate defects of the IL-12/IFN-γ axis in susceptibility to infections by mycobacteria and salmonella. J Interferon Cytokine Res 2014;34(5):307–17.

[7] Sivanesan D, Beauchamp C, Quinou C, Lee J, Lesage S, Chemtob S, et al. IL23R (Interleukin 23 receptor) variants protective against inflammatory bowel diseases (IBD) display loss of function due to impaired protein stability and intracellular trafficking. J Biol Chem 2016;291(16):8673–85.

[8] de Beaucoudrey L, Puel A, Filipe-Santos O, Cobat A, Ghandil P, Chrabieh M, et al. Mutations in STAT3 and IL12RB1 impair the development of human IL-17-producing T cells. J Exp Meditor 2008;205(7):1543–50.

[9] Guglani L, Khader SA. Th17 cytokines in mucosal immunity and inflammation. Curr Opin HIV AIDS 2010;5(2):120–7.

[10] Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics (Oxford, England) 2009;25(14):1754–60.

[11] McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 2010;20(9):1297–303.

[12] Cingolani P, Platts A, Wang le L, Coon M, Nguyen T, Wang L, et al. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. Fly 2012;6(2):80–92.

[13] McLaren W, Pritchard B, Rios D, Chen Y, Flicek P, Cunningham F. Deriving the consequences of genomic variants with the Ensembl API and SNP Effect Predictor. Bioinformatics (Oxford, England) 2010;26(16):2069–70.

[14] Vigeland MD, Gjotterud KS, Selmer KK. FILTUS: a desktop GUI for fast and efficient detection of disease-causing variants, including a novel autozygosity detector. Bioinformatics (Oxford, England) 2016;32(10):1592–4.

[15] Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc 2009;4(7):1073–81.

[16] Adzhubei I, Jordan DM, Sunyaev SR. Predicting functional effect of human missense mutations using PolyPhen-2. Curr Protoc Hum Genet 2013 Chapter 7:Unit7.20.

[17] Naung NY, Suttapreyasri S, Kamolmatyakul S, Nuntanaranont T. Comparative study of different centrifugation protocols for a density gradient separation media in isolation of osteoprogenitors from bone marrow aspirate. J Oral Biol Craniofac Res 2014;4(3):160–8.

[18] Satsangi J, Silverberg MS, Vermeire S, Colombel JF. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. Gut 2006;55(6):749–53.

[19] Houben RMGJ, Dodd PJ. The global burden of latent tuberculosis infection: a Re-estimation using mathematical modelling. PLoS Med 2016;13(10):e1002152.

[20] Rudko AA, Bragina EY, Puzyrev VP, Freidin MB. The genetics of susceptibility to tuberculosis: progress and challenges. Asian Pac J Trop Dis 2016;6(9):680–4.

[21] Fernández Echeverri D, González Loaiza D, Moncada-Vélez M, Orrego JC, Rojas JL, Arango Franco C, et al. Mendelian susceptibility to mycobacterial diseases (MSMD) in Colombian patients. Frontiers Immunol. 2015.

[22] Feinberg J, Fieschi C, Doffinger R, Feinberg M, Leclerc T, Boisson-Dupuis S, et al. Bacillus Calmette Guerin triggers the IL-12/IFN-gamma axis by an IRAK-4- and NEMO-dependent, non-cognate interaction between monocytes, NK, and T lymphocytes. Eur J Immunol 2004;34(11):3276–84.

[23] Arias AA, Perez-Velez CM, Orrego JC, Moncada-Velez M, Rojas JL, Wilches A, et al. Severe enteropathy and hypogammaglobulinemia complicating refractory mycobacterium tuberculosis complex disseminated disease in a child with IL-12Rbeta1 deficiency. J Clin Immunol 2017;37(7):732–8.

[24] Arend SM, Janssen R, Gosen JJ, Waanders H, de Boer T, Ottenhoff TH, et al. Multifocal osteomyelitis caused by nontuberculous mycobacteria in patients with a genetic defect of the interferon-gamma receptor. Neth J Med 2001;59(3):140–51.

[25] Bax HI, Freeman AF, Ding L, Hsu AP, Marciano B, Kristosturyan E, et al. Interferon alpha treatment of patients with impaired interferon gamma signaling. J Clin Immunol 2013;33(5):991–1001.

[26] Langrish CL, McKenzie BS, Wilson NJ, de Waal Malefyt R, Kastelein RA, Cua DJ. IL-12 and IL-23: master regulators of innate and adaptive immunity. Immunol Rev 2004;202:96–105.

[27] Ford NR, Miller HE, Reeme AE, Waukau J, Bengtson C, Routes JM, et al. Inflammatory signals direct expression of human IL12RB1 into multiple distinct isoforms. J Immunol 2012;189(9):4684–94.

[28] Oppmann B, Lesley R, Blom B, Timans JC, Xu Y, Hunte B, et al. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity 2000;13(5):715–25.

[29] Gajendran M, Loganathan P, Catinella AP, Hashash JG. A comprehensive review and update on Crohn's disease. Disease-a-month: DM 2018;64(2):20–57.

[30] Marks DJB, Rahman FZ, Sewell GW, Segal AW. Crohn's disease: an immune deficiency state. Clin Rev Allergy Immunol 2010;38(1):20–31.

[31] Kempski J, Brockmann L, Gagliani N, Huber S. T(H)17 cell and epithelial cell crosstalk during inflammatory bowel disease and carcinogenesis. Front Immunol 2017;8:1373.

[32] Robinson RT. IL12Rβ1: the cytokine receptor that we used to know. Cytokine 2015;71(2):348–59.

[33] Bull TJ, McMinn EJ, Sidi-Boumedine K, Skull A, Durkin D, Neild P, et al. Detection and verification of Mycobacterium avium subsp. paratuberculosis in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease. J Clin Microbiol 2003;41(7):2915–23.

[34] Collins MT, Lisby G, Moser C, Chicks D, Christensen S, Reichelderfer M, et al. Results of multiple diagnostic tests for Mycobacterium avium subsp. Paratuberculosis in patients with inflammatory bowel disease and in controls. J Clin Microbiol 2000;38(12):4373–81.

[35] Gitnick G, Collins J, Beaman B, Brooks D, Arthur M, Imaeda T, et al. Preliminary report on isolation of mycobacteria from patients with Crohn's disease. Dig Dis Sci 1989;34(6):925–32.

[36] Kirkwood CD, Wagner J, Boniface K, Vaughan J, Michalski WP, Catto-Smith AG, et al. Mycobacterium avium subspecies paratuberculosis in children with early-onset Crohn's disease. Inflamm Bowel Dis 2009;15(11):1643–55.

[37] Al-Shamali M, Khan I, Al-Nakib B, Al-Hassan F, Mustafa AS. A multiplex polymerase chain reaction assay for the detection of Mycobacterium paratuberculosis DNA in Crohn's disease tissue. Scand J Gastroenterol 1997;32(8):819–23.

[38] Baksh FK, Finkelstein SD, Ariyanayagam-Baksh SM, Swalsky PA, Klein EC, Dunn JC. Absence of Mycobacterium avium subsp. paratuberculosis in the microdissected granulomas of Crohn's disease. Mod Pathol 2004;17(10):1289–94.

[39] Bernstein CN, Nayar G, Hamel A, Blanchard JF. Study of animal-borne infections in the mucosas of patients with inflammatory bowel disease and population-based controls. J Clin Microbiol 2003;41(11):4986–90.

[40] Adegbola SO, Sahnan K, Warusavitarne J, Hart A, Tozer P. Anti-TNF therapy in Crohn's disease. Int J Mol Sci 2018;19(8):2244.

[41] Kelsen JR, Russo P, Sullivan KE. Early-Onset inflammatory bowel disease. Immunol Allergy Clin North Am 2019;39(1):63–79.

[42] Winthrop KL, Chang E, Yamashita S, Iademarco MF, LoBue PA. Nontuberculous mycobacteria infections and anti-tumor necrosis factor-alpha therapy. Emerg Infect Dis 2009;15(10):1556–61.

[43] Varfolomeev EE, Ashkenazi A. Tumor necrosis factor: an apoptosis junkie? Cell. 2004;116(4):491–7.